# ORAL ABSTRACTS

O-01

**Novel Alternatively Spliced Variant of Endothelin Converting Enzyme-1 Lacking a Transmembrane Domain**

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Endothelin-converting enzyme-1 (ECE-1) cleaves big-endothelins, bradykinin and beta-amyloid peptide. Several isoforms of ECE-1 (a-d) have been identified so far, they differ only in their N-terminus but share the catalytic domain located in the C-terminal end. Using quantitative PCR, we found ECE-1d to be the most abundant type in several endothelial cells (EC) types. In addition to full-length ECE-1 forms we have identified novel, alternatively spliced mRNA of ECE-1 b-d lacking exon 3', which codes for the a transmembrane TM region present in full-length forms. These ECE-1 splice variants (SVs) were highly expressed in EC derived from macro and microvascular beds but much less so in other cells expressing ECE-1, which suggests that the splicing mechanism is cell-specific. Full-length ECE-1d, SV and additional cDNA in which the 5 end of SV was deleted (SVcut) were stably expressed in HEK cells. Using these cells lines and a cell-free translation system we demonstrated that both SV- and SVcut cDNA translate into a protein of apparent MW of 75 kDa. This protein was recognized by C-terminal, but not by N-terminal specific anti-ECE-1 antibodies suggesting that it only share the C-terminal end with ECE-1d. In accordance, SV and SVcut expressing cells exhibited catalytic activity similar to that of full-length ECE-1d. The start site of this protein was identified using site directed mutagenesis; it lies in a region common to all ECE-1 forms which suggests that ECE-1b-d SV mRNAs are translated into one protein, designated ECE-1sv. We have also detected native expression of ECE-1sv protein in normal EC. Immunofluorescence staining demonstrated a distinct intracellular localization for ECE-1d and its SV form. The presence of ECE-1sv in different cellular compartments than full-length forms of the enzyme may suggest a distinct physiological role for these two proteins.

O-02

**Scavenging of Plasma ET-1 is Mediated by the Endothelial Cell Endothelin B Receptors**

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Pharmacological antagonists of $ET_B$ increase the plasma concentration of endothelin-1 (ET-1), suggesting that $ET_B$ plays an important role in the clearance of this peptide. By crossing floxed $ET_B$ mice (FF/--) with Tie2-Cre mice[1], we have generated an endothelial cell (EC)-specific $ET_B$ knockout (KO) mouse (FF/Tie2-Cre). We have examined the effect of selective KO of EC $ET_B$ receptors on basal plasma ET-1 and big ET-1 concentration, the clearance of ET-1 and on $ET_A$ expression. Plasma ET-1 and big ET-1 concentrations were measured by radioimmunoassay. Clearance of ET-1 was measured by injecting an i.v. bolus of [$^{125}$I]ET-1 (0.37 pmol/mouse; 28 kBq). Arterial blood was sampled over the following 2 minutes and radioactivity in each sample measured. $ET_A$ and $ET_B$ expression was assessed by quantitative autoradiography using the radiolabelled $ET_A$ specific ligand [$^{125}$I]-PD151242 and the $ET_B$ specific ligand [$^{125}$I]-BQ3020[2]. Autoradiography revealed downregulation of $ET_B$ in EC-rich tissues of FF/Tie2-Cre mice (8 ± 3 amol/mm$^2$) compared to controls (80 ± 21 amol/mm$^2$; n=4; $p$<0.05). $ET_A$ expression was unaltered despite a higher concentration of ET-1 in FF/Tie2-Cre mice (12.4 ± 3.0 pg.ml$^{-1}$) compared to controls (3.0 ± 0.8 pg.ml$^{-1}$; n=6; $p$<0.001) in all tissues studied. Plasma big ET-1 concentration was marginally increased in FF/Tie2-Cre animals (200 ± 8 pg.ml$^{-1}$) relative to controls (137 ± 13 pg.ml$^{-1}$; n=4; $p$<0.05). The area under the curve for the counts/minute (cpm)-time graph was significantly increased in FF/Tie2-Cre mice (235056 ± 21333 cpm.sec) and in control mice pre-treated with the selective $ET_B$ antagonist A192621 (199409 ± 20123 cpm.sec; n=4) compared to untreated controls (86726 ± 10968 cpm.sec; n=5; $p$<0.01). These results are consistent with the proposed role of EC $ET_B$ receptors as the predominant site of ET-1 scavenging. Increased plasma ET-1 does not result in down-regulation of $ET_A$ receptor expression in EC-specific $ET_B$ KO mice.

1. Kisanuki YY *et al. Dev Biol.* 2001;230:230. 2. Davenport AP *et al. Methods Mol Biol.* 2005;306:93.

O-03

**Agonist-dependent and agonist-independent proteolysis of the extracellular N terminus of the endothelin B receptor**

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The endothelin B (ETB) receptor belongs to the class I rhodopsin-like G protein-coupled receptors. Although class I receptors display a rather short N terminus, the ETB receptor has a cleavable signal peptide, which is cleaved off within the ER lumen during biosynthesis. A further cleavage of the N terminus occurs between residues 64 and 65 (SLAR/SLA) by a matrix-metalloprotease in an agonist-dependent manner (Grantcharova et al., 2002; J. Biol. Chem. 277:43933). Functional characterization of the wild-type ETB receptor and a genetically modified, N-terminally truncated ETB (∆2-64 ETB ) receptor revealed that the full-length ETB receptor elicited a biphasic, long-lasting ERK1/2 formation, whereas the ∆2-64 ETB receptor induced only a monophasic transient activation. As the location of the cleavage site of the ETB receptor is similar to that of the human PAR1 (LDPR/SFL), we hypothesized that the N terminus of the ETB receptor is susceptible to thrombin-mediated cleavage. In fact, thrombin also induced a cleavage of the extracellular N terminus in an agonist-independent manner, which was not inhibited in the presence of the metalloprotease inhibitor GM6001. Similarly, plasmin elicited cleavage of the N-terminus. When cells were pretreated for 30 min with plasmin (10 µg/ml), a condition sufficient to remove the extracellular N terminus quantitatively, subsequent ET-1 application induced only monophasic ERK1/2 activation. We show here for the first time that the full-length ETB receptor expressed in HEK cells is a substrate for serine proteases, e.g. thrombin and plasmin. More importantly, thrombin- and plasmin-induced cleavage alters the functional activity of the ETB receptor. Protease-induced switch in ETB receptor signaling could be of great importance in thrombosis and atherosclerosis, as these disease states are associated with increased plasma levels of thrombin and plasmin.

O-04

**Spatiotemporal *in vivo* imaging of the endothelin receptor system using microPET a dedicated positron emission tomography scanner for small animals**

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Positron emission tomography (PET) is the most sensitive technique available for imaging and quantifying receptor-bound radioligands *in vivo*. The recent development of dedicated PET scanners for small animals now allows functional imaging in rodents at high resolution (<2 mm) enabling delineation of receptor distributions within discrete organs and their larger substructures. Vascular receptors for peptides such as endothelin (ET) are a diverse group of potential drug targets that have not been studied extensively using PET. Our aim is to use microPET to image the ET receptor system *in vivo* to elucidate the fundamental processes involved in ET receptor pharmacology in normal and diseased tissue. For this purpose we have synthesized novel subtype selective PET radioligands as well as labeled the endogenous peptide ET-1 and its precursor peptide big ET-1 with $^{18}F$. With these radioligands we have imaged $ET_A$ receptors in the heart, rapid $ET_B$ receptor mediated clearance of ET-1 from the circulation by lung and kidney and followed enzyme conversion of big ET-1 and subsequent binding to ET receptors in the vasculature using anaesthetized rats. The microPET can delineate uptake in suborgan structures in the kidney as well as small structures below the notional resolution of the scanner such as thyroid and larger blood vessels. Our results clearly demonstrate that we can study ET receptor pharmacology *in vivo* using PET. Thus we have the potential to obtain pharmacodynamic information of novel drugs targeting the ET receptor system, image animal models and perform longitudinal studies in these models to monitor disease progression or effect of treatment to further clarify the significance of the ET receptor system in disease.

O-05

**Functional analysis of the endothelin-converting enzyme (ECE)-1a isoform-specific promoter reveals regulation by transcriptional repression**

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Isoform-specific expression of the human gene encoding endothelin-converting enzyme (ECE)-1 is regulated by alternative promoters residing within the 70 kbp 5' region of the ECE1 gene. Compared to isoforms ECE-1b and ECE-1c, ECE-1a has a highly restricted expression pattern. We, therefore, investigated the mechanisms by which the activity of the ECE-1a-specific promoter is controlled in more detail. Using computer-based analysis, 9 putative sites for putative binding of ETS transcription factors were identified within 1.2 kb of the human ECE-1a promoter. Among these, tandemly arranged ETS consensus sequences located at -638 and -658 were of particular interest because of inter-species conservation. Specific binding of nuclear protein to these cis-elements was demonstrated by gel shift assays. To further analyze functionality, point mutation in the core sequence of each of these ETS binding sites (EBS) were introduced into the wild-type ECE-1a promoter/luciferase construct and the mutated reporter constructs were transfected in endothelial and non-endothelial cells. Compared to the wild-type ECE-1a promoter, the construct mutated at EBS -638 was activated in human endothelial EA.hy926 and HMEC-1 cells about 3- and more than 10-fold, respectively ($p>0.001$). In human epithelial ECV304 cells, the mutation caused a more than 4-fold activation, whereas in bovine endothelial cells a 60% decreased promoter activity was observed. In contrast, mutation of EBS -658 uniformly resulted in significantly decreased promoter function suggesting species specific function of this cis-acting element. Mutation of the corresponding EBS conserved in the bovine ECE-1a promoter did not result in promoter activation. Moreover, mutation of EBS -638 in a 5' truncated ECE-1a promoter construct lacking the region upstream of -736 resulted in significantly decreased promoter activity in EA.hy926 cells. Our data show that ECE-1a promoter function is regulated by transcriptional repression in human cells and that the upstream promoter region is required for repressor function mediated by EBS -638.

O-06

**Involvement of Ca2 Channels in Endothelin-1 (ET-1)-Induced MAP Kinase Phosphorylation, Myosin Light Chain (MLC) Phosphorylation and Contraction in Rabbit Iris**

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Purpose: The goal of this study was to investigate the role and type of $Ca^{2+}$ channels involved in the stimulatory effects of ET-1 on the $Ca^{2+}$-dependent functional responses, p42/p44 MAP kinase phosphorylation, 20-kDa MLC phosphorylation and contraction, in rabbit iris sphincter. Methods: Myo-[3H]inositol phosphates production was measured by ion-exchange chromatography, MAP kinase phosphorylation was determined by Western blotting, MLC kinase (MLCK) activity was measured by incorporation of $^{32}Pi$ into MLC, and changes in muscle tension were recorded isometrically. Results: ET-1 induced inositol phosphates production, MAP kinase phosphorylation, MLC phosphorylation and contraction in a concentration-dependent manner with EC50 values of 71, 8, 6 and 25 nM, respectively. ET-1-induced MAP kinase phosphorylation, MLC phosphorylation and contraction were not significantly affected by nifedipine (1-60 μM), an L-type $Ca^{2+}$ channel blocker, or by LOE 908 (1-100 μM), a blocker of $Ca^{2+}$-permeable nonselective cation channels. However, SKF96365, a receptor-operated $Ca^{2+}$ channel (ROCC) blocker, inhibited MAP kinase phosphorylation, MLC phosphorylation and contraction in a concentration-dependent manner with IC50 values of 28, 30 and 42 μM, respectively. 2-APB, a store-operated $Ca^{2+}$ channel (SOCC) blocker, inhibited ET-1-induced MLC phosphorylation and contraction in a concentration-dependent manner with IC50 values of 12.7 and 19 μM, respectively, but was without effect on MAP kinase phosphorylation. Conclusion: The present study demonstrated that in rabbit iris (a) ET-1, through the ETA receptor, stimulates MAP kinase phosphorylation, MLC phosphorylation and contraction in a concentration-dependent manner, (b) that these $Ca^{2+}$-dependent functional responses are not significantly affected by nifedipine or LOE908, and (c) that ET-1- induced MLC phosphorylation and contraction are inhibited by SKF96365 and 2-APB, suggesting that these effects are mainly due to store-and/or receptor $Ca^{2+}$ entry.
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O-07

**Endothelin-1 induces ß1Pix translocation and Cdc42 activation via protein kinase A-dependent pathway**

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Pak-interacting exchange factor (Pix), a Rho family guanine nucleotide exchange factor (GEF), has been shown to co-localize with Pak and form activated Cdc42- and Rac1-driven focal complexes. In this study we present evidence that, treatment of human mesangial cells (HMC) with endothelin 1 (ET-1), forskolin, or with the cAMP analog 8-Br-cAMP, activated the GTP loading of Cdc42. Transient expression of constitutively active Gas also stimulated Cdc42. Over-expression of ß1Pix enhanced ET-1-induced Cdc42 activation, whereas the expression of ß1Pix SH3m(W43K) and ß1PixDHm(L238R, L239S) decreased ET-1-induced Cdc42 activation. Furthermore, ET-1 stimulation induced ß1Pix translocation to focal complexes. Interestingly, pretreatment of HMC with PKA inhibitors blocked both Cdc42 activation and ß1Pix translocation induced by ET-1, indicating the involvement of the PKA pathway. Through site-directed mutagenesis studies of consensus PKA phosphorylation sites and in vitro PKA kinase assay, we show that ß1Pix is phosphorylated by PKA. Using purified recombinant ß1Pix(wt) and ß1Pix mutants, we identify Ser516 and Thr526 as the major phosphorylation sites by PKA. ß1Pix(S516A/T526A) blocks ß1Pix translocation and Cdc42 activation. In conclusion, our results provide evidence that stimulation of PKA pathway by ET-1 or cAMP analog results in ß1Pix phosphorylation, which in turn controls ß1Pix translocation to focal complexes and Cdc42 activation.

O-08

**Transforming growth factor-ß-receptor signaling in endothelial cells. Requirements for the induction of the human ET-1 gene**

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Expression of the endothelin-1 (ET-1) gene is subject to complex regulation by numerous factors, among which transforming growth factor-ß (TGF-ß) is one of the most important. Our group has recently characterized the mechanism by which TGF-ß induces ET-1 expression in vascular endothelial cells. TGF-ß action is based on the activation of the Smad signaling pathway and the cooperation between Smad and AP-1 transcription factors at specific binding sites within the ET-1 promoter. TGF-ß signaling is initiated by binding of the cytokine to a heteromeric complex of type I and type II receptors. Most cell types contain a type I receptor known as ALK5. However, endothelial cells are unique as they coexpress an additional type I receptor named ALK1. These forms do not constitute redundant receptors with the same function, but they actually activate different Smad-mediated expression programs. TGF-ß/ALK5/Smad3 pathway is associated to a mature endothelium as it leads to inhibition of cell migration/proliferation, whereas TGF-ßALK1/Smad5 activates both processes and is more related to the angiogenic state. The aim of the present study was to investigate which TGF-ß receptor subtype leads to the activation of the ET-1 gene, i.e. the effect of ALK1 and ALK5 on ET-1 expression. For that purpose, we performed transient transfection experiments in bovine aortic endothelial cells using a human ET-1 promoter fused to a luciferase reporter and overexpressing wild type, constitutively active, and kinase-deficient forms of ALK1 and ALK5. Wild type and constitutively active ALK5 potentiated TGF-ß-induced and basal promoter activity, respectively; whereas ALK1 forms were with little or no effect. In the same way, kinase-deficient ALK5 was able to decrease activity up to 50% (ALK1 had no effect). Our preliminary experiments show that TGF-ß induces ET-1 expression preferentially through the activation of the ALK5 receptor. These findings may suggest that the TGF-ß/ET-1 signaling pathway may be related to the maturation state of the endothelium. Further experiments will be required to confirm this particular hypothesis and to evaluate its physiological relevance.

O-09

**Endothelin-1 activates NADPH oxidase in pulmonary arterial smooth muscle cells via a PI3 kinase mediated phosphorylation and translocation of the p47$^{phox}$ subunit**

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Our recent studies have identified endothelin-1 (ET-1) mediated generation of reactive oxygen species (ROS) as important mediators in the proliferation smooth muscle cells (SMC) within the pulmonary circulation. Our data also indicate that ROS are increased via an ETA receptor-mediated activation of the NADPH oxidase complex. However, the signaling molecules involved in this process have not been identified. Thus, in this study we have investigated the early events in ET-1-mediated ROS production using pulmonary artery SMC (PASMC) and focused on the potential role of PI3 kinase in linking the ETA receptor with NADPH activation and ROS generation. We found that ROS production in PASMC, estimated using dihydroethidium (DHE) oxidation, was increased following incubation with ET-1 and this could be blocked in cells pre-treated with the PI3 kinase inhibitor wortmannin. Further, we found that the exposure of PASMC to ET-1 resulted in an increase in PI3 kinase activity that was again sensitive to inhibition by wortmannin. To elucidate how PI3 kinase was exerting its effect on FPASMC ROS generation we focused on the p47$^{phox}$ subunit of the NADPH oxidase complex. Our data demonstrated that ET-1 produced an increase in Serine phosphorylation of the p47$^{phox}$ subunit and that this was associated with an increase in the translocation of p47$^{phox}$ to the plasma membrane. Further both phosphorylation and translocation of p47$^{phox}$ were reduced when PI3 kinase activity was inhibited. In addition, we used transient transfection of a p47$^{phox}$-GFP construct to confirm the PI3 kinase dependency of the p47$^{phox}$ translocation to the plasma membrane. Finally, we found that the activity of the NADPH oxidase complex, as determined by the rate of NADPH consumption, was increased in ET-1 treated cells and this was reduced by PI3 kinase inhibition. In conclusion, our data indicate that PI3 kinase is a key signaling molecule in the activation of NADPH oxidase in ET-1 stimulated FPASMC and identify the phosphorylation and translocation of the p47$^{phox}$subunit as the mechanism by which the NADPH oxidase complex is regulated.

O-10

**Molecular Mechanisms Controlling Activation of Rap1 by Endothelin-1 in Human Mesangial Cells**

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Endothelin-1 (ET-1) plays important role in the development of different types of renal disease. Calcium-regulated non-receptor Proline-rich Tyrosine Kinase 2 (Pyk2) is a critical mediator of ET-1 signaling in human glomerular mesangial cells (GMC). The aim of current study was to identify which small G-proteins serve as downstream effectors of Pyk2 and what molecular mechanism mediate ET-1-induced changes in GMC. Cells were infected by adenoviruses, encoding either Green Fluorescent Protein (control), or dominant interfering Pyk2 construct, termed Calcium Regulated Non Kinase (CRNK). Western blot (WB) analysis was used to verify expression of introduced constructs and to measure changes in activation of relevant signaling pathways. Endogenous Pyk2 activation was evaluated by WB with phospho-specific Tyr 402 antibody. Rap1 and RhoA activation was measured by affinity precipitation of activated GTP-bound form of small GTPases with fusion proteins of the Ral GDS-Rap binding domain and the Rho binding domain of Rhotekin, respectively, followed by WB with anti-Rap1 and anti-RhoA antibodies. ET-1 signaling via ETA receptor resulted in significant increase of Pyk2 autophosphorylation accompanied by the GTP-loading of Rap1 and RhoA. Adenovirus mediated transfer of CRNK into human GMC inhibited ET-1 induced autophosphorylation of endogenous Pyk2 and diminished activation of Rap1, but not RhoA. As demonstrated by co-immunoprecipitation followed by WB in control GMC after ET-1 stimulation, the mechanism linking Pyk2 and Rap1 included 1) increased p130Cas association with phosphorylated Pyk2; 2) augmented p130Cas Y165 and Y249 phosphorylation; 3) enhanced p130Cas-Crk complex formation. Inhibition of endogenous Pyk2 activation by CRNK expression prevented p130Cas phosphorylation and attenuated p130Cas association with Crk. C3G role in ET-1-induced Rap1 activation is under investigation using siRNA technique. Our data suggest that ET-1 stimulated GTPase Rap1 (but neither RhoA, nor Ras) by mechanism involving Pyk2 activation and recruitment of Crk/C3G complex to p130Cas in GMC.

O-11

**Signaling Pathways Regulating ICAM-1 Expression by Endothelin-1: Comparison with Interleukin-1ß in normal and scleroderma dermal fibroblasts**

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Endothelin-1 (ET-1), acting as a cytokine, is a mediator of fibrotic and inflammatory diseases including scleroderma. In addition to modulating vascular tone and extracellular matrix turnover, ET-1 up-regulates intracellular adhesion molecule-1 (ICAM-1), which is key to cell:cell/cell:matrix adhesions and leukocyte infiltration. These studies delineate the signal transduction pathways utilized by ET-1 and compares them with those adopted by interleukin-1ß (IL-1ß) in normal (NDF) and scleroderma dermal fibroblasts (SDF). Protein expression induced by ET-1 and IL-1ß on NDF, with or without signaling inhibitors, was detected via ELISA. mRNA levels were analyzed via lightcycler-PCR. Analysis of the ICAM-1 promoter region was achieved via transfection of deletion constructs into human dermal fibroblasts. Expression of PKCδ and ε protein in NDF and SDF was determined via western blot. In NDF ET-1 induces ICAM-1 mRNA and surface protein expression in a dose- and time-dependent manner via both receptor subtypes, ETA and ETB; bosentan abolishes the ET-1 response. MEK, but not PI-3 kinase or p38 MAPK is involved in the signaling cascade. Key to the cascade is activation of NFκB, achieved by ligation of either receptor subtype. IL-1ß signaling requires NFκB and MEK activation, along with activation of PKCδ. ET-1 and IL-1ß each utilize the same ICAM-1 promoter region, and the same NFκB site at -157bp. Responses to ET-1 and IL-1ß differ in SDF, with the sensitivity of ICAM-1 to ET-1 decreasing and IL-1ß responses remaining intact - yet the signaling pathways are very similar. However, we report altered expression of PKCδ and PKC ε in SDF. We conclude that differences in sensitivity to the two cytokines in SDF may be explained by altered expression of the PKC isoforms and cytokine receptors.

O-12

**Effects of hypoxia and inflammatory cytokines on endothelin-1 expression in T98G glioblastoma cells**

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Endothelin-1 (ET-1) is produced and secreted by glial cells and glioblastomas. We have previously shown that ET-1 was produced by T98G glioblastoma cells, and its production was increased by cytokines, such as tumor necrosis factor-alpha (TNF-alpha) and interleukin-1beta (IL-1b) (J Cardiovasc Pharmacol 2000; 36(5 Suppl 1):S390-2). ET-1 expression is known to be induced by hypoxia. We wished to clarify the effects of cytokines and hypoxia on ET-1 expression in T98G cells. Expression of ET-1 mRNA was examined by Northern blot analysis and immunoreactive (IR-) ET-1 levels in the medium were measured by radioimmunoassay in T98G glioblastoma cells cultured with cytokines (interferon-gamma 100 U/ml, TNF-alpha 20 ng/ml and IL-1b 10 ng/ml) under normoxia or hypoxia (1% O2). Northern blot analysis showed that TNF-alpha or a combination of three cytokines increased ET-1 mRNA expression in T98G cells and the increase was remarkable under hypoxia. IR-ET-1 levels in the medium were increased by treatment of TNF-alpha, IL-1b or a combination of three cytokines, but were decreased by interferon-gamma. Hypoxia, however, did not affect IR-ET-1 levels in the medium of T98G cells except the treatment of a combination of three cytokines. IR-ET-1 levels in the medium were rather decreased under hypoxia in the treatment of a combination of three cytokines. These findings indicate that hypoxia induces ET-1 mRNA expression, but IR-ET-1 levels in the medium do not reflect this induction. Hypoxia may affect the secretion of ET-1 from T98G cells or the consumption of the secreted ET-1.

O-13

**Upregulation of endothelin-converting enzyme (ECE)-1 in differentiation-induced human monocytic cells**

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Proteolytic processing of inactive big endothelins by endothelin-converting enzymes (ECEs) is essential for the synthesis of the biologically active peptide. Previously, a role for endothelin in the progression of atherosclerosis has been suggested because of increased ECE-1 expression in macrophages invading the atherosclerotic plaque in human coronary arteries. We, therefore, analyzed the regulation of ECE-1 mRNA expression in differentiation-induced human HL-60 monocytic cells. Using the phorbol myristate acetate (PMA) as differentiation-inducing agent, we found a dose-dependent increase in ECE-1 mRNA levels which reached its maximum at 300 nM PMA. Compared to DMSO-treated control cells, the ECE-1 mRNA levels were significantly increased to about 180% at 24 hours in the HL-60 subpopulation which became adherent upon PMA stimulation. In HL-60 cells which remained in the supernatant, the increase of ECE-1 mRNA was less pronounced (about 130% of controls). Pretreatment with the transcriptional inhibitor actinomycin D completely inhibited the increase in ECE-1 mRNA expression. Using RNase protection and RT/PCR assays, we found that the PMA-induced increase in ECE-1 mRNA levels was mainly based on a strong induction of ECE-1d isoform (along with a minor induction of ECE-1b), whereas the expression of ECE-1c and ECE-1a isoforms was unchanged. Using 5' RACE, the preferred initiation sites of transcriptional induction of ECE-1d could be determined. Association of induction of ECE-1d expression with the differentiation state was further confirmed by the detection of constitutive ECE-1d mRNA expression in peripheral blood monocytes. Our data demonstrate an association of increased ECE-1 expression with monocyte/ macrophage differentiation which may be of pathophysiological significance in human atherosclerosis.

O-14

**Medial Vascular Calcification (Elastocalcinosis) As A New Pathological Paradigm Involving Endothelin**

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Matrix Gla protein (MGP) knock-out mice develop massive medial arterial calcification. This can be reproduced in rats, albeit to a lesser extent, by administering warfarin, which inhibits the gamma-carboxylation of MGP and leads to medial elastocalcinosis (MEC) and stiffening of large arteries. This model mimics MEC and stiffening of large arteries observed during normal aging, and thus was used to test the effect of an endothelin (ET) receptor antagonist (ETRA, darusentan) in prevention and regression protocols. In addition, we used a rat vascular smooth muscle cell (VSMC) model of matrix calcification to study the involvement of ET receptors. Rat VSMC were cultured in growth medium (DMEM, 10% calf serum (CS), 1.2 mM Pi, 10 uM warfarin) in 6-well plates. At confluence (defined as day 0), the cells were switched to the calcification medium (DMEM, 5% CS, 1.6 mM Pi, 10 uM warfarin). In the in vivo model, darusentan completely prevented warfarin-induced MEC. When warfarin was started 4 weeks before darusentan, the ETRA regressed the accumulation of calcium in the vascular wall towards control values ($p<0.05$). As revealed by immunohistochemistry, ET was overexpressed throughout the media in rats treated with warfarin. Central pulse pressure, an index of arterial stiffness, was also reduced by darusentan. In the in vitro model, increasing Pi from 1.2 to 1.6 mM in the presence of warfarin elevated the extracellular calcium content from 4.5+/-0.5 to 13.0+/-1.1 ug/mg of protein. This elevation of extracellular calcification was prevented by the ETARA BQ-123 (7.7+/-0.6 ug/mg, $p<0.05$ at 1 uM), but not by the ETBRA BQ-788 (12.4+/-1.2 ug/mg at 1 uM). Taken together, our results suggest that ET is involved in the initiation as well as in the maintenance of MEC. The models developed here suggest a new therapeutic opportunity for ETRAs, and will allow the further investigation of signaling pathways involved in elastocalcinosis. (Supported by CIHR).

O-15

**Endothelin-1 induces endothelial dysfunction and interleukin-6 release in humans in vivo, inhibition by pre-loading of vitamin C**

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Background: Endothelin-1 (ET-1) may contribute to endothelial dysfunction and may induce interleukin-6 (IL-6) formation in vitro. Since inflammation of the vessel wall is a hallmark of atherosclerosis the objective was to test whether ET-1 induces IL-6 release in humans in vivo and whether this effect could be blocked by an anti-oxidant. Methods: Endothelium-dependent (EDV) and endothelium-independent vasodilatation were determined in 16 young healthy males during measurement of forearm blood flow (FBF) with venous occlusion plethysmography. Results: A 30 min infusion of ET-1 (20 pmol/min) into the brachial artery decreased EDV ($P<0.001$) and evoked an increase in deep venous IL-6 levels (0.96±0.14 vs 1.40±0.15 ng/mL; n=16; $P<0.001$) in the ipsi-lateral arm whereas there were no changes in IL-6 levels in the artery or venous levels of the contra-lateral arm. Administration of vitamin C (24 mg/min) following ET-1 infusion did not restore EDV. However, pre-loading of vitamin C before infusion of ET-1 inhibited the decrease in EDV and prevented any significant increase in deep venous levels of IL-6 in the ipsi-lateral arm (1.20±0.28 vs 1.29±0.27 ng/mL; n=11; $P=0.57$). Furthermore, infusion of a control vasoconstrictor substance, noradrenaline (80 ng/min) for 30 min did not evoke any significant changes in IL-6 levels. Conclusions: ET-1 induces reduced endothelium-dependent vasodilatation and evokes increase in IL-6 levels in humans in vivo. These effects could be inhibited by pre-loading of the anti-oxidant vitamin C. This suggests that the mechanism by which ET-1 induces endothelial dysfunction may in part be due to raised IL-6 levels and increased oxidative stress.

O-16

**Endothelin-1 Stimulates Pathologic Bone Formation through Repression of the Negative Wnt Regulator Dickkopf Homolog 1 (Dkk1)**

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Endothelin-1 (ET-1) stimulates osteoblast activity and is an important mediator of osteoblastic metastases in breast and prostate cancer. Tumor cells that secrete ET-1 into the local bone microenvironment activate the osteoblast through the endothelin A receptor (ETAR). This results in osteoblast proliferation and new bone formation. Blockade of ETAR inhibits the osteoblastic response and prevents the development of osteoblastic bone metastases in a mouse model. Moreover, ETAR antagonists reduce the progression of bone metastases in men with metastatic prostate cancer. To understand the role of ET-1 in bone metastases, secreted targets of ET-1 action in osteoblasts with the potential to enhance bone metastases were identified by gene microarray analysis and confirmed by real-time RT PCR. The transcript for the Wnt pathway inhibitor dickkopf homolog 1 (Dkk1) showed a significant decrease with ET-1 treatment at 24 hours. Dkk1 is implicated in suppressed bone formation of multiple myeloma. We hypothesized that the opposite may occur in osteoblastic disease, where ET-1 stimulates osteoblast activity by decreasing autocrine production of the negative regulator Dkk1. To test this hypothesis, the murine neonatal calvarial organ culture system was utilized to study the function of Dkk1. Recombinant Dkk1 blocked ET-1-mediated increases in osteoblast numbers and new bone formation but did not suppress basal levels of new bone formation or osteoblast numbers. Dkk1 also blocked the actions of two other potent stimulators of osteoblast activity, insulin and BMP2. Calvarial organ cultures treated with human anti-Dkk1 antibodies showed a marked increase in osteoblast numbers and new bone formation. Collectively, these data suggest that ET-1 mediates osteoblast activity via the Wnt signaling pathway. Previous work indicates that increased Dkk1 is responsible for the suppressed bone formation of myeloma. Our data indicate that the opposite may be responsible for the pathologic bone formation of osteoblastic metastases via tumor ET-1 suppression of Dkk1.

O-17

**ET-1 mediates O2- production and vasoconstriction through NADPH oxidase and NOS uncoupling**

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Experiments were designed to determine whether ET activation of NADPH oxidase and/or uncoupled NO synthase (NOS) are sources of O2- and contribute to vasoconstriction. Rat aortic rings were incubated with ET (0.001 μM to 1.0 μM) or vehicle in the presence and absence of superoxide dismutase (SOD; 300 U/ml), apocynin (NADPH oxidase inhibitor; 300 μM), ebselen (glutathione peroxidase mimetic; 50 μM), L-NAME (NOS inhibitor; 100 μM), tetrahydrobiopterin (BH4; 3 μM), or selective ET receptor antagonists (100 nM BQ-123; 100 nM A-192621). O2- production was monitored by dihydroethidine staining and/or lucigenin chemiluminescence. ET (0.1 and 1.0 μM) increased O2- production compared to vehicle ($p<0.05$). SOD, apocynin, and ebselen inhibited the ET induced increase in O2- in intact and endothelium-denuded aorta ($p<0.05$). L-NAME and BH4 also inhibited the ET induced increase in O2- in endothelium-intact tissue ($p<0.05$), while these two compounds had no effect on ET induced O2- in endothelium-denuded aorta. Preincubation of aortic tissue with BQ-123 or A-192621 had no effect on ET induced O2-, however preincubation with both antagonists inhibited the ET induced increase in O2- production ($p<0.05$). Additional experiments were designed to determine whether ET induced NOS uncoupling and/or activation of NADPH oxidase contribute to vasoconstriction. Rat aortic rings were incubated with ET (1.0 μM) or vehicle in the presence or absence of sepiapterin (BH4 synthesis substrate; 100 μM) or apocynin (300 μM). Rings were mounted on a wire myograph to determine isometric force generation in response to increasing KCl concentrations. ET increased the contractile response to KCl compared to vehicle treated rings ($p<0.05$). Treatment with either sepiapterin or apocynin attenuated the ET mediated increase in maximal vasoconstriction ($p<0.05$; $p<0.005$, respectively) with no effect of sepiapterin or apocynin alone. These data support the hypothesis that ET mediates increased vascular tone, in part, through ETA and ETB receptor activation of O2- production from NADPH oxidase and/or NOS uncoupling.

O-18

**Intact Splanchnic Sympathetic Innervation is Required for Hypertension during Chronic ETB Receptor Activation in Conscious Rats**

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Five-day iv infusion of ETB receptor (ETBR) agonist sarafotoxin 6c (S6c) into normal rats causes a significant increase in arterial pressure. In vitro studies show that S6c relaxes most arteries but constrict veins. We have hypothesized that venoconstriction is an important mechanism of S6c-induced hypertension. Veins hold about 70% of the total blood volume in the circulation, the majority of which is in the veins of the splanchnic circulation. Sympathetic nerves provide the dominant vasoconstrictor input to splanchnic veins. There is evidence that S6c may increase sympathetic input to the splanchnic vasculature. The goal of our study was to explore the role of the splanchnic sympathetic innervation in S6c-induced hypertension. We hypothesized that chronic activation of ETBRs induces hypertension in part by increasing sympathetic input to the splanchnic vasculature. In one group of rats, most sympathetic innervation to the splanchnic region was removed by surgical excision of the celiac and superior mesenteric ganglia (celiac ganglionectomy; CGX) (n=8), while the nerves stayed intact in control rats (n=8). All remaining experiments were conducted in conscious rats chronically instrumented with arterial and venous catheters and housed in metabolism cages. Rats received saline for 2 days (control period), then S6c (5 pmol/kg/min) for 5 days (infusion period), followed by a 3-day saline infusion. Blood pressure, heart rate, water intake, and urine volume were recorded daily. Blood pressures during the control period were not different in control and CGX rats. Mean arterial pressure (average of 127 mmHg) increased significantly in controls during S6c infusion when compared to the pressure on the 2nd control day (105 mmHg). In CGXs, mean arterial pressures were significantly higher only during the first 2 days of infusion (116 and 116 mmHg) when compared to the 2nd control day (104 mmHg). There were no significant differences in heart rate or water balance between the two groups of rats. These data indicate that the splanchnic sympathetic innervation plays a critical part in hypertension produced by chronic activation of ETBRs in rats.

O-19

**Combined Effects of Age and Salt-Loading on Endothelial Function, Vascular Remodeling and Oxidative Stress in a Murine Transgenic Model of Endothelial Cell Human Endothelin-1 Overexpression**

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We previously showed that young transgenic (TG) mice overexpressing prepro-endothelin (ET)-1 specifically in endothelial cells had increased tissue ET-1, endothelial dysfunction, increased vascular NAD(P)H oxidase activity and inflammation in mesenteric resistance arteries but no significant elevation of blood pressure (BP) compared to non-transgenic (WT) littermates. We now investigated effects of age and salt on vascular structure, function and oxidative stress in these TG mice. Ten-month old male TG and WT mice were studied with/without salt loading (4% NaCl) for 4 weeks. Systolic BP (SBP) was measured by tail-cuff, vascular reactivity of mesenteric resistance artery was studied on a pressurized myograph, and vascular NAD(P)H oxidase activity by lucigenin chemiluminescence. Salt-loaded TG mice had slightly but significantly increased SBP compared to non salt-loaded TG mice (106.0±4.2 vs. 100.1±3.1, respectively). Media/lumen ratio (%) was higher ($P<0.01$) in TG (7.8±0.4) compared to WT (6.0±0.3) and further increased by salt loading (9.2±0.7) whereas no differences were seen in cross-sectional area. Maximal relaxation to acetylcholine (Ach) was significantly impaired in TG mice ± salt excess compared to WT. The maximal relaxation to Ach after L-NAME was reduced ($P<0.001$) in TG (22.7±4.5) and in salt-loaded TG (7.9±1.2) compared to WT (61.9±5.8). This inhibition was partially restored by vitamin C pre-incubation but did not reach levels attained in WT mice. Response to sodium nitroprusside was similar in all groups. The contracting response to ET-1 (%) was totally absent in TG ± salt-loading compared to WT (20.6±5.5). Vascular NAD(P)H oxidase was significantly increased in TG mice compared to WT mice and was unaffected by salt loading. In conclusion, we found that aged TG mice with endothelial cell ET-1 overexpression have structural alterations of mesenteric resistance vessels, endothelial dysfunction due to reduced nitric oxide bioavailability, reduced responsiveness to ET-1 and enhanced vascular NAD(P)H oxidase activity some of which were exacerbated by salt loading.

O-20

**Targeted Disruption of the Endothelial Cell Endothelin B Receptor does not affect the BP Response to Salt**

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Rescued endothelin B ($ET_B$) receptor knockout (KO) mice and mice featuring renal inner medullary (IMCD) cell-specific knockout of ET-1 develop hypertension in response to increasing dietary salt. The cellular target for IMCD-derived ET-1 that mediates the natriuretic response has not been determined. We hypothesized that medullary endothelial cell (EC) $ET_B$ receptors are likely targets for IMCD-derived ET-1 and may determine salt sensitivity. Mice with loxP sites flanking $ET_B$ coding regions (FF/--) were crossed with Tie2-Cre mice to produce EC-specific $ET_B$ receptor KO mice (FF/Tie2-Cre). Mean arterial pressure (MAP) was continuously measured via telemetry implants in male FF/Tie2-Cre and FF/-- (control) mice. During week 1, the mice were fed a standard (0.8%) NaCl chow. In week 2 they were given low (0.08%) NaCl diet and in week 3 a high (2.5%) NaCl diet. Additionally during week 3 their drinking water was replaced with normal saline *ad libitum*. MAP of FF/Tie2-Cre mice [136.4 ± 4.2 mmHg] were not significantly different to that of FF/-- controls [133.2 ± 5 mmHg; n=6] during high salt feeding. Similarly, there were no differences in MAP between FF/Tie2 mice and controls during either normal salt diet [FF/Tie2-Cre: 128.9 ± 2.8 mmHg; FF/--: 129.6 ± 2.7 mmHg; n=13] or low salt diet [FF/Tie2-Cre: 123.6 ± 2.8 mmHg; FF/--: 124.5 ± 1.6 mmHg; n=13]. Treatment of FF/Tie2-Cre mice with the $ET_B$ antagonist A-192621 (30 $mg.kg^{-1}$), during 8% NaCl diet, resulted in a modest rise in MAP (145.2 ± 3.7 mmHg to 154.0 ± 2.9 mmHg; n=6; $p<0.05$) that was not significantly different to that seen in controls. In contrast to previous models of $ET_B$ deficiency and IMCD specific ET-1 KO, EC-specific $ET_B$ KO mice do not demonstrate raised BP compared to controls during high salt feeding. These observations are consistent with a role in BP regulation and responses to salt of a non-endothelial $ET_B$ receptor, such as that expressed on IMCD cells.

O-21

**Chronic high-sodium diet increases aortic wall ET1 protein and mRNA in a blood-pressure-independent fashion in Wistar-Kyoto rats**

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Inbred Wistar-Kyoto (WKY) rats exhibit mild hypertension when chronically fed a high-sodium diet (mean arterial pressure, MAP, increased by 10 mmHg). High sodium intake modulates renal ET1 production and vascular ET1 is upregulated in several forms of salt-induced hypertension. Therefore, we hypothesized that dietary sodium may regulate vascular ET1 production independent of hemodynamic changes. We investigated the effect of chronic high-sodium (HNa) diet with and without ETA blockade on circulating and aortic ET1 protein levels as well as aortic expression of ET1 and ETA mRNA. Comparing WKY rats fed a sodium-deficient (NaD) diet to those fed HNa diet for three weeks, we found that circulating ET1 protein levels were not affected by dietary sodium but aortic wall ET1 protein is significantly increased in response to HNa diet (331±43 pg/g tissue for NaD diet vs. 557 ±34 pg/gm tissue for HNa diet, $p=0.002$, n=6 each group). HNa diet also increases aortic wall ET1 mRNA levels by 40% as determined by quantitative RT-PCR. High dietary sodium tends to increase ETA transcripts in the aorta ($p=0.07$). To address the possibility that these changes were secondary to increased arterial pressures, we compared WKY rats chronically treated with the ETA-selective antagonist, ABT-672, while receiving either NaD diet or HNa diet. There were no differences in arterial blood pressure (MAP 89±1 mmHg on the NaD diet and 90±3 on the HNa diet) or heart rate. However, aortic wall ET1 protein levels are 4 fold higher in the HNa diet group. Interestingly, dietary sodium and ETA-blockade both, independently increase aortic wall ET1 mRNA (HNa diet increases levels $<1.5$ fold, $p<0.05$ by ANOVA, and ABT-627 treatment increases levels 2 fold, $p<0.01$ by ANOVA). HNa diet tends to increase ($p=0.07$) while chronic ETA blockade reduces aortic ETA transcripts (6-10 fold, $p<0.05$). Conclusions: The expression of ET1 mRNA by the aortic wall is increased in response to chronic high dietary sodium in WKY rats in the absence of changes in arterial blood pressure. This work was supported by NIH R01 HL64720.

O-22

**CGS 35601, a single molecule triple vasopeptidase inhibitor reduced hypertension in chronically instrumented conscious and unrestrained Dahl salt-sensitive rats on a high-salt diet**

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We previously reported that CGS 35601, a potent triple inhibitor of angiotensin converting enzyme (ACE), neutral endopeptidase (NEP), and endothelin converting enzyme-1 (ECE-1), completely normalized mean arterial blood pressure (MABP) in 36 weeks-old SHR, a normal renin model. The aim of the present study was to determine the effects of this triple vasopeptidase inhibitor (VPI) on the hemodynamic and biochemical profiles of instrumented, conscious and unrestrained Dahl salt-sensitive (DSS) rats. Methods: Diet-gene-prone hypertensive DSS rats (male, 385±10 g, n=8/gr) were fed a normal (Gr. 1) or high-salt (HS) diet (Gr. 2-3; NaCl: 8% in food and 1% in drinking water) for 6 weeks and then instrumented with a carotid catheter and placed individually in a metabolic cage for 30 days. The hemodynamic, hematological and biochemical profiles were assessed daily. The different treatment started after a 7-day stabilization period : Gr. 1 under normal diet (+ vehicle, 250 µl/h), Gr. 2 with HS diet (+ vehicle, 250 µl/h) and Gr. 3 with HS diet + CGS 35601 (0.1, 1 and 5 mg/kg/d x 6d each, i.a. constant infusion) followed by a 5d wash out period. Results: CGS 35601 dose-dependently reduced MABP toward baseline observed in DSS under normal diet or Wistar rats. Heart rate was unaffected. Circulating plasma concentrations of peptidic vasoconstrictors and vasodilators were modulated according to established and previously demonstrated concept related to VPI. The hemodynamic profile retuned to normal during the washout period. Pharmacotoxicology (>40 plasma and urinary parameters, mostly renal and hepatic) was normal in all groups. Conclusion: This novel single molecule triple VPI is a potent and effective anti-hypertensive agent with a safe short-term profile that may be of interest for treating hypertension and other cardiovascular diseases. Other hypertensive rat models are being tested. Support: FICQ, FMCQ, FRSQ (BB Scholar), IPS Pharma, Novartis.

O-23

**Dominant-negative PKC-ε inhibits ET-1-induced positive inotropy in adult ventricular myocyte**

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ET-1 plays important roles in several cardiac processes including inotropy, hypertrophy, and apoptosis. The acute effects of ET-1 on cardiac contractile responses have been linked to activation of protein kinase C (PKC). However, the specific PKC isoform mediating acute ET-1 response has not been clearly defined. Three major PKC isozymes are expressed in a normal mammalian heart; PKC-α, -δ, and -ε. In this study, we examined the hypothesis that PKC-ε mediates positive inotropy by ET-1 stimulation using adenovirus-mediated overexpression of dominant-negative (dn) PKC-ε. To monitor localization patterns and expression levels, dn constructs were fused to yellow fluorescent protein (YFP). YFP fused dn-PKC mutant allows PKC function to be inhibited in an isoform-specific and concentration-dependent manner. Double point mutations were introduced to generate dn-PKC mutants; one in the ATP binding site of the enzyme and one in the pseudosubstrate site. Dn-PKC mutants translocate constituitively because the mutation in the pseudosubstrate site promotes translocation and anchoring regardless of the presence of activators. Dn-PKC-ε-YFP localized to the sarcolemma and perinuclear region in adult rat ventricular myocytes in a short term culture (<40hrs). Myocyte contractile function was assessed by monitoring twitch shortening with field stimulation at 0.5 Hz, 22°C. Myocytes expressing GFP alone or wild-type PKC- ε responded to ET-1 with positive inotropism (≈50% increase in twitch amplitude). However, myocytes expressing dn-PKC-ε-YFP showed little or no positive inotropic response to ET-1. The role of another novel PKC isoform expressed in the heart, PKC- δ was tested in comparison with PKC-ε. Dn-PKC-δ-YFP did not abolish ET-1 mediated positive inotropic responses, despite robust expression and accumulation at transverse-tubules and the perinuclear region. The results demonstrate for the first time that PKC-ε is the isoform mediating ET-1-induced positive inotropy in adult ventricular myocytes.

O-24

**Blood pressure-Independent reversal of upregulated renin-angiotensin system by endothelin antagonism in the hypertrophied heart is specific in stroke prone spontaneously hypertensive rat (SHR-SP), not in SHR**

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It is unknown whether endothelin (ET)-1 affects the production of angiotensin II (AII) in the heart. We hypothesized that ET-1 may act on the upstream of renin-angiotensin system (RAS) in the hypertrophied heart. The aim was to investigate the effects of the ET blockade on the expression of the various components of RAS including AII in the heart of stroke prone spontaneously hypertensive rats (SHR-SP). SHR-SP were treated for 3 months with SB209670 (ET-A/-B dual receptor antagonist), or saline (vehicle) starting from the prehypertensive stage (6 weeks old). When compared with age-matched control WKY rats, peptide level of AII (15-fold higher), both peptide and mRNA levels of renin, angiotensinogen, angiotensin converting enzyme (ACE) and AII type 1 receptor (AT1R) were significantly upregulated in the heart of vehicle-treated SHR-SP, whereas all these upregulated components of RAS were remarkably reversed by long-term ET antagonism. AII type 2 receptor (AT2R) was 30% downregulated in vehicle-treated SHR-SP, and was reversed by ET blockade. Moreover, the higher plasma activity of renin and ACE, and plasma level of AII in SHR-SP were reversed by ET antagonism. The Chymase system, the alternate pathway for AII production, was 60% upregulated in vehicle-treated SHR-SP, and was reversed by ET blockade. Marked cardiac hypertrophy and fibrosis at histological level in SHR-SP was ameliorated by ET antagonism, and left ventricular hypertrophy demonstrated by echocardiography in SHR-SP was also suppressed by ET blockade. It should be noted that similar treatment protocol in spontaneously hypertensive rats (SHR) could not reverse the upregulated RAS and cardiac hypertrophic changes although blood pressure was slightly reduced to the similar extent as in SHR-SP. Thus, these findings indicate that ET antagonism suppressed upregulated RAS in SHR-SP heart independently of blood pressure. Furthermore, it is suggested that ET system may act on the upstream of RAS in the heart, and that ET has some regulatory mechanisms on cardiac RAS in SHR-SP, not in SHR.

O-25

**Clinical trials of endothelin antagonists in heart failure: A question of dose?**

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Circulating endothelin (ET)-1 concentrations are substantially elevated and correlate both with the haemodynamic severity and NYHA class of patients with chronic heart failure (CHF). In preclinical studies, involving different models of experimental heart failure, both mixed and selective endothelin antagonists reduce cardiac pressures and increase cardiac output, as well as reducing survival. ET receptor antagonists impressively improve haemodynamics of patients with CHF without causing neurohormonal activation. However, recent clinical trials, such as the ENABLE and EARTH [1] studies, have shown neutral effects in terms of mortality and symptoms. ETB blockade, but not ETA antagonism, results in impaired clearance of ET-1 [2] and hence raised tissue and plasma ET-1 concentrations. ET antagonists are arbitrarily classified as ETA selective if they demonstrate more than 100-fold selectivity for the ETA over the ETB receptor [3]. However, even relatively ETA selective antagonists result in ETB receptor blockade [4] if given at a high enough dose. ETB antagonism has been shown to result in a deleterious effect in both healthy volunteers [5] and patients with vascular disease[6]. Treatment with ET antagonists in one study [1] was associated with elevated plasma concentrations of ET-1, and other studies have not been fully reported. We believe that, at the doses used in clinical trials, the ET antagonists used may all have exhibited significant ETB receptor blockade. Indeed, if the trials were repeated with a highly selective ETA antagonist, at appropriate doses, a benefit in mortality and morbidity might be found. Full publication of the other trials of ET antagonists in CHF will help address this and other reasons for failure of the concept.

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O-26

**An endothelin converting enzyme-1 inhibitor prevents cardiomyopathy in mice over-expressing Big-ET-1**

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Background: We generated mice with conditional cardiac over-expression of human big-endothelin-1 (ET-1) using $\alpha$-MHC-driven tetracycyline-responsive transactivator (tTA) and tTA-dependent ET-1 transgenes. This model is associated with an inflammatory cardiomyopathy characterized by contractile dysfunction leading to heart failure and death as early as 5 wks post-ET-1 induction. Attempts to rescue this phenotype with an ETA/ETB receptor antagonist led to only a partial response, possibly due to limitations in the ability of this agent to compete for binding in this model's high cardiac ET-1 levels and tight interactions between ET-1 and its receptors. Therefore, we hypothesized that inhibition of ET-1 production using an inhibitor of the endothelin converting enzyme-1 (ECE-1) would be a more effective treatment strategy. Objective: To prevent heart failure in tTA::big-ET-1 binary transgenic (BT) mice by inhibiting active ET-1 synthesis with ECE-1 inhibitor CGS 26303. Methods and Results: Osmotic mini-pumps delivering CGS 26303 (5 mg/kg/day) were implanted subcutaneously in BT and non-binary transgenic (NBT) controls at the time of ET-1 induction (8 wks of age). Invasive hemodynamic assessments were performed in BT (n=5) and NBT (n=4) mice after 28 days of treatment, under gas anesthesia using a Millar mikro-tip transducer. Peak arterial systolic and diastolic pressures, heart rate, and peak LV systolic, diastolic, end diastolic, and LV contractile parameters (peak +dP/dt) were measured. Preliminary data indicate that arterial and LV pressures were similar in BT and NBT mice. As well, there were no differences in measures of LV contractility between these two groups. Summary: Unlike untreated or ETA/ETB antagonist-treated historical controls, treatment with an ECE-1 inhibitor prevented hemodynamic deterioration at 4 wks of ET-1 over-expression. Conclusion: Abrogating ET-1 production by inhibiting ECE-1 activity prevented the phenotype observed in mice over-expressing big-ET-1. Longer treatment with CGS 26303 is required to determine whether this early hemodynamic improvement will prolong survival in this otherwise lethal model of ET-1-induced cardiomyopathy.

O-27

**Genetic inactivation of vascular endothelial endothelin-1 in mice is protective against angiotensin II-induced vascular remodeling and cardiac fibrosis, but not cardiac hypertrophy**

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Although it has been known that endothelial cell is the major site of production of Endothelin-1 (ET-1), the specific role of endothelial ET-1 and its interaction with other important peptide, such as Angiotensin-II (AII), in cardiovascular disease has not been explored. To test the hypothesis that vascular ET-1 contributes significantly to ATII-induced hypertension and cardiac damage, we performed subcutaneous ATII infusion on Vascular Endothelial Endothelin-1 Knock Out (VEETKO) mice. In these mice, the peptide level of ET-1 in major organ including the heart is reduced as much as 80%, providing a good model for studying the specific role of vascular endothelial ET-1. Animals were divided into 4 groups of treatment: 2 groups of ATII-infused mice (KO, n=7 and WT, n=9) and 2 groups of vehicle treated mice (KO, n=5 and WT, n=5), which served as control. Initial blood pressure (BP) of VEETKO mice was slightly but significantly lower than WT mice)(KO Vs. WT: 105.14+4.67 vs.116.14+7.99 mmHg, p:0.02). AII infusion caused a similar increase of BP between WT and VEETKO mice (143.25+5.96 vs. 141+7 mmHg, p= 0.66). Despite the similar increase in blood pressure aortic media/lumen ratio, an indicator of vascular remodeling, is markedly higher in WT mice. Area of interstitial and perivascular fibrosis caused by AII is significantly higher in WT mice than VEETKO mice. Supportively, left ventricular expression level of Collagen 1 and 3 increased significantly in AII-infused WT mice, while in VEETKO mice the expression level remained indistinguishable from that of Control mice. Although slightly lower in VEETKO mice, LV weight/body weight following AII infusion increased similarly in both group. A-II infusion also increased the expression level of ANP and BNP in both group, but it was still significantly lower in VEETKO mice. These findings indicate that vascular endothelial ET-1 is involved in AII-induced vascular remodeling and cardiac fibrosis, but not in AII-induced cardiac hypertrophy.

O-28

**Endogenous endothelin signaling is essential for the activated phenotype of lung fibroblasts in scleroderma**

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Purpose: Endothelin-1 expression is elevated in fibrotic disease. When added to normal lung fibroblasts, endothelin-1 (ET-1) induces the expression of pro-fibrotic genes. ET-1 is overproduced by lung scleroderma (FASSc) fibroblasts, but the contribution of endogenous ET-1 to the phenotype of lung fibroblasts is unknown. Methods: The impact of the dual ET receptor antagonist bosentan on the expression of genes in normal and FASSc lung fibroblasts (N=5) was assessed by Affymetrix gene chip, real-time polymerase chain reaction (RT-PCR) and Western blot analysis. In addition, the effect of bosentan on the phenotype of FASSc lung fibroblasts was assessed by functional assays. Results: Affymetrix gene array analysis revealed that FASSc lung fibroblasts overexpressed over 350 genes ($p<0.001$), of which approximately half depended on endogenous ET-1 signaling. RT-PCR and Western analysis confirmed that the overexpression of type I collagen, fibronectin and connective tissue growth factor by FASSc lung fibroblasts was blocked by bosentan ($p<0.05$). Similarly, elevated cell migration by FASSc lung fibroblasts was blocked by bosentan. Conclusion: Elevated endogenous ET-1 signaling contributes to the pro-fibrotic phenotype observed in FASSc lung fibroblasts. Dual ETA/ETB antagonism is likely to be of benefit in chronic fibrotic disease.

O-29

**Low dose of an inhaled endothelin A receptor antagonist in experimental acute lung injury: effects on ET-1 plasma concentration and pulmonary inflammation**

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Inhalation of endothelin A (ETA) receptor antagonists has been shown to improve gas exchange in experimental acute lung injury (ALI) but may induce side effects by increased circulating ET-1 levels. We investigated whether a low dose of an inhaled ETA receptor antagonist (LU-135252) improves gas exchange without effecting ET-1 plasma concentrations and lung injury in an animal model of ALI. Twenty-two piglets were examined in a prospective, randomized, controlled study. In anaesthetized animals, ALI was induced by surfactant depletion. Animals received either LU-135252 at a dose of 0.3 mg;kg$^{-1}$ over 20 minutes (*LU group*, n=11) or nebulization of saline buffer (*Controls*, n=11). In *LU group* arterial oxygenation and mean pulmonary artery pressure improved compared to *Controls* ($PaO_2$: 319±44 vs. 57±3 mmHg; MPAP: 32±2 vs. 41±2 mmHg, values at 6h after induction of ALI, $p<0.05$). Mean arterial pressure and cardiac output showed no significant changes between both groups. Endothelin-1 plasma concentrations increased from 0.96±0.06 after induction of ALI to maximum 1.17±0.09 fmol/ml 3h after ALI in *LU group* and did not differ significantly compared to *Controls* (1.21±0.08 fmol/ml). Regarding histological examination, we found no differences in total lung injury score between both groups. However, the *LU group* revealed significantly reduced interstitial inflammation and hemorrhage ($p<0.05$ vs. *Controls*). In this animal model of ALI, inhalation of LU-135252 at a dose of 0.3 mg;kg$^{-1}$ induced a significant and sustained improvement in gas exchange, while there were no changes in endothelin-1 plasma concentrations. Furthermore, our data indicate a trend towards decreased pulmonary inflammation in the group receiving the inhaled ETA receptor antagonist.

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**STRIDE-2 Trial: A placebo-controlled study for sitaxsentan in pulmonary arterial hypertension**

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Rationale: The first approved, oral treatment for PAH was bosentan (BOS), a twice-daily, oral endothelin receptor antagonist (ETRA) that blocks both the ETA and ETB receptors. Sitaxsentan (SITAX), a once daily, oral, ETA selective ETRA (ETA:ETB >6500:1), has a pharmacological profile distinct from BOS. STRIDE-2 was designed to evaluate the safety, efficacy and dose response of SITAX; an open label BOS arm was also included in the study design. Methods: STRIDE-2 was a multi-center, placebo-controlled study that randomized 246 patients (pts) 1:1:1:1 to placebo, SITAX 100 mg, SITAX 50 mg or open label BOS for 18 weeks. Pts were WHO Class II-IV with PAH (IPAH or related to CTD or CHD). The primary endpoint was distance walked in six minutes (6MW). Efficacy measures were double blind for SITAX and placebo; 6MW was third party blind for BOS treated pts. Results: Overall discontinuations (n) were placebo (11), SITAX 100mg (4), SITAX 50mg (8), and BOS (9). Placebo-subtracted 6MW treatment effects were 31.4m (p=0.03) for SITAX 100mg, 24.2m (p=ns) for SITAX 50mg, and 29.5m (p=0.05) for BOS. WHO functional class improvement was significant for SITAX 100mg (p=0.04). Liver function (LFT) abnormalities >3x ULN occurred in 6.5% of patients randomized to placebo, 3.2% for SITAX 100mg, 4.9% for SITAX 50mg, and 11.5% for BOS. Conclusion: SITAX 100mg improved 6MW and WHO functional class. SITAX 100mg was well tolerated with a low incidence of LFT abnormalities. SITAX 50mg was not an effective dose. Once daily SITAX 100mg appears to be a safe and effective therapy for the treatment of PAH.

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**Regulation of Endothelin-1 by Angiopoietin-1: Implications for Inflammation**

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Endothelin-1 (ET-1) is increasingly recognized as a pro-inflammatory mediator in various diseases, such as atherosclerosis and acute respiratory distress syndrome (ARDS). Angiopoietin-1 (Ang1), a ligand of the endothelial receptor Tie2, is an angiogenic factor that also inhibits endothelial apoptosis, reduces vascular leakage and suppresses the induction of inflammatory markers, suggesting that it has diverse vasoprotective, anti-inflammatory actions. Thus, we examined the effects of Ang1 on ET-1 production. **Methods:** Cultured human endothelial cells were treated with recombinant Ang1 with or without TNF$\alpha$ (100 U/mL). ET-1 release into the culture medium after 24h was determined by ELISA. Preproendothelin-1 (ppET-1) mRNA levels were measured by quantitative RT-PCR. Fisher344 rats were subjected to cell-based gene transfer to the lung circulation by injecting syngeneic fibroblasts transfected with Ang1 cDNA or a null plasmid. After 24h, LPS (100 µg/kg BW) was instilled intratracheally to induce pulmonary inflammation. Bronchoalveolar lavage was performed 6h later, and lungs were harvested for histological and molecular analyses. **Results:** ET-1 release from cultured endothelial cells was dose-dependently reduced by Ang1, which also prevented induction of ET-1 release by TNF$\alpha$ ($p<0.05$). RNA expression of ppET-1 was similarly reduced. In LPS-challenged lungs, ppET-1 RNA was induced by 3.4 fold and ET-1 protein in lavage fluid was increased by 5.6 fold ($p<0.05$). Ang1 gene transfer attenuated the LPS-induced increases in ppET-1 RNA and lavage ET-1 protein by 34% and 33%, respectively ($p<0.05$). The downregulation of ET-1 correlated with the amelioration of pulmonary inflammation, as indicated by reductions in leukocyte infiltration (by 43%), intra-alveolar septal thickening (by 40%), and E-Selectin expression (by 75%, $p<0.05$ vs null). **Conclusions:** These results show that ET-1 transcript and protein levels are downregulated by Ang1 in both *in vitro* and *in vivo* systems, and that cell-based Ang1 gene transfer markedly ameliorated inflammation *in vivo* in an experimental model of ARDS. The suppression of ET-1 production by Ang1 may be a new strategy for the treatment of vascular inflammatory diseases.

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**Role of endothelin-converting enzyme-1 and bradykinin in hypoxia-induced pulmonary hypertension**

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Though the beneficial effects of endothelin receptor blockade for the treatment of pulmonary hypertension have been established, little is known regarding the consequence of endothelin-converting enzyme-1 (ECE-1) inhibition to the pathogenesis of this fatal disorder. ECE-1 is a metallopeptidase with broad substrate specificity. This enzyme not only produced vasoconstrictor endothelin-1 (ET-1), but also inactivated vasodilator bradykinin in vitro. This study investigated the effect of ECE-1 deficiency on the pulmonary vascular response to hypoxia in mice. Here, we report that ECE-1 heterozygous mice (n=16) developed less hypoxic pulmonary hypertension and vascular remodeling than paired controls (n=16). After 3 weeks of hypoxia, ECE-1+/- mice had lower RV systolic pressure (33.2±3.88 in WT, 20.29±2.03 mmHg in KO; $P<0.005$), RV/BW (1.51±0.09 in WT, 1.25±0.03 in KO; $P<0.01$), RV/(LV+S) (0.41±0.023 in WT, 0.33±0.01 in KO; $P<0.05$) and % pulmonary artery wall thickness (46.18±2.44 in WT, 35.05±0.88 in KO; $P<0.05$) than the WT mice. Although the levels of ET-1 peptide in serum and lung increased ~3-4 folds in both groups after 3 weeks of hypoxic exposure ($P<0.001$ vs. normoxic), the ET-1 level was not different between the two genotypes under both normoxic and hypoxic conditions (P=NS). Surprisingly, we found that at the normoxic state the levels of bradykinin peptide was ~3-folds higher in lungs of ECE-1+/- than WT mice ($P<0.001$). Chronic hypoxia dramatically reduced the lungs bradykinin in WT mice to almost undetectable levels ($P<0.005$ vs. WT normoxic) but only slightly did so in KO mice ($P<0.05$ vs. KO normoxic). These results suggest a novel pathophysiological role of ECE-1 in the pathogenesis of hypoxic pulmonary hypertension. The beneficial effects of ECE-1 deficiency in this model can be attributed to the preservation and potentiation of bradykinin action and not to reduced ET-1 production.

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**Collecting duct-specific knockout of the endothelin A receptor alters renal vasopressin responsiveness, but not sodium excretion or blood pressure**

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Collecting duct (CD)-specific knockout (KO) of endothelin-1 (ET-1) causes hypertension, impaired ability to excrete a Na load, and enhanced CD sensitivity to the hydroosmotic effects of vasopressin (AVP). CD express the two known ET receptors, ETA and ETB; in the current study, the role of the CD ETA receptor in mediating ET-1 actions on this nephron segment was evaluated. The ETA receptor gene was selectively disrupted in CD (CD ETA KO). CD ETA KO mice had no differences in systemic blood pressure, Na or K excretion, and plasma aldosterone or renin activity in response to a normal or high Na diet as compared to controls. During normal water intake, urinary osmolality (Uosm), plasma Na concentration, and plasma osmolality were not affected, but plasma AVP concentration was increased in CD ETA KO animals (0.57 ± 0.25 pg/ml in controls and 1.30 ± 0.29 pg/ml in CD ETA KO mice). CD ETA KO mice had a modestly enhanced ability to excrete an acute, but not a chronic, water load. DDAVP infusion increased Uosm similarly, however CD ETA KO mice had a more rapid subsequent fall in Uosm during sustained DDAVP administration. CD suspensions from CD ETA KO mice had a 30-40% reduction in AVP- and forskolin-stimulated cyclic AMP accumulation. These data indicate that CD ETA KO decreases renal sensitivity to the urinary concentrating effects of AVP, and suggest that activation of the ETA receptor downregulates ET-1 inhibition of AVP actions in the CD. Furthermore, the CD ETA receptor does not appear to be involved in modulation of systemic blood pressure or renal Na excretion under physiologic conditions.

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**Acute intravenous and renal medullary interstitial infusions of hypertonic saline stimulate renal ET-1 production in rats**

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Results of studies conducted *in vitro* suggest that exposure of renal tubular epithelial cells to high $Na^+$ concentrations or hyperosmolar solutions enhances ET-1 production and release. Whilst providing a plausible mechanism for the increased urinary ET-1 excretion observed during high salt intake, it is not known whether infusion of hypertonic saline into the renal medulla would produce a similar effect *in vivo*. Therefore we tested whether acute intravenous and renal medullary interstitial infusions of hypertonic saline enhanced urinary excretion of ET-1 in anesthetized rats. Rats received infusions of isotonic saline (NaCl at 284 mOsmol/kg $H_2O$) either intravenously (25 µl/min) or into the renal medullary interstitium (8.33 µl/min) during a 1 h equilibration period and 30 min baseline period. Rats then received infusions of NaCl either intravenously (284, 921 or 1664 mOsmol/kg $H_2O$; n = 5, 3, 3) or into the renal medulla (284 or 1714 mOsmol/kg $H_2O$; n = 5, 5) for two further 30 min periods. Urine was collected from either the bladder (intravenous infusions) or left ureter (renal medullary infusions) during the 30 min baseline period and two subsequent 30 min infusion periods. Compared to isotonic saline, infusion of a hypertonic NaCl solution (1714 mOsmol/kg $H_2O$) into the renal medulla significantly increased the rate of ET-1 excretion in the urine ($P<0.05$; from 0.34±0.03 to 0.46±0.05 fmol/min), increased urine flow ($P<0.05$) and increased the rate of $Na^+$ excretion ($P<0.01$). Intravenous infusion of NaCl at 1664 but not 921 mOsmol/kg $H_2O$ significantly increased urinary ET-1 excretion rate (from 0.81±0.11 to 1.92±0.18 fmol/min) and urine flow compared to infusion of isotonic NaCl ($P<0.01$). The hypertonic NaCl solutions had no significant effects on mean arterial pressure, which remained constant. Thus exposure of the renal medulla to hypertonic concentrations of NaCl acts as a stimulus for renal ET-1 production in vivo, consistent with the hypothesis that medullary $Na^+$ concentration may be an important regulator of renal medullary endothelin synthesis.

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**Dual endothelin (ET) -A/B receptor antagonist is better than the selective ET-A receptor antagonist in ameliorating the decreased VEGF signaling and inadequate coronary collateral development in early diabetic hearts**

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Decreased vascular endothelial growth factor (VEGF), the key angiogenic factor, has been documented in inadequate collateral formation in the heart in diabetic (DM) animals and patients, but no study yet has focused on the therapeutic implication to improve the defect in VEGF signaling in DM hearts. The present study investigated whether ET-A/B dual receptor antagonist (SB209670, 1 mg/rat/day) and selective ETA-receptor antagonist (TA-0201, 1 mg/kg/day) would reverse the downregulated VEGF signaling in early streptozotocin (STZ)-induced diabetic hearts with no obvious cardiac dysfunction in echocardiography. Male Sprague-Dawley rats were administered citrate saline (vehicle) or STZ (65 mg/kg IP). Diabetes was confirmed by hyperglycemia and after 1 wk of diabetes, animals were separated into those receiving SB209670, TA-0201 or vehicle by osmotic mini pump for 2 weeks and sacrificed. Glucose levels in DM rats were greatly increased (405±103 mg/dL) than in non-DM rats (120±8 mg/dL). Cardiac ET-1 level was significantly increased in DM (2.1±0.3, pg/mg) than in non-DM rats (1.4±0.5, pg/mg) and SB209670 treatment completely reversed the higher ET-1 levels in DM heart than the TA-0201 treatment. VEGF level in DM heart was significantly decreased (7.8±1, pg/mg) than in non-DM rats (12±1.3, pg/mg), SB209670 and TA-0201 treatments completely prevented this VEGF downregulation in DM hearts (13.0±3.8 and 13.2±3.5 pg/mg, respectively). Although VEGF was almost equally recovered in both treatment groups, ET-A/B dual receptor blocker was much more effective in reversing the alterations in phosphorylated Akt and eNOS, the two important downstreams of VEGF angiogenic signaling, in DM heart than the selective ETA-receptor antagonist. In conclusion, ET antagonism is potentially effective in preventing the downregulation of VEGF in early DM heart, and the dual ET-A/B receptor antagonist is better than the selective ETA-receptor antagonist in ameliorating the decreased VEGF signaling and inadequate coronary collateral development in early DM hearts.

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**Genetic inactivation of vascular endothelial cell endothelin-1 is protective against high-fat diet induced obesity, hypertension and insulin resistance**

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Epidemiological and experimental data have shown that the existence of obesity is frequently coincides with hypertension and insulin resistance, which strongly suggests that these conditions share a common pathophysiological root. We hypothesized that endothelin-1 (ET-1) is involved in the development of diet induced-obesity, insulin resistance and hypertension. To seek the evidence for this hypothesis, we use vascular endothelial cell ET-1 knockout (VEETKO) mice. Animals were divided into 4 groups of treatments: 2 groups of high-fat diet (HFD) fed mice (VEETKO / WT n=7 each) and 2 groups of normal chow (NC) fed mice (VEETKO / WT n=5 each). Body weights of VEETKO mice are slightly but significantly lower than that of their WT littermates. The difference in the body weight is attributed only to the difference in weight of white adipose tissue. Plasma level of leptin, which is significantly lower in VEETKO mice, confirmed this finding (1868.4±159.4 vs. 3410.7±237.9 pg/ml, $p<001$). HFD increased both body weights and adipose tissue weights in the much greater extend in WT mice than in VEETKO mice. Neither food consumption nor energy expenditure was different between two genotypes, leaving adipogenesis as the only possible mechanism of the difference in body weight gain. Matrigel plug assay revealed defective angiogenesis in VEETKO mice as hemoglobin content recovered from these mice was lower than from WT mice. Initial blood pressure (BP) of VEETKO mice was slightly but significantly lower than that of WT mice (VEETKO vs. WT :105.14±4.67 vs.116.14±7.99 mmHg, $p<0.05$). Following HFD, the difference was significantly widened. These findings indicate that ET-1 is a key player in the development of HFD induced obesity, most probably by its role in angiogenesis, hypertension and insulin resistance and provide the basic rationale for the use of ET-1 antagonism in metabolic syndrome.

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**The renal endothelin system in diabetic endothelin-transgenic rats**

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Background: Diabetic nephropathy is one of the leading causes of end stage renal disease in developed countries. The mechanisms of diabetic nephropathy are not fully understood. The physiology and pathophysiology of the renal endothelin-system as well as its relationship with the renin-angiotensin-system led us to hypothesize a potential role of endothelin for development and progression of diabetic nephropathy. Methods: Four weeks old Sprague Dawley (SD)-rats transgenic for human endothelin-2 [TGR(hET2)L37] (TG+) and non-transgenic litter-mates (TG-) were studied. Half of the respective groups were made diabetic by injection of streptozotozin (STZ). Proteinuria and creatinine-clearance were analyzed using monthly monitoring of all rats in metabolic cages. By the end of the six months study all animals were sacrificed in order to determine the influence of diabetes and the status of the renal endothelin-system on functional, structural and molecular markers of renal damage. Results: Six months duration of diabetes did not lead to a significant reduction of creatinine-clearance in SD-rats (TG-/STZ+) compared to normoglycemic SD-rats (TG-/STZ-). By contrast, transgenic diabetic rats (TG+/STZ+) were still hyperfiltrating after 6 months of hyperglycemia. Diabetes did not aggravate the proteinuria induced by transgenic overexpression of endothelin. The results of glomerular expression of (endogenous) endothelin-1 (real time-RT-PCR) explain these observations (see Table 1). Discussion: Six months duration of diabetes is not sufficient to induce advanced diabetic nephropathy in SD-rats. Transgenic overexpression of endothelin seems to prolong hyperfiltration in hyperglycemic rats. The different regulation of endogenous endothelin-1 and its receptors may be the explanation for this unexpected result.

Table 1: The results of glomerular expression of (endogenous) endothelin-1 (real time-RT-PCR)

| | TG-/STZ- | TG+/STZ- | TG-/STZ+ | TG+/STZ+ |
|---|---|---|---|---|
| ET-1 | 37.6+/-5 | 64.2+/-12* | 38.8+/-8.3 | 34.5+/-6.3# |
| ETA-Rec. | 42.9+/-6.2 | 97+/-30 | 148.1+/-35.8# | 71.4+/-28.6 |
| ETB-Rec. | 85.9+/-11,2 | 139.2+/-30,5 | 188.9+/-45# | 108.3+/-19.9 |

* p≤0.05 vs. TG-; # p≤0.05 vs. STZ-

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**PKC and MAPK Inhibition blocks upregulation of ETB receptors in cerebral arteries after subarachnoid hemorrhage**

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Introduction: The cerebral ischemia that occurs after a subarachnoid hemorrhage (SAH) often results in death or severe disability. Previous studies have observed upregulation of endothelin B receptors (ETB) in cerebral arteries following experimental induced SAH. The purpose of this study was to examine whether protein kinase C (PKC) and mitogen activated protein kinase (MAPK) in rats could alter the SAH induced ETB receptor upregulation and prevent the associated cerebral blood flow (CBF) reduction. Methods: SAH was induced by injecting 250 µl blood into the prechiasmatic cistern. In conjunction and after the induced SAH, the PKC inhibitor RO-31-7549 or the MAPK inhibitor SB386023-b was injected intracisternally. After two days the basilar arteries (BA) and middle cerebral arteries (MCA) were harvested and contractile responses to endothelin-1 (ET-1) were investigated by myographs. ETA and ETB receptor mRNA and protein levels were analyzed by real-time PCR and immunohistochemistry. To investigate if PKC and MAPK influenced the global and regional CBF after SAH we used an autoradiographic technique. Results: SAH resulted in enhanced contraction to ET-1, as well as increased levels of ETB receptor mRNA and protein in both MCA and BA. Administration of the MAPK inhibitor or PKC inhibitor during the SAH decreased the maximum contraction elicited by application of ET-1 considerably compared to SAH. The MAPK and PKC inhibition downregulated ETB receptor mRNA and protein levels compared to that seen after SAH only. No differences were observed in the ETA receptor mRNA levels. The reduction in global and regional CBF observed after SAH were significantly prevented by treatment with PKC or MAPK inhibition. Conclusion: The results indicate that MAPK and PKC inhibition downregulate contractile ETB receptors and may have a therapeutic potential in the treatment of cerebral ischemia associated with SAH.

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**Endothelin and light-induced retinal degeneration**

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Exposure to excessive light induces degeneration of photoreceptors accompanied by reactive gliosis. We have described the presence of endothelin-1 (ET-1) and its receptors (ETA and ETB) in different sites of the mouse retina, including the retinal pigment epithelium, the outer plexiform layer (OPL), astrocytes, the ganglion cell layer and endothelium. After light induced degeneration ET-1 and ETB are overexpressed in astrocytes, and endothelinergic structures disappear from the OPL. Therefore, we asked whether blocking of endothelinergic receptors would modify the course of light induced degeneration. BALB-c mice were kept under basal illumination conditions (60 lux:darkness, 12:12 hours), or exposed to constant illumination (1,500 lux) during 4 days. Animals from both groups received Tezosentan (10 mg/kg SC) or saline. Retinas from the four experimental groups were studied by immunohistochemistry and Western blots using antibodies against ET-1, receptors ETA and ETB, glial fibrillary acidic protein (GFAP) or cleaved caspase 3 (CC3). Changes in retinal astrocytes were evaluated in retinal wholemounts immunostained for ET-1, ETB or GFAP. Quantitative image analysis showed a large increase of these markers after 4 days of constant illumination. Tezosentan treatment significantly reduced the GFAP increase, both in wholemounts and Western blots. Dying photoreceptor cells were estimated by the amount of CC3 positive nuclei within the outer nuclear layer (ONL). The number of CC3-labeled nuclei significantly decreased after Tezosentan treatment. Our observations show that Tezosentan decreases the GFAP response of retinal glia to photoreceptor degeneration. Immunohistochemical and Western blot evidence indicates that both the astrocytic and the Müller cell responses are quenched by blockade of endothelinergic receptors. Decrease of the glial response is probably the sum of at least two different mechanisms: (a) the inhibition of ETB receptors in astrocytes, which are upregulated in the degenerating retina and (b) the reduction of photoreceptor death shown by CC3 labeling. These results provide experimental evidence that ET-1 may modulate photoreceptor survival and the gliotic response to photoreceptor degeneration.

O-40

**Endothelin-A/-B dual antagonism reverses the angiogenic growth factor alteration in the frontal cortex of SHR-SP without changing the regional cerebral blood flow**

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Stroke-prone spontaneously hypertensive rats (SHR-SP) exhibiting prehypertensive, typical hypertensive and malignant hypertensive stages suffer spontaneous stroke in part due to abnormal cerebrovascular development. We have reported that endothelin-1 (ET-1), its type A receptor ($ET_AR$), VEGF and its receptors were upregulated in SHRSP frontal cortex at typical hypertensive stage, whereas ET type B receptor ($ET_BR$), eNOS and Akt, which acts on downstream of VEGF, were downregulated compared to its genetic control normotensive WKY rats. The current study investigated whether long-term treatment with the ET-A/-B dual receptor antagonist SB209670, or saline (vehicle), starting from prehypertensive stage (6 weeks old) can reverse these alterations in the SHRSP brain. ET antagonism suppressed serum and cerebral ET-1 levels and $ET_AR$ expression in the SHRSP brain. A 46% upregulation of VEGF and its receptors (KDR and Flt-1) observed in vehicle-treated SHRSP brain compared with age-matched WKY was remarkably reversed by ET antagonism. The 38% downregulated eNOS expression in vehicle-treated SHRSP was recovered by treatment with the ET antagonist. ET antagonism for 12 weeks in SHRSP could also ameliorate the 25% decrease in $ET_BR$ expression in the frontal cortex of vehicle-treated SHRSP. Akt, downregulated in vehicle-treated SHRSP, was normalized by ET antagonism. At the typical hypertensive stage VEGF and its receptors were upregulated in the SHRSP brain, whereas Akt and eNOS, the downstream components of VEGF signaling, were downregulated; these alterations were reversed by ET antagonism. Thus, $ET_BR$ and eNOS were altered in parallel in the SHRSP brain. Cerebral blood flow of SHRSP was tended to be decreased at typical hypertensive stage associated with upregulated ET-1 and $ET_AR$ but was not reversed by ET antagonism. Therefore, it was suggested that ET antagonism in SHRSP from the prehypertensive stage prevents the progression of hypertension-induced neurovascular remodeling in the frontal cortex without altering the cerebral blood flow.

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**Role of endothelin-1 in ocular blood flow regulation in humans**

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In the recent years we have performed a number of studies investigating the role of endothelins in the control of ocular blood flow in humans. Several randomized placebo-controlled clinical trials were performed in healthy humans using exogenous intravenous administration of ET-1 and the specific ETA-receptor antagonist BQ-123. Using non-invasive technology retinal, optic nerve head and choroidal blood flow were studies separately. Exogenous ET-1 induced a dose-dependent decrease in blood flow in all three vascular beds under study. This effect was almost completely abolished by pre-treatment with BQ-123 indicating that the vasoconstrictor response is mainly mediated via the ETA receptor subtype. BQ-123 alone did not influence blood flow in the posterior pole of the eye. Accordingly, ET-1 seems to contribute only little to ocular vascular tone under physiological conditions. In addition, it appears that ET-1 plays a role in choroidal and retinal blood flow regulation during isometric exercise and contributes to hyperoxia-induced vasoconstriction in the retina.

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**Activation of PPARα attenuates ET-1 production from cerebrovascular endothelial cell**

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Elevated ET-1 has been implicated in brain injury and stroke. Inhibition of ET-1 biosynthesis has been reported to ameliorate cerebral complications following brain trauma. The mechanism involved in increased ET-1 is still far from being understood. However, PKC is involved in ET-1 and NO production. Moreover, expression of PPARα has been reported in the brain microvessels and PPARα activation has been reported to improve peripheral vascular function in vascular diseases. We tested the hypothesis that activation of PPARα will attenuate ET-1 production from cerebrovascular endothelial cell (CVEC) by a mechanism involving NO and PKC. CVEC culture was established from piglet brain. Quiescent confluence cells were incubated with buffer or clofibrate (10 μM) for 18 hr to activate PPARα before exposing the cell to buffer, PMA (1μM), bradykinin (BK, 1μM), angiotensin II (AII, 1μM), or hemoglobin (Hem, 10 μM) for 4 hr. ET-1 and NO levels as well as eNOS, PPARα and endothelin converting enzyme (ECE) expressions were determined. To determine a role for PKC, cells were pretreated with calphostin C (1μM), a PKC inhibitor 15 min before clofibrate or PMA. PMA, BK, AII or Hem increased ET-1 levels by 24, 11.4, 3.6, or 2 folds ($p<0.05$)from 0.36±0.08 to 8.6±0.8, 4.1±0.7, 1.30±0.1, or 0.47±0.03 (fmol/μg protein), respectively. Clofibrate reduced basal ET-1 level and blunted increased ET-1 level by the vasoactive agents. NO level was increased by clofibrate from 18±2 to 25±2 and was not affected by addition of PMA (30±0.5 pM/μg protein) or PMA alone. Calphostin C blocked clofibrate-induced increase in NO production but not eNOS expression. Clofibrate increased PPARα and eNOS but reduced ECE protein expression. These data suggest that activation of PPARα attenuated ET-1 production by agents that are involved in mediating brain injury and inflammatory processes. The reduction in ET-1 production may have resulted from PKC-mediated increase in NO production which may be mediated via activation of PPARα. In conclusion, PPARα activators may help ameliorate cerebrovascular dysfunction by preserving endothelial cell NO production following conditions that result in elevated ET-1.

O-43

**Roles of local $ET_A$ and $ET_B$ receptor-operated mechanisms in carrageenan-induced articular incapacitation, edema and inflammation in rat knee joint**

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Carrageenan (CG) causes sustained nociceptive and priming effects in rat knee joint that are potentiated by intraarticular (i.a.) endothelin-1 (ET-1), but inhibited by sarafotoxin S6c (SRTX; Daher et al., Eur J Pharmacol, 496, 77, 2004). This study assesses the relation between these actions and the ability of $ET_A$ and $ET_B$ receptors to trigger joint edema and inflammation. Male Wistar rats (200-250 g) were given ET-1 or SRTX (30 pmol; i.a.) 24 h prior to CG (300 µg, i.a.) or PBS into the same joint. Nociception was estimated by measuring paw elevation time (PET; see details in above reference) while walking on a revolving cylinder, edema as increases in articular diameter (IAD) and inflammation using an index comprising the sum of scores (SS) for monocyte and neutrophil infiltration into synovial membrane and underlying epithelium, all at 4 h after CG (i.e. at time point of peak nociception). CG caused nociception (PET from 10 ± 1 to 30 ± 6 s), edema (IAD from 0.1 ± 0.05 to 2.5 ± 0.5 mm) and inflammation (SS from 0 to 8). ET-1 potentiated CG-induced increases in PET and IAD (but not SS), whereas SRTX reduced all 3 effects of CG (PET 14 ± 3 s, IAD 0.6 ± 0.2 mm and SS 3.3). SRTX also suppressed CG-induced priming of naive joints to all 3 effects of a second CG challenge given 72 h after the first (PET 43 ± 4 to 28 ± 2 s, IAD 5 ± 0.2 to 2 ± 0.2 mm, SS 8.5 to 4.7). All effects of ET-1 or SRTX were prevented by BQ-123 or BQ-788 (10 nmol, i.a.), respectively. In presence of BQ-123, ET-1 reduced SS in SRTX-like fashion. The priming effect of CG in naive joints was potentiated by BQ-788, but unaltered by BQ-123. Thus, the nociceptive and priming actions of CG are highly correlated to edema and inflammation of the synovial capsule. Local $ET_B$ receptors mediate prolonged inhibition of all these CG effects, whereas $ET_A$ receptors counteract the $ET_B$ receptor-mediated actions. Finally, CG in the naive joint recruits endothelins to selectively promote $ET_B$ receptor-operated inhibition of priming to effects of subsequent inflammatory insults. Support: CNPq, CAPES and PRONEX (Brazil).

O-44

**Upregulation of Endothelin-Converting-Enzyme-1 (ECE-1) in host liver during chronic allograft rejection**

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Plasma endothelin-1 (ET-1) is increased in chronic rejection and contributes to cell activation, growth induction, and inflammation. Chronic allograft rejection is an unsolved problem in solid organ transplantation and remains the major cause for graft loss. We have recently shown that cytokines in host organs are transcriptionally regulated during chronic rejection and that endothelin receptors contribute to this process (1). Since endothelin-converting-enzyme-1 (ECE-1) contributes to ET-1 production by cleaving its precursor big-endothelin-1, and the liver is a major site of cytokine production, we postulated that hepatic ECE-1 gene expression and ET-1 uptake are altered during chronic rejection. The Fischer F344/Lewis chronic cardiac transplant model was used, without immunosuppression. ECE-1 gene expression in host liver was measured by real-time PCR and hepatic ET-1 organ uptake and ET-1 clearance were assessed after injection of radiolabelled 125I-ET-1. Blood samples were collected and tissue samples were taken to determine hepatic 125I-ET-1 uptake. Chronic allograft rejection was associated with severe graft arteriosclerosis and myocardial fibrosis indicating chronic rejection. Host hepatic ECE-1 gene expression increased during chronic rejection ($p<0.001$ vs. control). In contrast hepatic 125I-ET-1 uptake was decreased by more than 63 percent in transplanted animals ($p<0.05$ vs. control). No effect regarding to endothelin-1 plasma clearance were observed. These results show that during chronic rejection ECE-1 gene expression is upregulated in host liver. Furthermore, the decreased ET-1 uptake suggests a possible reduction of ET-receptor distribution in host liver under chronic rejection. These results indicate that the hepatic ET-system is activated in the hosts reaction to chronic rejection. Since the liver is a key organ for cytokine-production and ET metabolism it may contribute to host responses and cytokine production via endothelin-1 during rejection. (1) Am J Transplant. 2005; 5:1042-9

O-45

**Involvement of endothelin in morphine tolerance in neuroblastoma (SH-SY5Y) cells**

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Long-term use of morphine in pain management leads to adverse effects such as development of antinociceptive tolerance. We have previously shown the involvement of central endothelin (ET) mechanisms in morphine analgesia and development of tolerance in vivo. The present study was conducted to investigate in vitro mechanism of interaction of ETA receptor antagonist, BMS182874, and morphine during acute and chronic morphine tolerance in SH-SY5Y cells. SH-SY5Y cells were exposed to acute and chronic treatment with vehicle, morphine, ET-1, BMS182874, or morphine+BMS182874). Activation of G-protein-coupled receptors in SH-SY5Y cells was determined using [35S]GTPγS binding assay. Morphine produced a concentration-dependent increase in [35S]GTPγS binding in SH-SY5Y cells after acute morphine treatment. EC50 values after acute morphine (100nM, 1μM and 10μM) treatment were significantly decreased suggesting sensitization of opioid receptors. Chronic morphine treatment produced significantly lower maximal response of GTP binding compared to both control and acute morphine treatment, indicating uncoupling of G-proteins. Acute and chronic exposure of cells to ET-1 did not produce any change in ET-1-induced GTP binding. BMS182874 treatment alone (acute or chronic) did not produce G-protein activation. However, in cells co-treated chronically with morphine (10μM) and BMS182874 (1μM), morphine-induced GTP stimulation was significantly higher than control. EC50 value after control treatment was 414nM and significantly increased in chronic morphine treated cells (>1000nM). However, EC50 value was significantly reduced in cells with chronic treatment of BMS182874 and morphine (63nM) compared to control and chronic morphine). ETA antagonists significantly enhance coupling of G-protein to opioid receptors. Therefore, we propose that restoration of morphine antinociception by ETA antagonists in morphine-tolerant animals is likely by a G-protein mediated mechanism.

O-46

**Endothelin-1 potentiates TRPV-1 currents in primary sensory neurons via ETA receptor-mediated PKC activation**

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Endothelin-1 (ET-1) was originally cloned from endothelial cells, but is produced by a number of cell types including macrophages and tumor cells. Apart from its cardiovascular effects, ET-1 is involved in inflammatory and neuropathic pain. To date, only few studies have addressed the cellular effects of ET-1 in primary sensory neurons. ETA receptors are expressed in small sensory neurons of the dorsal root ganglion (DRG). However, the molecular effectors and the underlying signaling pathways of ETA receptor-mediated nociception remain elusive. TRPV1 is an important effector protein of painful and inflammatory stimuli. Therefore, we studied the effects of ET-1 on TRPV1 in freshly dissociated small diameter neurons derived from the DRG and in HEK293 cells co-expressing TRPV1 and the ETA receptor. Whole cell patch clamp analyses revealed that ET-1 strongly potentiated responses to capsaicin in a subpopulation of neurons and in HEK293 cells. ET-1-mediated potentiation of capsaicin-evoked currents was observed for up to 15 min. In HEK293 cells, stimulation of PKC by phorbol myristate acetate induced a potentiation of capsaicin-evoked currents similar to that elicited by ET-1. In contrast, activation of PKA by forskolin or dibutyryl cAMP, did not potentiate capsaicin-induced TRPV1 currents. When HEK cells coexpressing ETA receptors and TRPV1 were pretreated with bisindolylmaleimide X (inhibition of PKC), the ET-1-mediated potentiation of capsaicin responses was blunted, whereas inhibition of PKA with H89 had no influence on ET-1-induced potentiation. The data demonstrate that ET-1 potentiates capsaicin-induced TRPV1 currents via the ETA receptor depending on the activation of PKC. The ETA receptor/PKC signaling pathway is likely to play a major role in the pain-producing and pain-potentiating effects of ET-1.

O-47

**Attenuation of angiotensin II-induced body weight loss in endothelin-1 deficient mice**

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Cardiac cachexia, which is one of the most important determinants of exercise capacity and mortality in congestive heart failure, has been shown to be partially caused by increased circulating level of angiotensin II (ATII). While many studies focused on the interaction between ATII and endothelin-1 (ET-1) in cardiovascular remodeling, ours focused on the interaction between the two in body weight loss. To test the hypothesis that these two potent peptides work synergistically to cause body weight loss, we performed subcutaneous ATII infusion on vascular endothelial specific ET-1 targeted mice (VEETKO mice). After 2 weeks of infusion the body weight of WT mice decreased dramatically (n=9). Although normal weight gain was significantly prevented, body weight loss did not occur in VEETKO mice (n=7)(VEETKO mice gained 2.54+5.98 % of body weight, WT mice lost 20.76+5.98%, $p<0.0001$). Skeletal muscle weight, which contributes largely to the sum of body weight, was similarly decreased in both genotypes. In contrast, adipose tissue weight was markedly decreased only in WT mice. As clearly shown by the amount of daily food consumption following infusion, ATII caused anorexia excessively in WT mice. Almost no difference in the amount of daily food consumption was observed in VEETKO mice (Daily food intake before infusion KO Vs WT: 4.4+0.11 Vs 4.26+0.27 g/day (p:NS), during infusion: 4.26+0.27 Vs. 2.79+0.53 g/day ($p<0.0001$)) . To investigate whether the protective effect of ET-1 deficiency was exerted solely on the preservation of food consumption we performed pair feeding experiment. Despite the exactly same amount of daily food consumption, body weight loss of WT mice was significantly higher than VEETKO mice. These findings indicate that ET-1 deficiency is protective against ATII induced body weight loss not only through the prevention of anorexia but also through the modulation of ATII direct catabolic effect.

O-48

**Strategies to target the endothelin system in human cancer**

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The endothelin (ET) system has been linked to cancer progression and may be important in the functions of cancer cells themselves, or of cancer-associated stromal cells. The active peptide of the ET system, ET-1, is biosynthesized as an inactive precursor, which is activated by the metalloprotease endothelin converting enzyme-1 (ECE-1). ET-1 acts on two cell membrane receptors of the GPCR family, ETA and ETB, which selectively activate several intracellular signaling pathways. Therefore, these various steps offer strategies to control cancer progression. Our aims are to determine which of all these steps, ET-1 activation, receptor activation and/or intracellular signaling, and which cells, cancer cells or cancer-associated stromal cells, are the most appropriate targets of the ET-system in human cancer. We demonstrated that the ET system is widely expressed in surgical specimens of human cancers, in the cancer cells and in cancer-associated stroma. Using antagonists to ETA and/or ETB receptors we have shown that these drugs can control the apoptosis of carcinoma and non-carcinoma human cancer cells, but not all cell lines responded to these drugs. Combination of antagonists to ET-1 receptors and various drugs targeting intracellular pathways demonstrated either additive or synergistic effects, suggesting that drug combination should be used in order to enhance efficacy. Using a series of thiol-based synthetic ECE-1 inhibitors, we demonstrated that these drugs may have a more general efficacy than receptor antagonists in controlling the survival of human cancer cells. Cancer-associated fibroblasts selectively expressed the ETB receptor, and in ETB-expressing human fibroblasts, ET-1 was not involved in cell growth, survival or the production of proteases, but was decreasing the contractile properties of these cells. In summary, antagonists or inhibitors of the various components of the ET system selectively control the functions of human cancer cells and cancer-associated stromal cells.

O-49

**IRL 1620, a tumor selective vasodilator, enhances the uptake and efficacy of anti-neoplastic agents in breast tumor rats**

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ETB receptors are over expressed in invasive as well as ductal and lobular breast carcinoma in humans. ETB receptors predominating on endothelial cells produce vasodilation. We found that IRL 1620, a potent endothelin B receptor agonist, selectively enhanced breast tumor perfusion in breast tumor rats. The present study was conducted to evaluate the effect of IRL 1620 on the tumor delivery and efficacy of paclitaxel in rats. Pre-clinical model of MNU-induced primary mammary tumor model in rats were used for the study. Rats with a tumor volume of 200-500mm3 were selected and administered with [3H]paclitaxel (40 µCi/rat) 15 min after the administration of IRL 1620 (3 nmol/kg). Rats were sacrificed 3 h after [3H]paclitaxel administration and tumor and organs were excised and concentration of [3H]paclitaxel was determined. For efficacy study, paclitaxel (5 mg/kg, i.v) was administered every third day for a total of 5 doses, 15 min after the administration of IRL 1620 (3 nmol/kg, i.v). Body weight of rats and tumor size were monitored on every third day and continued for a total of 30 days after the last dose of paclitaxel. Progression, stasis, partial or complete remission of tumors were monitored. Tumor [3H]paclitaxel concentration was increased by 308.5% when [3H]paclitaxel was administered 15 min after IRL 1620 compared to vehicle treated rats. However, IRL 1620 did not increase [3H]paclitaxel concentrations in other organs. Paclitaxel administration on every third day for a total of 5 doses produced 60.0% reduction in tumor volume, 9.5% tumor regression and 0% complete tumor remission compared to saline treated rats. However, paclitaxel when administered 15 min after IRL 1620 produced 268.9% reduction in tumor volume, 48.7% tumor regression and 13.3% complete remission of tumors compared to saline treated rats. IRL 1620 significantly enhanced delivery and effectiveness of paclitaxel in an animal model of breast cancer and can be used an adjuvant to increase the efficacy of blood-borne antineoplastic agents.

O-50

**The endothelin axis is a target of the metastasis suppressor gene RhoGDI2**

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Half of patients treated for locally advanced bladder cancer relapse with often fatal metastatic disease to the lung. We have recently shown that reduced expression of the GDP dissociation inhibitor, RhoGDI2, is associated with decreased survival of patients with advanced bladder cancer. However, the effectors by which RhoGDI2 affects metastasis are unknown. Here we use DNA microarrays to identify genes suppressed by RhoGDI2 reconstitution in lung metastatic bladder cancer cell lines. We identify such RNAs and focus only on those that also increase with tumor stage in human bladder cancer samples in order to discover only clinically relevant targets of RhoGDI2. Levels of Endothelin-1 (ET-1), a potent vasoconstrictor, were affected by both RhoGDI2 reconstitution and tumor stage. To test the hypothesis that the endothelin axis is important in lung metastasis, lung metastatic bladder carcinoma cells were injected in mice treated with the endothelin receptor specific antagonist, Atrasentan, thereby blocking engagement of the up-regulated ET-1 ligand with its cognate receptor. Endothelin antagonism resulted in a dramatic reduction of lung metastases, similar to the effect of re-expressing RhoGDI2 in these metastatic cells. Taken together, these experiments demonstrate a novel approach of identifying therapeutic targets downstream of metastasis suppressor genes. The data also suggest that blockade of the ET-1 axis may prevent lung metastasis, a new therapeutic concept that warrants clinical evaluation.

O-51

**Endothelin-1 promotes epithelial to mesenchymal transition in human ovarian cancer cells**

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The endothelin-1 (ET-1)/endothelin A receptor ($ET_AR$) pathway has been shown to have a significant role in ovarian carcinoma by promoting tumorigenesis. Here we studied the role of ET-1 in promoting epithelial-to-mesenchymal transition (EMT) in ovarian carcinoma, a key event in cancer metastasis. We demonstrate that the ET-1/$ET_AR$ axis in HEY and OVCA 433 ovarian carcinoma cells drives fibroblast-like morphological changes in 3-D cultures, associated with enhanced cell invasion, down-regulation of E-cadherin, increased levels of ß-catenin, Snail and other mesenchymal markers and suppression of E-cadherin promoter activity. The $ET_AR$ activation by ET-1 triggers a phosphatidylinositol-3 kinase (PI-3K) and ILK-mediated signaling pathway leading to glycogen synthase kinase-3ß (GSK-3ß) inhibition, Snail and ß-catenin stabilization and transcriptional programs that control EMT. Transfection of dominant negative integrin-linked kinase (ILK) or exposure to an ILK inhibitor suppresses the ET-1-induced phosphorylation of GSK-3ß as well as Snail and ß-catenin protein stability, activity and invasiveness, indicating that ET-1/$ET_AR$-induced effects depend on ILK. $ET_AR$ blockade by specific antagonists, or reduction by $ET_AR$ siRNA reverses EMT by inhibiting autocrine signaling pathways leading to E-cadherin down-regulation and cell invasion. In ovarian carcinoma xenografts, ABT-627, a specific $ET_AR$ antagonist, suppresses EMT determinants and metastatic potential. Finally, expression of $ET_AR$ significantly correlates with E-cadherin down-regulation and N-cadherin expression in human advanced-stage ovarian tumors. In conclusion, we demonstrate that $ET_AR$ activation by ET-1 is a key mechanism of the complex signaling network that promotes EMT, as well as ovarian cancer cell invasion and metastasis. These findings provide a wider window of therapeutic intervention, in which targeting ILK and the related GSK-3ß signaling cascade via $ET_AR$ blockade may be advantageous in the treatment of ovarian carcinoma. Supported by MIUR, AIRC

O-52

**Combined endothelin A receptor antagonist and bisphosphonate treatment more effectively reduces prostate cancer growth in bone than either alone**

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Endothelin-1 (ET-1) secreted by prostate cancers stimulates osteoblastic responses via the endothelin A receptor (ETAR). ETAR blockade reduces bone metastases in animal models, as well as in men with prostate cancer. Markers of bone resorption are increased and bisphosphonate antiresorptive drugs reduce skeletal morbidity in patients with osteoblastic disease. We hypothesized that osteolysis contributes to skeletal morbidity in patients with prostate cancer, as much as pathologic bone formation. We used an animal model to test the effect on tumor growth in bone of osteoblast inhibition with ETAR receptor antagonist (atrasentan) or osteoclast inhibition with bisphosphonate (zoledronic acid ZA), as single agents or in combination. Male nude mice were inoculated with LuCaP 23.1, a prostate xenograft that secretes ET-1 and prostate specific antigen (PSA). Mice were treated for 24 wks with vehicle, atrasentan (20mg/kg/day), ZA (5ug/kg, 3X/week) or the combination, starting one week before tumor inoculation. Tumor progression was followed by radiographs and serum PSA. Mice treated with atrasentan or ZA alone had less osteoblastic response on x-ray compared to vehicle ($p<0.05$) but did not differ from each other. Combination therapy was significantly more effective than vehicle or single treatments ($p<0.01$). PSA concentrations were similar between the vehicle and single treatment groups but were significantly lower in mice treated with both atrasentan and ZA. Histomorphometry demonstrated less tumor burden in bone ($p<0.001$) as well as new bone area ($p<0.05$) in mice treated with the combination compared with those treated with vehicle or ZA. Single agent atrasentan caused a greater reduction in tumor burden and new bone area than ZA alone. The data suggest that osteoblast and osteoclast activities cooperate to drive the progression of prostate cancer growth in bone. Thus, combination therapy targeting the two major bone cell types should be an effective treatment for osteoblastic bone metastases.

O-53

**Knockout of endothelin-1 in vascular endothelial cell protects against insulin resistance induced by high-salt diet in mice**

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Insulin resistance is associated with vascular dysfunction, hypertension and cardiovascular disease. To elucidate the precise pathogenesis of insulin resistance and to establish novel therapeutic strategies towards cardiovascular diseases, identification of molecular link between insulin resistance and cardiovascular diseases is inevitable. Endothelin (ET)-1 has an important role in maintaining vascular tone and plasma ET-1 level is elevated in patients with insulin resistance, suggesting that ET-1 participates in insulin-regulated glucose homeostasis. To assess the role of ET-1 derived from endothelial cells in the progression of insulin resistance, we treated the vascular endothelial cell ET-1 knockout (VEETKO) mice (n=5) and wild type (WT) mice (n=5) with high-salt diet (HSD: 8% NaCl) for 2 weeks to induce insulin resistance. Prior to HSD, blood pressure (BP) in VEETKO mice was slightly but significantly lower than that of WT mice (VEETKO:105.0±0.6mmHg, WT:120.6±1.2mmHg,$p<0.001$), and there was no difference on the levels of fasting glucose and insulin, glucose tolerance test (GTT) and insulin tolerance test (ITT). At the end of HSD, BP increased by approximately 10 mmHg in both groups, but BP in VEETKO mice remained lower. GTT and ITT showed that glucose tolerance and insulin sensitivity were impaired in WT mice as expected, whereas VEETKO mice were more glucose tolerant ($p<0.01$) and insulin sensitive ($p<0.005$) compared with WT mice. Furthermore, HSD increased serum TNF-alpha level and decreased serum adiponectin level in WT mice, which was explained by impaired insulin sensitivity, but those changes in VEETKO mice were almost half of WT mice (TNF-alpha: WT 21.7±5.6 to 84.2±16.1pg/ml,$p<0.001$, VEETKO 15.74.3 to 43.0±3.6pg/ml,$p<0.05$, adiponectin: WT 4.30±0.46 to 2.68±0.51μg/ml,$p<0.05$, VEETKO 4.98±0.36 to 4.03±0.63μg/ml,p=n.s). Thus, inactivation of ET-1 in endothelial cell protects against insulin resistance induced by HSD in mice and ET-1 blockade may provide a novel strategy of prevention and therapy in insulin resistance in human.

O-54

**Endothelin-like actions of an N-terminal fragment of parathyroid-hormone related protein (PTHrP)**

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The 1-23 peptide of PTHrP stimulates new bone formation. The responses are blocked by the ETA antagonist, atrasentan. PTHrP1-23 and ET-1 share limited sequence identity, providing an explanation of why prostate cancers cause osteoblastic metastases despite making the osteolytic factor PTHrP. Prostate cancer cells frequently express PSA, which cleaves after residue Y23, inactivating the osteolytic activity of PTHrP. We tested binding of PTHrP to transfected ETA, ETB, or PTH1R, +/- RAMPs 1-3 in competitive ligand-binding or receptor activation assays. We found no significant affinity of PTHrP1-23 for the receptors. We compared the actions of 100nM ET-1 or PTHrP1-23 on mouse calvarial ex vivo organ cultures (n=3) for 24hrs and analyzed RNAs by Affymetrix microarrays. The two peptides altered significantly different sets of genes. PTHrP1-23 stimulated many transcription factors and structural proteins involved in muscle differentiation, as well as genes involved in protein degradation and vesicular sorting/endocytosis. PTHrP1-23 increased 140 genes >2X and decreased 30 genes >2X. ET-1 regulated genes known to affect osteoblast function (IL-6, Cyr61, CTGF, Dkk1), but few genes with known function in osteoblasts were changed in the PTHrP1-23-treated RNA. Changes including several transcription factors, Nfatc2 (+6X), Dlx3 (+3X), and secreted proteins, TIMP1 (+2.8X) and COMP (+2.1X). Of 43 genes selected for roles in osteoblasts, only 4 were also affected by ET-1, but 21 of them (49%), were altered by the peptide adrenomedullin (AM), although AM did not stimulate myogenic mRNAs. AM, like PTHrP 1-23, potently stimulates new bone formation, but its mechanism of action on osteoblasts is also unknown.

The results suggest that PTHrP1-23 stimulates bone formation through a novel mechanism and at the molecular level acts via a unknown receptor signaling mechanism, which could still involve ETA, given the ability of atrasentan to block the biological responses to 1-23.

O-55

**Shigatoxin (Stx)-2 upregulates endothelin (ET)-1 gene in glomerular podocytes and promotes cytoskeleton dysfunction: implications for glomerular hemodynamics of HUS**

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Shigatoxin (Stx) is the offending agent of post-diarrheal HUS, characterized by glomerular ischemic changes preceding microvascular thrombosis. Since podocytes are highly sensitive to Stx cytotoxicity and represent a source of vasoactive molecules, we studied whether Stx-2 modulated the production of endothelin (ET)-1, taken as candidate mediator of podocyte dysfunction. Differentiated podocytes exposed to Stx-2 (50pM, 1nM) showed a significant time-dependent increase of ET-1 mRNA accompanied by elevated release of the peptide into the cell supernatant. Transfection with a dominant-negative mutant of IkB kinase2 or with Ap-1 decoy oligodeoxynucleotides reduced ET-1 mRNA levels. Further, inhibition of p38 and p42/44 Mitogen Activated Protein Kinases (MAPKs), which were markedly phosphorylated at early-time exposure to Stx-2, reduced transcription of NF-kB promoter/luciferase reporter gene construct induced by Stx. Differentiated podocytes possess a contractile structure composed of F-actin fibers extended across the cell body. Stx-2 caused actin-based cytoskeleton alteration associated with the formation of intercellular gaps. F-actin redistribution and gap formation induced by Stx-2 were prevented by the blockade of ETA receptor, proving a role of ET-1 via ETA receptor. Consistent with Stx-2 effect, addition of ET-1 (100nM) to cultured podocytes induced cytoskeleton alterations and intercellular gap formation. Exogenous ET-1 also increased protein permeability across the cell monolayer. Injection of Stx-2 in mice caused typical HUS ultrastructural changes with focal foot process effacement in concomitance with peripheral bundles of actin. ET-1 staining was also increased over control mice. In summary, our data show that podocyte is a functionally relevant target of Stx, a novel stimulus for ET-1 synthesis and cytoskeletal changes that might play a pivotal role in glomerular ischemia and severe hemodynamic derangement in HUS.

O-56

**Endothelin antagonism prevents diabetic retinopathy in NOD mice. Potential role of the angiogenic factor adrenomedullin**

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Introduction: Alterations in retinal endothelin-1 and nitric oxide synthase (NOS) activity may play a causal role in the hemodynamic and histopathological changes of diabetic retinopathy. Aim: To evaluate the therapeutic potential of long term endothelin antagonism in preventing the development of retinopathy in a genetic mouse model of non-obese Type 1 diabetes (NOD). Methods: NOD mice, implanted subcutaneously with insulin pellets or wild type controls were treated for five months with the selective ETA-receptor antagonist BSF 20875 (30mg/kg/day ) in the drinking water. Non-treated NOD mice received only water. At the end of the study blood glucose levels were evaluated and animals were anaesthetized then perfused intracardially with FITC labeled dextran. Retinas were removed and either formalin fixed for confocal microscope evaluation of retinal vascular filling or transferred to RNALater for quantitative PCR (Taqman) to evaluate expression of NOS-3, NOS-2, ET-1, ETRA, ETRB and the angiogenic factor adrenomedullin. Results: Compared to wild type controls, expression of ET-1, ETRA, ETRB and adrenomedullin were markedly up-regulated in retinas of non-treated NOD mice (delta Ct values, 14.8 vs 13.7, 18.57 vs 17.51, 10.76 vs 9.9 and 11.7 vs 9.1 respectively). Mean integral fluorescence intensity (MIFI) of retinal vascular filling was reduced from normal values of 24 to 12.5 in non-treated animals. BSF 20875 treatment normalized the upregulated expression of ET-1 and adrenomedullin and the deficit in MIFI but did not affect the increased ETRA and ETRB expression or the elevated plasma glucose levels found in non-treated NOD animals. NOS2 and NOS3 expression was unchanged from wild type control values in all groups. Conclusion: These results show that selective ETA receptor antagonists may be useful therapeutic tools in preventing diabetic retinopathy associated with Type-1 diabetes. Part of this beneficial effect may be mediated by preventing the upregulated expression of angiogenic peptides including adrenomedullin.

# POSTER ABSTRACTS

P-001

**Effects of adipokines on expression of endothelin-1 and adrenomedullin in bovine brain microvascular endothelial cells**

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Obesity is one of the major background factors for hypertension and type 2 diabetes mellitus, which constitute metabolic syndrome. Adipokines, such as leptin, resistin and tumor necrosis factor-alpha (TNF-alpha), have been implicated in the pathogenesis of hypertension and type 2 diabetes mellitus in obesity. It was reported that leptin or resistin induced endothelin-1 (ET-1) expression in vascular endothelial cells (Circ Res 2002; 90:711-8 and Circulation 2003; 108:736-40), whereas we could not find any significant effects of leptin or resistin on ET-1 expression in human umbilical vein endothelial cells (HUVECs) (Peptides 2005; 26:845-851). In the present study, we studied effects of three adipokines (leptin, resistin and TNF-alpha) and hypoxia (1% O2) on expression of ET-1 and adrenomedullin (AM) in bovine brain microvascular endothelial cells (BBMVECs). AM is a potent vasodilator peptide, which is secreted by various types of cells including vascular endothelial cells. Expression of ET-1 and AM mRNAs was studied by Northern blot analysis, and immunoreactive (IR)-ET-1 and IR-AM levels in the medium were measured by radioimmunoassay after the Sep-Pak C18 extraction. Leptin or resistin had no significant effects on expression of ET-1 or AM in BBMVECs. Expression of both ET-1 and AM mRNAs was induced by hypoxia, whereas IR-ET-1 levels in the medium were rather decreased by hypoxia. TNF-alpha decreased expression levels of ET-1 and AM mRNA, but did not affect IR-ET-1 or IR-AM levels in the medium. The present study has shown that leptin or resistin did not affect expression of ET-1 or AM in BBMVECs, consistent with our previous report on HUVECs (Peptides 2005; 26:845-851). Decreased expression of ET-1 and AM mRNAs by TNF-alpha in vascular endothelial cells may be related to some pathophysiology of metabolic syndrome.

P-002

**Phosphorylation of endothelin converting enzyme-1 isoforms: relevance to subcellular localization**

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Endothelin converting enzyme-1 (ECE-1) is a metalloenzyme with four subisoforms, which differ only in their N-terminal domain. ECE-1a and c are the most common isoforms and found at the plasma membrane and in the Golgi complex whereas ECE-1b displays lysosomal localization. We have recently shown that ECE-1a but not ECE-1b also colocalizes with nuclear membrane markers and maintenance of cells in high glucose (25 mM) promotes relocalization of ECE-1a from the membrane to the intracellular compartment. In order to investigate the mechanisms involved in this process, we conducted a search for potential phosphorylation sites, which yielded a different number of putative sites for PKC and PKA in the N-terminal region. Stimulation of Chinese Hamster Ovary cells expressing a green fluorescent protein (GFP)-tagged human ECE-1a or ECE-1b with 100 nM PMA resulted in phosphorylation of ECE-1a as determined by immunoprecipitation with an anti-GFP antibody followed by immunoblotting with an anti-phosphoserine antibody. Stimulation of cells with PMA also promoted intracellular relocalization as seen in cells grown under high glucose conditions. Incubation of cells grown in 25 mM glucose with the PKC inhibitor Calphostin C (100 nM) partially prevented the relocalization of ECE-1a from plasma membrane to intracellular compartments. Stimulation of cells with 100 nM forskolin caused phosphorylation of ECE-1b and not ECE-1a, which is consistent with the lack a putative PKA site in the ECE-1a N-terminal sequence. While phosphorylation is not required for ECE-1 enzymatic activity, these results suggest that ECE-1 isoforms are phoshorylated and that phosphorylation might play an important role in the regulation of intracellular trafficking of ECE-1 subisoforms.

P-003

**Expression and localization of endothelin converting enzyme-1 (ECE-1) isoforms in human endothelial cells**

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ECE-1 is an important metalloprotease responsible for production of vasoactive endothelin-1 (ET-1) from inactive big endothelin-1. ET-1 is involved in numerous disease states including hypertension and atherosclerosis, renal disease and many cancers, including prostate cancer. In humans, ECE-1 exists as four separate isoforms, ECE-1a, b, c and d. Previously it has been shown that ECE-1a and ECE-1c localize to the plasma membrane, ECE-1b to late endosomes and multivesicular bodies, and ECE-1d to recycling endosomes. Much controversy still exists over isoform localization, however. Recent research has shown the presence of both ECE-1a and ET-1 in the nucleus of endothelial cells. ECE-1 has also been shown to be present in Weibel-Palade bodies, endothelial cell-specific storage granules. The aim of this study, therefore, was to conclusively determine expression and localization of the ECE-1 isoforms in human endothelial cells. Isoform-specific polyclonal antibodies were used for immunofluorescence microscopy analysis of human umbilical vein endothelial cells (HUVECs) and EAhy926 cells (a transformed endothelial cell line); double labeling with organelle-specific markers was used to identify sub-cellular localization. Nuclear staining of both HUVEC and EAhy926 cells was seen using a monoclonal antibody which recognizes all ECE-1 isoforms. The ECE-1a antibody also showed nuclear staining in both cell lines. Intracellular staining was seen using the ECE-1c antibody but nuclear staining was absent, implying the nuclear isoform to be ECE-1a. The ECE-1b antibody showed intracellular staining consistent with lysosomes/endosomes; no nuclear staining was seen. Nuclear fractionation was used to isolate nuclei for western blotting to confirm microscopy findings. von Willebrand Factor (vWF) was used as a marker for Weibel-Palade bodies in HUVECs but no colocalization with ECE-1 was seen during this study using either monoclonal or polyclonal antibodies.

P-004

**Assay of endothelin converting enzyme using western blotting and autoradiography**

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A simple protocol to measure the activity of endothelin converting enzyme (ECE) is described herein. ECE enzyme converts Big endothelin (Big-ET-1, 4300 daltons, 38 amino acids) into endothelin-1 (ET-1, 22 amino acids, 2500 daltons). Endothelin is potent vasoactive peptide implicated in a number of disease states especially those of the cardiovascular system. The following study presents a new assay for assessing ECE activity, the rate limiting step in the formation of ET-1. Using special urea-acrylamide gels, small peptides can be resolved followed by electrotransfer of peptides to nitrocellulose membrane then immunoblotting using polyclonal anti Big ET-1 antibodies. In addition, iodinated radiolabeled Big ET-1 or custom-made substrate peptide can be used in the urea gels followed by exposing to film without the need for Western blotting. The procedure may be useful for other works in the endothelin arena.

P-005

**Control of the expression of endothelin-1 by AU-rich elements in the 3'-untranslated region of the gene**

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The regulation of the synthesis of the endothelial-derived vasoconstrictor endothelin-1 (ET-1) is a complex process that occurs mainly at the mRNA level. Transcription of the gene accounts for an important part of the regulation of the expression, as already described for different modulators such as the cytokine transforming growth factor-ß (TGF-ß). However, very little is known about mechanisms governing ET-1 expression at the post-transcriptional level. The aim of this study was to investigate the regulation of the ET-1 expression at this level. Since the 3'-untranslated region (3'-UTR) of mRNAs commonly contains genetic determinants for the post-transcriptional control of gene expression, we focused on the potential role of the 3'-UTR of ET-1 mRNA. Experiments performed with luciferase reporter constructs containing the 3'-UTR showed that this region exerts a potent destabilizing effect (80% reduction compared to constructs without the 3'-UTR). Deletional analyses allowed us to locate this activity within a region at positions 924-1127. Some (but not all) of the AU-rich elements (AREs) present in this region were found to be essential for this mRNA destabilizing activity. Cytosolic proteins from endothelial cells interact specifically with these RNA elements, and a close correlation exists between the ability of the AREs to destabilize ET-1 mRNA and the binding of proteins to these elements. Our results are compatible with the existence of a strong repressional control of ET-1 expression mediated by destabilization of the mRNA. This is probably exerted through the interaction between the AREs present in the 3'-UTR and specific cytosolic proteins whose identity is currently being analyzed. Moreover, regulation of ET-1 expression at the post-transcriptional level adds a new order of complexity and offers new targets to understand the balance of this vasoconstrictor at controlling vascular tone.

P-006

**Upregulation of endothelin receptor B in human endothelial cells by low-density lipoproteins: role of protein kinase C**

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Low-density lipoproteins (LDL) represent the most important treatable risk factors for coronary artery disease. Although it has been previously shown that hypercholesterolemia stimulates the endothelin system, the effects of increased levels of LDL on endothelial endothelin receptors have not been previously studied. In particular, the influence of native and oxidatively modified low-density lipoprotein and the regulatory mechanisms in endothelial cells are currently unknown. Human endothelial cells almost exclusively express the endothelin receptor type B ($ET_B$). Therefore, the effect of native and oxidized low-density lipoprotein (nLDL, oxLDL) on the expression of endothelin receptor B was studied in primary cultures of human umbilical vein endothelial cells (HUVEC). HUVEC were stimulated by nLDL and oxLDL in a time (1 to 24 h) and dose (25 to 100 µg/mL)-dependent manner. In order to analyze signal transduction pathways involved in the regulation of $ET_B$, protein kinase C was inhibited using 100 nM Ro-31-8220. The mRNA expression of endothelin receptor B was determined by quantitative RT-PCR and $ET_B$ protein expression by Western blot. Native LDL induced $ET_B$ mRNA after 1 h reaching its maximum at 100 µg/mL (199±35%, n=15, $P<0.05$ vs. control). Stimulation of HUVEC with oxLDL increased $ET_B$ mRNA expression (max. 1 h, 100 µg/mL oxLDL: 308±48%, n=15, $P<0.05$ vs. control) as well. The induction of $ET_B$ was also found on the protein level. Induction of endothelin receptor B expression by oxLDL is mediated by PKC. These data demonstrate that low-density lipoproteins even independent of oxidative modification are potent inducers of endothelial $ET_B$ receptors at the mRNA and protein level. Given the NO releasing capacity of endothelial $ET_B$ receptors, this effect may represent a possible vasoprotective mechanism in the endothelium of patients with hypercholesterolemia.

P-007

**Differential trafficking and desensitization of human ETA and ETB receptors in HEK 293 cells**

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Endothelin-1 (ET-1) is a potent vasoconstrictor acting on ETA and ETB receptors localized to smooth muscle cells. As vascular smooth muscle cells express both subtypes, it is difficult to study the subcellular localization and functional properties of each subtype or interactions between receptors. Therefore, we investigated the localization and function of ETA and ETB receptors transfected into HEK 293 cells. Confocal microscopy was used to examine co-localization of ET receptors with the plasma membrane marker, pan-cadherin, with the endosome marker, early endosomal antigen 1 (EEA1), and with the endoplasmic reticulum (ER) marker, calnexin. In cells transfected with ETA receptors, 83±2% of these receptors co-localized with pan-cadherin. In cells transfected with ETB receptors, 54±2% of the receptor co-localized with pan-cadherin; the remainder co-localized with EEA1 and calnexin. When ETA and ETB receptors were co-transfected, 97±1% of ETB receptors co-localized with ETA receptors and 84±2% of ETB receptors co-localized with pan-cadherin. ET-1 and sarafotoxin 6c (S6c, ETB receptor agonist) dose-dependently increased $[Ca^{2+}]_i$, (Fluo-4 fluorescence) in cells transfected with ETA or ETB receptors, in which 100nM of ET-1 and S6c resulted in maximal responses. When stimulated with ET-1(100nM), ETB receptors desensitized faster ($t_{1/2}$=21±1s) than ETA receptors ($t_{1/2}$=48±1s). S6c(100nM)-induced increases in $[Ca^{2+}]_i$ desensitized in cells expressing ETB receptors only ($t_{1/2}$=17±1s); S6c did not increase $[Ca^{2+}]_i$ in cells transfected with ETA receptors only. The $t_{1/2}$ for S6c- or ET-1-induced desensitization in cells co-transfected with ETA and ETB receptors was >400s. The data indicate that ETA receptors localize to the cell membrane while ETB receptors are in the cell membrane and intracellular compartments. Co-expressed ETA and ETB receptors are in the cell membrane. ETB receptors desensitize faster than ETA receptors but receptor co-expression eliminates desensitization. It is concluded that ETA and ETB receptors interact to change receptor trafficking and function. This interaction may modify ET receptor function in vascular smooth muscle cells that co-express these receptors.

P-008

**Effect of gene transfer of pro-opiomelanocortin on endothelin-1 release in cultured endothelial cells**

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Pro-opiomelanocortin (POMC) is the precursor of many neuropeptides like adrenocorticotropin hormone (ACTH), melanocyte-stimulating hormone (α-MSH), and the endogenous opioids, ß-endorphin (ß-EP). It is well known that many other cells like endothelial cells are both source and target of the POMC peptides. Besides having the ability to suppress local inflammatory reaction, these locally released POMC peptides can also cause local vasodilation, analgesia, as well as to promote wound healing. Endothelins are a family of endogenous peptides, mainly secreted by endothelial cells. ET-1 is also known to participate in vasoconstriction and angiogenesis during the wound healing and may have a role in pain signaling. It is believed that ET-1 almost exclusively act through autocrine/paracrine mechanisms. Aim: We attempted to employ gene delivery technique to transfer the POMC gene into the endothelial cells and explore the effect of such gene transfer on the alteration of ET-1 secretion. Method: ACTH overproduction was achieved in endothelial cells using adenovirus-mediated gene delivery of ACTH precursor gene, POMC. The cultured media of EA.hy926 endothelial cells were collected for ET-1, ACTH, and ß-EP assays after gene transfer. Result: The POMC-transduced EA.hy926 cells released significantly elevated levels of ACTH (274.5±13.6 pg/ml) and ß-EP (1992.3±76.7 pg/ml), which were 20-100 folds of that in cells of control groups ($P< 0.001$). These results indicate that POMC gene was effectively transduced by adenovirus vectors and processed into various neuropeptides in endothelial cells. POMC gene delivery also significantly attenuated the release of ET-1 in endothelial cells. The ET-1 level in the cultured media of Ad-POMC-infected cells (23.7±2.33 ng/ml) was significantly lower than that in control groups (42.15±0.93 and 43.7±3.7 ng/ml for control and Ad-GFP group, respectively; $P<0.001$). Conclusion: Interaction between POMC and ET-1 exists in endothelial cells and POMC gene transfer may be a tool to explore such interaction in vitro.

P-009

**Evaluating the effect of PKC, JNK, and ERK inhibitors on the upregulation of ETB receptor in rat basilar and mesenteric arteries**

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Introduction: We have previously showed that protein kinase C (PKC) and extracellular-regulated kinase (ERK 1/2) are involved in upregulation of ETB receptors in an organ culture model. The present study was designed to evaluate the importance of c-Jun N-terminal kinase (JNK) and the time dependency of the inhibitory effect of PKC, JNK, and ERK inhibitors on upregulation of endothelin B (ETB) receptors.

Methods: Rat basilar arteries were incubated for 24 hours and the PKC inhibitor RO-31-7549, the ERK 1/2 inhibitor SB386023, and the JNK inhibitor SP600125 were added after 3, 6, or 12 h of incubation. Vessel segments were mounted in myographs and the contractile responses to endothelin-1 (ET-1; ETA and ETB receptor agonist) and sarafotoxin 6c (S6c; ETB receptor agonist) were studied. The ETB and ETA receptor mRNA levels were determined with a real-time polymerase chain reaction (PCR). Results: The PKC and ERK inhibitors attenuated the contraction induced by S6c but not ET-1 in the basilar artery. This effect was less pronounced in mesenteric arteries. The efficiency of the inhibitors was proportional to the incubation time. The real-time PCR showed a decrease of the ETB receptor mRNA levels in arteries treated with PKC or ERK inhibitors, while the ETA mRNA levels were unchanged. The JNK inhibitor had a significant inhibitory effect on ETB receptor upregulation in the basilar artery but not in the mesenteric artery. The ETB receptor mRNA levels were decreased in basilar arteries treated by JNK inhibitor. Conclusion: Our results show that the PKC, ERK, and JNK are more important in the upregulation of contractile ETB receptors in cerebral arteries compared to mesenteric arteries. Since there is similar upregulation in cerebral arteries in models of focal ischemia and subarachnoid haemorrhage, this inhibition may provide a new therapeutic approach. Furthermore, the evaluation of the time dependency of the inhibitors is of great importance, when considering therapeutic possibilities.

P-010

**Post-transcriptional regulation of endothelin-1: role of verotoxins**

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*E. coli* O157:H7 derived verotoxins (VTs) can activate the microvascular endothelium of VT-target organs, leading to vascular injury and hemolytic uremic syndrome (HUS). A hallmark of endothelial activation is the induction of the vasoconstrictor and cellular mitogen, endothelin-1 (ET-1). Consistent with these observations, our lab previously found that VT-1 and VT-2 can induce ET-1 expression through stabilization of the short-lived ET-1 mRNA (Ref 1). In other studies, we found that baseline ET-1 mRNA turnover is regulated by an AU-rich element (ARE) within its 3'-untranslated region (3'-UTR) (Ref 2). We therefore hypothesized that VTs may stabilize the ET-1 transcript by disrupting ARE-dependent mRNA turnover pathways. To test this hypothesis we expressed ET-1 3'-UTR reporter transcripts in the presence or absence of VT-2. VT-2 induced reporter expression in an ET-1 3'-UTR-dependent manner. Mutation of a functionally important 3'-UTR $AU_nA$ motif (nucleotides 978-987) attenuated VT-2-mediated reporter induction whereas mutation of a closely juxtaposed $AU_nA$ motif (938-947) had no effect, suggesting VT-2 stabilizes the ET-1 mRNA via a specific 3'-UTR verotoxin response element (VRE). Because ET-1 mRNA stability is regulated by the ARE RNA binding protein AUF1, heat shock, and the ubiquitin-proteasome pathway (Ref 2), we studied the effect of VT-2 on the expression of AUF1, HSP70 (heat shock protein 70), and protein-ubiquitin conjugates. Although VT-2 did not change steady-state AUF1 or HSP70 protein levels, VT-2 did elicit a transient and reversible decrease in total cellular protein-ubiquitin conjugates, suggesting that VT-2 may stabilize ET-1 mRNA through dynamic modulation of the ubiquitin proteasome pathway. Taken together, our data suggests VTs may contribute to HUS by modulating ET-1 post-transcriptional regulatory pathways via the ubiquitin-proteasome pathway.

Ref 1. Bitzan et al, J Clin Invest 1998, 101(2).

Ref 2. Mawji et al, J Biol Chem 2004, 279(10).

P-011

**Cerivastatin, a hydroxymethylglutaryl coenzyme A reductase inhibitor, suppresses endothelin-1 production through the activation of endothelial nitric oxide synthase**

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It has been reported that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) produce a variety of cardiovascular protective effects independent of their ability to lower total and low-density lipoprotein (LDL) cholesterol. Recent studies have also reported that statins produce pleiotropic effects through an improved endothelial functions, enhanced fibrinolysis, and antithrombotic actions. In the present study, we examined the effects of pitavastatin, pravastatin, atorvastain, and cerivastatin on edothelin-1 (ET-1) production in cultured porcine aortic endothelial cells (PAECs). Treatment with cerivastatin but not pitavastatin, pravastatin, and atorvastatin decreased basal and TNF-$\alpha$-stimulated ET-1 release from PAECs in dose-dependent manner (1-10 μM). Although a brief exposure (1, 2, and 6 hours) of cerivastatin (10 μM) failed to reduce basal and TNF-$\alpha$-stimulated ET-1 release from PAECs, pretreatment of cerivastatin for 24 hours produced significant decreases in ET-1 release in basal and TNF-$\alpha$-stimulated conditions. Northern blot analysis showed that cerivastatin markedly suppressed ET-1 mRNA expression in both conditions. In addition, these inhibitory effects of cerivastatin on ET-1 release and preproET-1 mRNA expression were completely abolished by the combination of mevalonate (200 μM). We next investigated the mechanism which cerivastatin suppresses ET-1 production in PAECs. Cerivastatin had no inhibitory effects on the activation of nuclear factor-kappa B which plays an important role in the regulation of ET-1 gene expression. On the other hand, cerivastatin did not have any effects on endothelial nitric oxide synthase (eNOS) protein levels but induced eNOS phosphorylation at Ser-1177. Taken together with our present results, it is most likely that cerivastatin suppresses ET-1 production possibly through an increase in eNOS activity and the subsequent enhanced NO production in PAECs. These findings also suggest that cerivastatin may have beneficial effects on various ET-1-related diseases.

P-012

**Adenosine triphospate, via the P2Y2 receptor, inhibits endothelin-1 release from inner medullary collecting duct cells**

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Adenosine triphosphate (ATP) and endothelin-1 (ET-1) have both been shown to inhibit vasopressin-stimulated water reabsorption in the inner medullary collecting duct (IMCD). Since both ATP and ET-1 are released by the IMCD and can act in an autocrine manner to regulate IMCD water transport, we sought to determine if these factors can modulate the other's production. To begin such studies, the effect of ATP on IMCD ET-1 production was examined. ATP caused a dose-dependent inhibition of immunoreactive ET-1 release and ET-1 mRNA levels in primary cultures of rat IMCD cells. This effect was first manifest after 4 hr of exposure to ATP and persisted for at least 24 hr. The lowest concentration of ATP that was inhibitory was 10 μM and the maximal response was seen at 100 μM (46% reduction of ET-1 release). ATP acted through the P2Y2 receptor since its effect was mimicked by UTP, but not by the P2X agonist $\alpha$,ß-methylene-ATP. L-NMMA, indomethacin, U-73122, or calphostin C did not block the ATP inhibitory effect, indicating ATP acts through NO-, prostaglandin-, phospholipase C-, and PKC-independent pathways, respectively. There was no evidence that alterations in calcium signaling were involved since BAPTA-AM, nifedipine, or W-7 (calmodulin inhibitor) did not affect the ATP response. SP600125 (JNK inhibitor) and SB203580 (p38 MAPK inhibitor) did not affect the ATP response, however PD98059 (MEK inhibitor) did significantly abrogate ATP inhibition of ET-1 release. In summary, these data demonstrate the ATP inhibits IMCD ET-1 release associated with reduced mRNA levels and that this effect is dependent, at least in part, upon MEK activation. These findings suggest that ATP and ET-1, while both antagonizing AVP action in the IMCD, may have a complex interaction that ultimately determines the degree to which they each participate in modulating collecting duct function.

P-013

**cDNA cloning and sequence analysis of preproendothelin-1 from salmon, Oncorhynchus keta**

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Endothelin- (ET) like immunoreactivity has been observed not only in mammals but also in fish. The biological actions of ET are similar in fish and mammals. To identify ET-related peptides in fish, we screened the salmon stomach cDNA library using the rapid amplification of cDNA ends method and cloned cDNAs encoding preproendothelin-1 (PPET-1). The deduced amino acid sequence of salmon PPET-1 comprises 244 amino acids, including a putative signal sequence of 23 amino acids and a mature ET-1 of 21 amino acids, as well as big ET-1 and ET-1-like sequences. The salmon stomach ET-1 sequence is identical to the ET-1 peptide recently purified from trout, Oncorhynchus mykiss, kidney by Wang Y et al.(1999). This sequence, together with other published PPET sequences, was used to analyze the phylogenetic relationship among all ET family genes.

P-014

**The combined ECE and neutral endopeptidase (NEP) inhibitor SLV306 inhibits systemic endogenous conversion of infused big endothelin-1 in human volunteers**

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The aim of this study was to determine whether SLV306 inhibited the systemic conversion of big ET-1 to the mature peptide and the C-terminal fragment (CTF). On 4 separate occasions, following the oral administration of one of three increasing doses of SLV306 (to reach an average target concentration of 75, 300, 1200 ng ml-1 of the active metabolite KC12615) or a placebo, in a randomized, double blinded regime, big ET-1 was infused into 13 healthy male volunteers (mean age 23 years). Big ET-1 was administered at 8 and 12 pmol kg-1 min-1(20min each). Plasma samples were collected pre, during and post big ET-1 infusion. Immunoreactive (IR) ET, big ET-1 and CTF was measured by RIA. The infusion of big ET-1 resulted in an increase in a systemic IR big ET-1 by two orders of magnitude above basal levels in the placebo group. SLV306 dose dependently caused a significant rise in circulating big ET-1 levels (compared with placebo) indicating that at the two highest doses, SLV306 was inhibiting an increasing proportion of endogenous conversion activity. In the placebo group, levels of the CTF increased an order of magnitude above basal confirming a proportion of the infused big ET-1 was being selectively converted as expected. In the presence of the highest two doses of SLV306, a small increase in the CTF was observed, consistent with our previous studies with the dual NEP/ECE inhibitor, phosphoramidon since CTF is also a substrate for metabolism by NEP and inhibition of this enzyme by SLV306 may reduce proteolysis. NEP is also thought to metabolize ET-1 to biologically inactive fragments. Intriguingly, despite inhibition of NEP activity, there was no increase in levels of mature ET. These results suggest that in the presence of SLV306, the biologically active peptide may continue to be removed beneficially from the circulation by clearing receptors. Overall, the results suggest that SLV306 can inhibit the conversion of big ET-1 and may be of benefit in cardiovascular disease where big ET-1 levels are elevated, particularly in human atherosclerosis where enzymatic conversion of big ET-1 is significantly upregulated .

P-015

**MAPK, PKC and PI3-K are Involved in endothelin-1-induced astrocyte proliferation**

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Purpose: ET-1 is a potent mitogen for many cells especially when ET-1 levels are elevated under pathophysiological conditions. However, the signal transduction pathway utilized by ET-1 in astrocyte proliferation is not clear. In the present study, the signaling pathways involved in ET-1-mediated astrocyte proliferation were determined. Methods: Specifically, we focused on the involvement of the MAPK, PKC and PI3 kinase signal pathways in cell proliferation after treatment with ET-1 in U373MG astrocytoma in culture. A formazan MTT assay and [3H]-thymidine incorporation assay were used for quantifying cell proliferation. Phosphorylation of ERK1/2, PKC, and Akt was detected by western blot analysis. A kinase assay was employed to detect the activities and translocation of PKC in membrane and cytosolic fractions. Changes in [Ca2+]i levels were determined by Fura-2 calcium imaging. Results: ET-1 caused a rapid phosphorylation of ERK1/2, which could be blocked by treatment with PD98059 and U0126 (MEK inhibitors). While PKC inhibitor chelerythrine attenuated ET-1-induced cell proliferation, it was unable to block ET-1-induced ERK phosphorylation. In U373MG cells, ET-1 did not activate PKCs (c- and n-PKCs) and did not elevate [Ca2+]i. U73122 (a phospholipase C inhibitor) also had no effect on ET-1-induced ERK1/2 phosphorylation. FTI-277 and genistein, AG82 and herbimycin A did not abolish the ERK1/2 phosphorylation. LY294002 completely blocked the phosphorylation of Akt and cell proliferation, but did not block the phosphorylation of ERK1/2. Conclusions: ET-1 activates phosphorylation of ERK1/2, which plays an important role in astroglial proliferation for U373MG astrocytoma cells. Conventional and novel PKCs appear not to be involved in astrocyte cell proliferation in U373MG cells. The PI3 kinase pathway is involved in signal transduction induced by ET-1, but it does not appear to participate in crosstalk with the MAPK pathway. The results suggest that ET-1 stimulates cell proliferation by the activation of MAPK-, PKC- and PI3K-dependent pathways that appear to function in a parallel manner. There is no apparent, direct crosstalk between these three pathways in U373MG cells.

P-016

**The extracellular N terminus of the human endothelin B receptor is required for biphasic ERK1/2 activation and re-differentiation of vascular smooth muscle cells**

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Protein analysis of the human endothelin B (ETB) receptor, which consists of 442 amino acids (aa), demonstrated the existence of two different isoforms: i) a full-length ETB receptor lacking the signal peptide (aa 27-442); ii) an N-terminally truncated ETB receptor lacking further 38 aa C-terminal of the signal peptide cleavage site (aa 65-442). IRL 1620 treatment of vascular smooth muscle cells (VSMCs) expressing the full-length or the N-terminally truncated ETB ($\Delta$2-64) receptor (corresponds to the short isoform) induced a biphasic and monophasic ERK1/2 activation, respectively. The second phase, but not the first was mediated via ß$\gamma$-subunits of Gi proteins and required transactivation of the epidermal growth factor (EGF) receptor. EGF receptor transactivation was due to the release of heparin-binding EGF (HB-EGF), as it was abolished by inhibitors of matrix-metalloproteases and by downregulation of HB-EGF with CRM197. The ETB receptor-mediated biphasic ERK1/2 activation was linked to re-differentiation of VSMCs as IRL1620 induced an increase in smooth muscle myosin heavy chain (SM-MHC) promoter activity and promoted smooth muscle myosin, SM22$\alpha$ and $\alpha$-actin protein expression. In contrast, no differentiation was observed in VSMCs, expressing the N-terminally truncated ETB receptor. The finding that the ETB receptor stimulated re-differentiation of VSMCs depended on the transactivation of the EGF receptor and biphasic ERK1/2 activation was confirmed by experiments using tyrphostin AG1478 (an inhibitor of EGF receptor tyrosine kinase), pertussis toxin (PTX) and matrix-metalloprotease inhibitor, which abrogated both the second phase of ERK1/2 activation and re-differentiation of VSMCs.

P-017

**Involvement of Rho-associated kinase in endothelin-induced contraction in rat aorta**

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In this study, we used isolated rat aorta without endothelium. In $Ca^{2+}$-free solution or in the presence of the myosin light chain kinase (MLCK) inhibitor wortmannin (WM) of 3 μM, 100 nM endothelin-1 (ET-1) evoked a sustained contraction of 26 % of the ET-1 (100 nM)-induced control contraction. This 26 % contraction was completely inhibited by the Rho-kinase inhibitor Y-27632 of 10 μM and also by 1 μM sodium nitroprusside (SNP). Post-application of 3 μM WM incompletely attenuated the ET-1 (100 nM)-induced contraction in the normal physiological salt solution (PSS), leaving the contraction of 12 %. This remaining contraction was inhibited by 10 μM Y-27632 or 1 μM SNP. Post-application of 1 μM SNP inhibited the ET-1 (100 nM)-induced contraction in the PSS by only 13 % and the remaining contraction was entirely inhibited by 3μM WM. These results suggest that SNP specifically inhibits the $Ca^{2+}$-independent and WM-insensitive contraction induced by ET-1 in rat aorta. However, the inhibition amplitude of the ET-1-induced contraction in the PSS by SNP was a half of that of the contraction in the $Ca^{2+}$-free solution or in the presence of WM. Pre-treatment with Y-27632 in the normal PSS allowed the production of a short-lasting contraction by ET-1 which was finally attenuated until the base level. On the other hand, pre-treatment with Y-27632 in the $Ca^{2+}$-free solution or combination of WM and Y-27632 completely prevented the ET-1-induced contraction. These results suggest that the contraction by MLC-phosphorylation occurred as an initial event, thereafter the contraction was relaxed by activation of MLC phosphatase (MLCP) by inhibition of Rho-kinase, and the contraction independent of MLCK pathway is caused by the MLC-phosphorylation by Rho-kinase. In conclusions: Rho-kinase plays a role in $Ca^{2+}$ sensitization by both MLCP inhibition and MLC-phosphorylation in ET-1-induced contraction of rat aorta. Contribution of the MLC-phosphorylation by Rho-kinase to the contraction is only 13 % in the normal ET-1-induced contraction, but as the MLCK pathway is inhibited, the MLC-phosphorylation activity of Rho-kinase increases.

P-018

**Gene expression mediated by endothelin-1 in normal and scleroderma dermal fibroblasts**

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Endothelin-1 (ET-1) is important in the early pathogenesis of fibrotic and inflammatory diseases such as scleroderma. In addition to modulating vascular tone and extracellular matrix turnover, ET-1 released from activated endothelium may have a role in altering the phenotype of fibroblasts in the underlying connective tissue, by upregulating key genes. These studies analyze the effect of ET-1 exposure on normal (NDF) and scleroderma (SDF) dermal fibroblasts on the expression of cytokine and extracellular matrix genes. SDF were exposed to ET-1 [100nM] for increasing periods of time, and mRNA extracted and analyzed via lightcyling PCR. Samples were compared with age-matched controls. 9 genes were examined: ET-1, IL-1ß, TGFß, CTGF, TSP-1, ICAM-1, MMP-1, TIMP-3, and COL1A1. Analysis found that ET-1 is capable of directly up-regulating matrix and, importantly, cytokine genes in NDF. The ability of ET-1 to upregulate these genes is greatly diminished in SDF, highlighting previous exposure to the cytokine during pathogenesis. Also, the genes show two distinct patterns of regulation, with cytokines genes and ICAM-1 displaying different responses to the extracellular matrix genes. ET-1 upregulates ICAM-1 via binding of an NFkB site <200bp from the TATAA box. In silico analysis of the gene promoters reveals that NFkB sites exist within 500bp of the TATAA in the cytokine genes, but over 1500bp in the ECM genes. This may be important regarding the efficiency with which ET-1 mediates expression. In future studies we will use ChIP to examine NFkB sites to these sites. Comparative analysis of ET-1 responses in NDF versus SDF highlighted different signal transduction pathways controlling the two sets of genes. This expands our knowledge of both endothelin biology, and has an impact of our understanding of scleroderma pathology and the role of endothelin within it.

P-019

**Multiple signaling pathways of endothelin type B receptor in rat median eminence**

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Endothelins (ETs) are a family of isopeptides (ET-1, ET-2 y ET-3), which exhibit distinct pharmacological actions throughout the activation of two receptor subtypes. The pathways stimulated downstream by ETs appear to be complicated and diverse; but commonly, stimulation of ETA and ETB receptor induces the well-known phosphoinositides (PI) metabolism. Additionally, the mechanism of nitric oxide synthase (NOS) activation is diverse and could be associated to calcium mobilization induced by receptor mediated PI breakdown. We assessed the possible link between ET-receptor mediated PI turnover and NO/cGMP signaling pathways, in rat median eminence (ME) fragments, a brain structure known to contain a rich plexus of NOS-containing neurons and fibers, together with densely arranged endothelin ETB-receptors-like immunoreactive fibers. Male Sprague-Dawley rats were sacrificed and tissues microdissected. NOS activity was assayed by monitoring the conversion of radiolabelled L-arginine to L-citruline. cGMP was determined by radioimmunoassay and the phosphoinositide hydrolysis was assessed as accumulation of inositol monophosphates (InsP1) in the presence of LiCl. Our data show that ET-1, ET-3 and IRL 1620 increase InsP1 accumulation, NOS activity and cGMP formation, in a similar degree. ETs-stimulatory effect on InsP1 accumulation and cGMP formation was inhibited by neomycin and the absence of extracellular calcium, suggesting that calcium is involved in endothelin receptor-induced phospholipase C activation. L-NAME, inhibited ET-1 or IRL1620-stimulated cGMP formation. BQ 123 did not alter, and BQ788 inhibited ETs-induced increase in the PI metabolism, NOS activity and cGMP generation. Our data indicate that in the ME, ETB receptor signals through receptor mediated PI breakdown and activation NOS/cGMP signaling pathway.

P-020

**Activation of protein kinase-A by endothelin-1 in vascular smooth muscle cells**

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Endothelin-1 (ET1) stimulates hypertrophy of vascular smooth muscle cells (VSMC) through diverse signaling pathways. We have found that despite its minimal effect on cAMP levels, ET1 stimulates the activity of protein kinase A (PKA) in VSMC, as profoundly as the beta-adrenergic agonist, isoproterenol (ISO). However, PKA activation by ET1 differs from that induced by ISO in the following key ways. 1) The duration of PKA activation by ET1 is much shorter than that induced by ISO. 2) The transient PKA activation by ET1 occurs by a distinct (from that of ISO) mechanism involving Gq/Gi heterotrimeric G proteins (assessed by using pertussis toxin or adenovirus-mediated expression of the regulator of G protein signaling, RGS3), MAP kinase-dependent cPLA2 activation and COX1-mediated prostaglandin release; this is accompanied by a simultaneous activation Ca/Calmodulin-dependent phosphodiesterase that contributes to a transient duration of PKA activation by ET1. 3) A transient PKA activation by ET1 promotes the hypertrophic growth of VSMC (a new finding), whereas a sustained PKA activation by ISO inhibits VSMC growth as expected (the role of PKA was assessed by using adenovirus-mediated expression of PKA inhibitor, PKI, as well as by pharmacological inhibition of COX1. 4) The PKA-dependent component of the hypertrophic signaling of ET1 is partially mediated by PKA-dependent phosphorylation and inactivation of glycogen synthase kinase-3 (GSK3), an enzyme that regulates cell growth by multiple mechanisms. 5) Finally, when activated by ET1, PKA phosphorylates a complement of other proteins, some of which are different from those phosphorylated by PKA during ISO stimulation, as assessed by phospho-proteomics approach. Moreover, the same proteins, in the same VSMC, can be phosphorylated by PKA at different sites, dependent on the mode of PKA stimulation (ET1 vs. ISO), as assessed by 2D-electrophoresis. We have developed a method for isolation and identification of the proteins that are phosphorylated by PKA in intact cells, which will lead us to (i) identification of the novel PKA substrates, and (ii) understanding a new, PKA-dependent mechanism, by which ET1 stimulates hypertrophy of VSMC.

P-021

**The Role of Nitric Oxide and ET-1 in the Pathobiology of Cardiovascular Diseases, Tumors and Neurodegeneration**

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The exact role of nitric oxide (NO) and endothelin-1 (ET-1) in the pathogenesis of human diseases especially atherosclerosis, tumors growth and neurodegeneration such as AD remains unclear and controversial. The development and progression of atherosclerotic lesions in human as well as in WHHL rabbits and YOS rats is associated with increase of NOS2 and ET-1 immunoactivity. In contrast, there is a significant reduction of immunoreactivity for NOS1 in aortic endothelial cells (EC), but no change in NOS3 immunoreactivity in human and WHHL rabbits. However, aortic EC from YOS rats shows significantly decrease in NOS3 immunoreactivity. The subendothelial macrophages and smooth muscle cells (SMC) showed a different pattern of immunoreactivity of NOS1, NOS2 and ET-1. The lipid-rich macrophages in the intima and SMC in the subendothelium and the medial layers of the vascular wall were also positive for these markers. We have also extended our study to human tumors. Dramatically lower NOS1 immunoreactivity was observed in colorectal liver tumor vascular endothelium. As compared to control groups there were significantly less NOS3 immunopositive EC in metastatic tumor vessels. A striking rise of NOS2 was observed in tumor vessel endothelium. ET-1 immunoreactivity level was also significantly higher in tumor vessel endothelium and was accompanied by the increased expression of NOS1-3 and ET-1 immunoreactivity in liver parenchymal and malignant brain tumor cells. Research exploration supports the theory that this molecule appears to be one of the key factors for the disruption of normal brain homeostasis which causes the development of brain lesions and pathology such as AD. Especially the vascular content of NO activity appears to be a major contributor to this pathology before the overexpression of NOS activity in other brain cellular compartment develops. We speculate that pharmacological intervention using NO donors and/or suppressors and selective ET-1 receptors antagonist drugs could delay or minimize the human pathologic process and may eventually be considered as a specific treatment for diseases conditions at their earlier stages.

P-022

**The role of endothelin-1 in extracellular matrix remodeling**

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Background: Systemic sclerosis is a chronic autoimmune multisystem disorder characterized by excessive accumulation of extracellular matrix (ECM) and fibrosis of skin and internal organs such as the heart and lungs. There is a bunch of evidence implicating the vaso-constrictive peptide endothelin-1 (ET-1) as a pro-fibrotic mediator. CD40/CD40L system has been known to be involved in the pathogenesis of various fibrotic diseases. We and others have shown that CD40 expression is elevated in SSc skin fibroblasts. Interferon gamma (IFN-γ) is the well-known powerful stimulator of CD40 expression. However, IFN-γ expression in involved skin of SSc is not elevated. Objective: The objective was to demonstrate the role of ET-1 in terms of CD40 expression on skin fibroblasts and ECM contraction. Methods: We conducted FACS analysis for CD40 expression on skin fibroblasts and collagen gel contraction assay for ECM remodeling with or without pre-treatment of ET-1. Results: ET-1 (100nM) up-regulates intercellular adhesion molecule, but not CD40 on normal skin fibroblasts. Normal skin fibroblasts incubated with ET-1 enhances significantly collagen gel more than cells without ET-1. ETA receptor antagonist, BQ-123, and ET A+B antagonist, but not ETB receptor antagonist BQ-610 or BQ-788 blocked ET-1 mediated gel contraction. Conclusion: ET-1 promotes the ability of skin fibroblast to contract ECM through ETA receptor. Therefore, neutralizing ET-1 or blocking ETA receptor may be beneficial in decreasing skin fibrosis in patients with systemic sclerosis.

P-023

**Endothelins and extracellular matrix protein production in diabetes**

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Increased extracellular matrix (ECM) protein production is a characteristic feature of diabetic angiopathy. We have previously demonstrated that endothelin (ET) blockade may prevent capillary basement membrane thickening and increased fibronectin (FN) production in diabetic retinopathy. In this study, we investigated the mechanisms of glucose-induced increased FN production by endothelial cells. Human Umbilical Vein Endothelial Cells (HUVECs), exposed to various glucose levels, lead to significant increase in FN mRNA and protein expression peaking at 24 hours. Similar upregulation was noted when HUVECs were exposed to ET-1. Glucose- as well as ET-induced FN upregulation was associated with mitogen-activated protein kinase (MAPK) and protein kinase B (PKB) activation. Furthermore, transcription co-activator p300 was upregulated in association with activation of transcription factors, NF-kB and AP-1. To further investigate the mechanisms leading to glucose-induced and ET-mediated FN synthesis, we used several inhibitors of specific pathways. Dual ET receptor antagonist bosentan prevented increased FN synthesis and activation of MAPK, PKB, and transcription factors NF-kB and AP-1. Such normalization of FN was also achieved by dominant negative PKB transfection and by PKB inhibitor ML-9 and by MAPK blockers. Interaction of these pathways with protein kinase C (PKC) in FN synthesis was also observed following specific PKC inhibition. Furthermore, p300 gene silencing prevented transcription factor activation and increased FN synthesis. Data from this study suggests that interaction of multiple signaling pathways lead to the activation of transcription factors and increased glucose-induced, ET-mediated, FN synthesis. ET may, therefore, be a potential treatment target to prevent such alterations. Supported by grants from Canadian Diabetes Association and the Canadian Institutes of Health Research.

P-024

**Constrictor Responses to ET-1 are Increased in Human Atherosclerotic Coronary Artery and in Aorta from a Novel Mouse Model for Conditional Ablation of Vascular Smooth Muscle Cells**

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Apoptosis of vascular smooth muscle cells (VSMC) occurs in human atherosclerosis resulting in a thinned media and plaque rupture. Tissue and plasma levels of endothelin-1 (ET-1) are increased in human atherosclerosis with enhanced endothelin-converting enzyme activity in human coronary artery (CA). We have discovered there is no compensatory down-regulation of ET receptors in the media of diseased CA and no decrease in vasoconstrictor response to ET-1 despite a thinned medial layer. Indeed vasoconstriction to ET-1 in atherosclerotic CA is enhanced compared to normal CA from donor hearts. To further understand the role of VSMC loss on vascular reactivity we have investigated the consequence of VSMC apoptosis in transgenic mice (SM22$\alpha$DTR) expressing human diphtheria toxin receptor under the minimal SM22$\alpha$ promoter on the response to ET-1. Exposure of these mice to 1ng/g diphtheria toxin (DT) for 42 days results in a loss of VSMC from the media of arteries. Aortae were compared from SM22$\alpha$DTR mice not exposed (controls) or exposed (ablated) to DT using wire myography. Following normalisation, cumulative concentration response curves were constructed to ET-1 (0.1-300nM) and experiments terminated by addition of 95mM KCl to determine the maximum possible contractile response in mN/mm. ET-1 responses were expressed as a % of this KCl response. Data were analysed to determine values of potency ($pD_2$) and maximum response ($E_{max}$). Values are mean±sem from n mice. There was a significant loss of VSMC in ablated (n=8) compared to control (n=5) mice (2366±110 vs 4149±122 cells/mm$^2$). The response of aortae from control and ablated groups to KCl was not different (0.99±0.17 vs 1.06±0.19mN/mm). ET-1 produced little or no response in aorta from control mice with an increase in maximum response in aorta from the ablated group that reached significance at 10nM ET-1 (control 0.99±0.99% KCl: ablated 31.35±13.39% KCl). These data suggest that this model of VSMC ablation may be of use in exploring the mechanisms underlying the augmented vasoconstrictor response to ET-1 in human atherosclerosis.

P-025

**Effect of endothelin (ET) on Na/H exchanger (NHE) activity of human monocytes and atherosclerosis-related functions**

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The aim of the present study is to investigate the influence of high concentrations of ET on human monocyte sodium/hydrogen exchanger (NHE) activity and on the atherosclerosis-related monocyte functions. NHE is an integral membrane protein that exchanges one intracellular H+ ion for an extracellular Na+ ion. It plays an essential role in all cell types regulating cell volume and internal pH (pHi). ET is a potent vasoconstrictive and mitogenic 21-amino-acid peptide produced by endothelial cells that has been implicated in the pathogenesis of atherosclerosis. Monocytes are key contributors to the atherosclerotic process. Monocytes attach to endothelial cells, migrate into the subendothelial space and differentiate into macrophages. Uptake of oxidized low-density lipoproteins (LDL) via scavenger receptors CD36 leads to foam cell formation, the initiating stage of the atheromatic lesion. We studied the effect of increased ET concentrations (10pg/ml) on the pHi of monocytes using the fluorescent indicator BCECF-AM. The effect of high ET concentrations (10pg/ml) on the ability of monocytes to bind on laminin and to migrate on trans-well culture inserts was estimated. We also measured the density of CD36 receptor using a fluorescein isothiocyanate (FITC)-linked anti CD36 monoclonal antibody. ET caused an increase in pHi ($p<0,05$). The stimulation of NHE was verified by measurements of the effect of ET in the presence of the NHE inhibitor, cariporide (20nM). In this case a reversal of ET effect on pHi was observed ($p<0,05$). Furthermore, after ET treatment an increase ($p<0,005$) of the adhesive capacity of monocytes was observed, but by using cariporide this increase was abolished ($p<0,005$). Similarly, ET also enhanced ($p<0,0001$) monocyte migration on laminin. When cariporide was added, this effect of ET was abolished ($p=0,0001$) as well. Finally, ET significantly increased ($p<0,005$) the number of CD 36 on the surface of monocytes as compared to controls. This effect was not seen after inhibition of NHE ($p<0,0005$).

P-026

**Endothelin-A receptor blockade does not impair the cardiovascular and hormonal adaptation to anesthesia with xenon or isoflurane in dogs**

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The objective of this study was to investigate whether circulatory and hormonal changes during xenon/remifentanil or isoflurane/remifentanil anesthesia are altered by endothelin-A ($ET_A$) receptor blockade. **Methods:** Nine beagle dogs were studied in 4 protocols (n=6). Controls: after a 30-min awake period, anesthesia was induced iv and maintained with isoflurane/remifentanil (Protocol 1) or xenon/remifentanil (Protocol 2) for one hour. Time course in protocols 3 and 4 was equal to protocols 1 and 2 but was preceded by $ET_A$ blockade with ABT-627 (Atrasentan®) iv. **Results:** Irrespective of $ET_A$ blockade, mean arterial blood pressure (MAP) ranged between 92 and 96 mmHg in the awake state and fell to 67±3 (Controls; mean±SEM) and 64±2 mmHg (ABT) during isoflurane/remifentanil anesthesia, whereas MAP remained constant during xenon/remifentanil anesthesia. Heart rate decreased during either form of anesthesia, but to a greater extent during xenon/remifentanil anesthesia. In Controls as well as in ABT-treated dogs, the decrease in cardiac output from a baseline of about 2.3 L/min to 1.2 L/min during isoflurane/remifentanil was greater during xenon/remifentanil anesthesia where cardiac output fell from 2.3 L/min to 0.9 L/min. Systemic vascular resistance increased by 30 % during isoflurane/remifentanil and by 120% during xenon/remifentanil anesthesia with and without $ET_A$ blockade. Hormonal alterations during anesthesia remained unaffected by $ET_A$ blockade. Angiotensin II increased similarly in all protocols, vasopressin increased to a greater extent during xenon/remifentanil anesthesia, and adrenaline and noradrenaline plasma concentrations rose during xenon/remifentanil anesthesia only. Endothelin plasma concentrations were not substantially changed in response to $ET_A$ blockade or following anesthesia. **Conclusions:** The hemodynamic and hormonal reactions following xenon/remifentanil and isoflurane/remifentanil anesthesia do not depend on the endothelin system, since they are unaffected by $ET_A$ receptor blockade. Therefore, the use of Atrasentan does not impair cardiovascular stability during xenon or isoflurane based anesthesia in our dog model.

P-027

**Endothelin Converting Enzyme (ECE) is Present and Functional in Rat Thoracic Aorta and Vena Cava**

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In DOCA-salt hypertension, aortic but not venous ET-1 levels are elevated. A historical report suggests that ECE, a metalloprotease that cleaves big ET-1 to ET-1, is functional in rabbit saphenous artery but not saphenous vein. We challenge this hypothesis by proposing that ECE is present and functional in both thoracic aorta and vena cava from normotensive sham and DOCA-salt hypertensive rats. Immunohistochemically, we detected ECE on adventitial and endothelial cells of thoracic aorta from sham and DOCA-salt rats, and throughout the vessel wall of thoracic vena cava from sham and DOCA-salt rats. ET-1 staining was present throughout sham aorta and vena cava, suggesting ET-1 is synthesized via ECE in these tissues. We examined the effects of two ECE/neutral endopeptidase (NEP) inhibitors, phosphoramidon and CGS-26393 (CGS), on big ET-1-induced contraction in sham aorta and vena cava. Phosphoramidon (10 μM) significantly reduced maximal aortic big ET-1-induced contraction [control: 48.6±16.0% of initial phenylephrine (PE, 10 μM) contraction; phosphoramidon: 20.1±9.6%], and venous contraction [control: 478.1±61.5% of initial norepinephrine (NE, 10 μM) contraction; phosphoramidon: 176.5±35.2%]. CGS (10 μM) significantly reduced maximal aortic big ET-1-induced contraction (control: 83.9±2.9%; CGS: 21.5±1.6%), but not maximal venous contraction (control: 349.6±43.9%; CGS: 281.5±53.4%). Thiorphan (10 μM), a specific NEP inhibitor had no effect on maximal aortic or venous ET-1-induced contraction. We compared big ET-1-induced contraction of aorta and vena cava from sham and DOCA-salt rats. Maximal big ET-1 (100 nM) -induced contraction was significantly reduced in aorta from DOCA-salt rats [DOCA: 17.7±5.3%; sham: 60.7±17.8%], but not vena cava from DOCA-salt rats [DOCA: 479.1±51.3%; sham: 478.1±61.5%], suggesting that aorta from DOCA-salt rats are desensitized to big ET-1, while vena cava are not. Our findings suggest that ECE is present in aorta and vena cava, and that differences in ET-1 synthesis between aorta and vena cava of hypertensive rats are not caused by differences in ECE activity.

P-028

**Potent Contractions and Distinct Contractile Dynamics in Response to Endothelin-1 in Murine Arteries**

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Although endothelin-1 (ET-1) is one of the strongest known vasoconstrictors in most species, we have previously found that it is only weakly effective in the mouse[1]. The aim of this study was to further investigate its vasoactive effects in vascular beds known to be sensitive to ET-1 in rats and humans, including the renal artery. Experiments were performed to determine the vasoconstrictor responses of the thoracic aorta, carotid, femoral and renal arteries. Isolated vascular rings of C57BL/6J male mice (40 wks) were placed in organ chambers, or a Mulvany myograph, and exposed to ET-1 ($10^{-11}$-$10^{-7}$ mol $l^{-1}$) in the presence of the nitric oxide synthase inhibitor L-NAME ($3x10^{-4}$ mol $l^{-1}$) to exclude interference of NO. Vessels from different vascular beds demonstrated distinct patterns in potency of the responsiveness to ET-1 and the dynamics of this response. The maximal contraction to ET-1 was significantly greater in the carotid (42±14% KCl) and femoral (117±16% KCl) arteries than in the aorta (5±1% KCl) (both $p<0.05$); the maximal contraction of the femoral artery being significantly greater than that of the carotid artery ($p<0.05$). The dynamics of contractile responses to ET-1 varied between the different vessels, with the renal arteries showing a rapid, strong vasoconstriction, followed by a near complete loss of tension, while the aorta, carotid, and femoral arteries showed a sustained vasoconstriction, followed by a slight loss of tension in the carotid and femoral arteries ($p<0.05$, for end-contraction). In conclusion, the data demonstrate, although contractility to ET-1 in mice is generally thought to be low, the femoral artery exhibits the most potent contractions seen to date. Moreover, the highly sensitive responsiveness in the renal artery, may play a role in the development of hypertension, where ET-1 production as well as renal vasoconstriction are increased.

1. Traupe T, et al. J. Hypertens., 20: 2239-45, 2002.

P-029

**Endothelial ETB receptor expression is not changed in small mesenteric blood vessels of DOCA-salt hypertensive rats**

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Endothelial ETB receptors are important in the clearance of circulating endothelin-1 (ET-1). Our previous studies suggested that this clearance activity was impaired in the splanchnic vasculature of DOCA-salt hypertensive rats. We tested the hypothesis that this was due to fewer ETB receptors on endothelial cells in DOCA-salt mesenteric arteries and veins. We examined the expression of endothelial ETB receptors in cross sections of small (150-250 micron) mesenteric blood vessels from normotensive and DOCA-salt hypertensive rats by double-label immunofluorescence and confocal microscopy. Von Willebrand Factor (vWF) was used as an endothelial cell marker. The fluorescence intensity of endothelial ETB receptors was quantified using Image J software. There were no differences in vWF staining between DOCA-salt and sham tissues. There was significantly greater ETB receptor fluorescence intensity in endothelial cells of small mesenteric veins than small mesenteric arteries of sham rats. However, no significant difference in ETB receptor fluorescence intensity was found between DOCA-salt and sham tissues (sham arteries: 25.98 +/- 7.01; sham veins: 35.47 +/- 5.70; DOCA arteries: 28.18 +/- 4.20; DOCA veins: 30.42 +/- 2.13). In addition, no significant difference in ETB receptor protein level was found between DOCA-salt and sham small mesenteric blood vessels by western blot analysis (sham arteries: 50.44 +/- 7.51; sham veins: 37.61 +/- 8.88; DOCA arteries: 60.45 +/- 7.24; DOCA veins: 53.52 +/- 4.18) . We conclude that impaired uptake activity by ETB receptors in the splanchnic bed of DOCA-salt rats is not due to a decreased number of endothelial ETB receptors. Other possible explanations include decreased ETB receptor binding affinity, altered cellular localization (intracellular vs. membrane) or altered post receptor disposition mechanisms in DOCA-salt rats.

P-030

**Impaired Endothelial Function, Oxidative Stress and Vascular Remodeling in Resistance Arteries of DOCA-Salt Osteopetrotic Mice: Role of Inflammation**

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Vascular damage in deoxycorticosterone acetate (DOCA)-salt hypertensive rats may be partially mediated by vascular inflammation and reactive oxygen species generation. Homozygous osteopetrotic mice (Op/Op), deficient in macrophage colony-stimulating factor (m-CSF), exhibit reduced inflammation. However, whether the osteopetrotic mutation confers microvascular protection in hypertension remains to be determined. We therefore investigated the effects DOCA-salt (DS) hypertension on vascular structure, function and oxidative stress. Adult Op/Op, heterozygous (Op/+), and wild type (+/+) mice were treated with DOCA (200mg/mice) and saline for 14 days. Systolic blood pressure (SBP) was measured by tail-cuff, vascular reactivity of mesenteric resistance artery was studied on a pressurized myograph, and vascular NAD(P)H oxidase activity by lucigenin chemiluminescence. DS +/+ and Op/+ mice had significantly increased SBP (145±2 and 135±1 vs. 108±3 and 112±2 mmHg in controls, $P<0.01$) whereas there was no rise in Op/Op (130±1 vs. basal 131±1). Response to norepinephrine (NE) was enhanced ($P<0.01$) and endothelium-dependent vasodilation to acetylcholine (Ach) was impaired ($P<0.05$) in +/+ and Op/+ DS mice compared to controls. Relaxation to Ach was unaffected by L-NAME in DS +/+. However, NE-induced contraction and Ach-induced relaxation were similar in DS and untreated Op/Op, and was abolished by L-NAME. Vessels from +/+ and Op/+ DS had significantly increased media-to-lumen ratio and media thickness of mesenteric resistance arteries ($P<0.01$), neither of which was altered in Op/Op mice compared to untreated littermates. Vascular media cross-section area was significantly increased only in +/+ DS. NAD(P)H oxidase was significantly increased in +/+ and Op/+ DS. These results demonstrate that m-CSF-deficient mice develop less endothelial dysfunction, vascular remodeling and oxidative stress induced by DS than +/+ and Op/+, suggesting a critical role of m-CSF and pro-inflammatory mediators in mineralocorticoid-salt hypertension.

P-031

**Transgenic rat models of the human ETA receptor develop arterial hypertension and show blunted response to adrenergic receptor stimulation**

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Pharmacological studies in rat models have revealed contradictory results with respect to the role of the ETA receptor in arterial hypertension and associated vascular hypertrophy. To address a putatively causal role of ETA in hypertension and vascular remodelling, we established transgenic models overexpressing the human ETA receptor in vascular smooth muscle cells. An expression construct containing human ETA cDNA under control of a 1,3 kB fragment of the murine SM22αpromoter was microinjected in fertilized oocytes of Sprague-Dawley rats. Five transgene-positive (tg) founders were identified by genomic PCR and two established tg lines (L6351 and L6878) were investigated in detail. (1) Construct copy number and transgene-specific transcript levels were analysed by real-time PCR. (2) Despite significant downregulation of endogenous ETA mRNA, we found 9-fold increased ETA-specific binding to mesenteric artery, but not to aortic membranes of tg L6351 rats. Unexpectedly, mesenteric ETB-specific binding was also more than 6-fold increased in tg L6351 transgenic rats. (3) Compared to controls, tg animals (L6351) showed significantly ($p< 0.001$) increased basal blood pressure (e.g., +73 mm Hg systolic). Maximum increments in blood pressure after ET-1 bolus injection were not different. However, phenylephrine (PE) bolus injection showed significantly ($p>0.001$) attenuated systolic arterial pressure response in tg animals (L6351: -45%, L6878: -77%). (4) Pressurized (70 mm Hg) isolated mesenteric arteries (L6351) showed significantly increased sensitivity against ET-1, but maximal constriction was unchanged. Mesenteric artery constriction to PE was significantly decreased (-65%) in tg rats (L6351, L6878), whereas constriction to TXA2 agonist or Angiotensin II remained unaltered. The functional and structural vascular phenotype of our ETA transgenic rats not only provides evidence of its pathophysiological role in vivo, but has revealed a specific interaction of the endothelin with the adrenergic system.

P-032

**Pharmacological characterization, cloning and sequencing of the endothelin ETA receptor in the aorta of the poisoning snake *Bothrops jararaca***

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Terrestrial snakes are characterized by a number of attributes that are deficient or lacking in species that do not experience the hydrostatic stress of gravity. Because of their elongated body shape, they are highly susceptible to disturbance of hydrostatic pressure. Since one of the main physiological functions of the endothelin (ET) system is to modulate the vascular tonus, and considering that there is very few evidence on ET receptors in tissues from reptiles, we evaluated the presence of ET receptors in the vascular system of the terrestrial poisoning snake *Bothrops jararaca*. To functionally characterize vascular ET receptors, cumulative concentration-response curves to ETs and sarafotoxins (SRTXs), in the absence and presence of selective receptor antagonists, were performed in isolated aortic rings. Vascular ET receptors mRNA expression was evaluated by RT-PCR analysis and a fragment of the $ET_A$ receptor was cloned and sequenced. ET1, SRTX-b, ET3, SRTX-c and IRL1620 induced concentration-dependent vasoconstriction, with a potency order typical of $ET_A$ receptors. BQ123, a selective $ET_A$ antagonist, inhibited contractions induced by ET1, SRTX-b, ET3 and SRTX-c with expected pKb values for mixed $ET_A/ET_B$ receptor populations. The non-selective $ET_A/ET_B$ receptors antagonist PD142893 produced similar responses. Whereas the $ET_B$ antagonist IRL1038 potentiated contractile responses to SRTX-c, BQ788 inhibited both ET1- and SRTX-c-induced vasoconstriction. Processing of the *Bothrops jararaca* aortic first-strand complementary DNA, by RT-PCR with primers designed from the Gallus gallus $ET_A$ receptor sequence, allowed isolation, purification, cloning and sequencing of a single band. The partial sequence of *Bothrops jararaca* $ET_A$ receptor showed very high homology with $ET_A$ receptor sequences from chicken, rat, human and Xenopus. In conclusion, pharmacological and molecular characterization suggest that both $ET_A$ and $ET_B$ receptors mediate vascular responses to SRTXs/ETs in *Bothrops jararaca*. One may speculate that activation of ET receptors in reptiles contributes to the regulation of vascular tonus and/or local blood flow.

P-033

**ET-1-induced MAP kinases activation involves c-Src independent mechanisms in DOCA-salt hypertension: Role of oxidative stress**

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We recently demonstrated that increased vascular oxidative stress in DOCA-salt rats is associated with activation of the endothelin system via ETA receptors. Endothelin-1 (ET-1) and reactive oxygen species are known to be critically involved in vascular smooth muscle cell (VSMC) growth, apoptosis and collagen deposition. c-Src, a tyrosine kinase activated by G-protein coupled receptors, is a critical proximal regulator of NAD(P)H oxidase-driven superoxide anion generation. In addition, c-Src induces activation of MAPKs associated with molecular and cellular processes underlying vascular changes that occur in hypertension. In the present study we investigated whether MAPKs are upregulated in DOCA-salt hypertension, and whether c-Src plays a role in this process. DOCA-salt and control uninephrectomized rats (UniNX) were treated with the ETA antagonist BMS182874 (40 mg/kg per day), Vitamin E (200 mg/kg per day) or vehicle. Activation of c-Src, p38MAPK and ERK1/2 in isolated mesenteric beds was assessed using phospho-specific antibodies. Both ERK1/2 (1.03±0.04 vs UniNX:0.69±0.05, $p<0.05$) and p38MAPK (1.23±0.10, n=6 vs UniNX: 0.80±0.09, $p<0.05$) phosphorylation were increased in DOCA-salt. c-Src phosphorylation was not altered in DOCA-salt rats (0.58±0.16, vs UniNX:0.57±0.08). In DOCA-salt, ETA antagonism blunted the increased activation of ERK1/2 (0.47±0.15 vs UniNx:0.38±0.04) and p38MAPK (0.75±0.05 vs UniNX:0.79±0.06). Vitamin E prevented the increased phosphorylation of ERK1/2 (0.52±0.07 vs UniNX:0.40±0.11) and p38MAPK (0.45±0.07 vs UniNX:0.55±0.05) in DOCA-salt. Our findings demonstrated that MAPKs upregulation in DOCA-salt hypertension is mediated by ET-1/ETA receptors. Moreover, the activation of these redox-sensitive pathways involves c-Src-independent mechanisms

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P-034

**How does $ET_B$ receptor activation in vivo increase $O_2$- levels in sympathetic ganglia?**

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Elevated superoxide ($O_2$-) anion production in sympathetic neurons may be associated with the pathogenesis of endothelin (ET) dependent hypertension. We recently found that in vivo activation of the $ET_B$ receptor significantly increased mean arterial pressure (MAP) as well as augmented $O_2$- expression in both neurons and glial cells of the inferior mesenteric ganglia (IMG) when compared to control rats. The objective of our present study was to determine if the alteration in $O_2$- levels was a direct effect of $ET_B$ receptor activation on sympathetic ganglia or indirectly as a consequence of ET induced hypertension. Male Sprague-Dawley rats were assigned to one of 3 treatments: they received iv infusions of 1) the specific $ET_B$ receptor agonist sarafotoxin 6c (S6c; 5 pmol/kg/min; n=5), 2) isotonic saline at 0.01 ml/min (control; n=5), or 3) phenylephrine (PE; 10 µg/kg/min) for 2 hrs to evaluate the effect of elevated blood pressure on $O_2$- production independent of $ET_B$ receptor activation. MAP increased 29.9$\pm$0.1 mmHg in S6c, 31$\pm$1.2 mmHg in PE and 1.7$\pm$1 mmHg in control rats. To measure $O_2$- levels, we removed the IMG immediately following infusion and stained them with dihydroethidine (DHE). The results confirmed our previous findings that the DHE fluorescence intensities (FI) of ganglionic neurons and surrounding glial cells were significantly greater in S6c than control rats, 215.5% and 197.6%, respectively. Moreover, FI of PE rats were also significantly greater than controls, 137.7% in neurons and 104.6% in glia, but significantly lower than in S6c rats. Based on the present data, we conclude that the increase in $O_2$- following $ET_B$ receptor activation can only be partially attributed to associated pressor effects. Our research is supported by NIH P01HL-70687.

P-035

**PPARγ activator, Rosiglitazone, Prevents Endothelin-1-Induced Inflammation in Vascular Smooth Muscle Cell from Normotensive and Hypertensive rats**

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Endothelin (ET)-1 may be involved in hypertension-associated inflammation. Since peroxisome proliferator-activated receptor (PPAR)g activators decrease blood pressure and improve structural and/or functional changes in ET-1-dependent hypertension, we investigated whether the PPARg activator rosigliatzone modulates ET-1-induced inflammation in cultured vascular smooth muscle cells (VSMC) from Wistar-Kyoto (WKY) and stroke-prone spontaneously hypertensive (SHRSP) rats. VSMC were stimulated with ET-1 (100 nmol/L), rosiglitazone (10 mmol/L) and ET-1 + rosiglitazone for 6-48 hours and protein expression of inflammatory markers were evaluated by immunoblotting. Basal levels of VCAM-1 and ICAM-1 were significantly higher in SHRSP than WKY VSMCs. ET-1-induced increase in protein expression of VCAM-1 and ICAM-1 was inhibited by rosiglitazone ($P<0.05$) in both WKY and SHRSP VSMC. Enhanced protein expression of COX-2 was only observed in SHRSP VSMC, and was reduced by rosiglitazone ($P<0.05$). Rosiglitazone-induced increase in PPARg expression was significantly attenuated by ET-1 in WKY and SHRSP VSMC. ET-1 stimulation was associated with enhanced ETA receptor protein expression in WKY (24h) and SHRSP (12-48 hours), which was reduced by rosiglitazone ($P<0.05$). Rosiglitazone abrogated ET-1-induced NFkB activation in WKY and SHRSP VSMC ($P<0.05$). In conclusion, rosiglitazone modulated ET-1 pro-inflammatory responses in VSMCs from normotensive and hypertensive rats through modulation of ET-1 effects and reduction of the NFkB activation.

P-036

**Endothelin-1 is an ubiquitous constrictor of human coronary artery *in vitro*: increased responsiveness in disease and comparison to other vasoconstrictors**

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Atherosclerosis is associated with endothelial cell dysfunction, narrowing of the lumen, up-regulation of the endothelin system and loss of the opposing vasodilator nitric oxide (NO). We have tested the hypothesis that there is a compensatory down-regulation of response to ET-1 in human atherosclerotic coronary artery (CA) *in vitro*. We have also compared the response of human CA to ET-1 and established vasoconstrictors; angiotensin-II (A-II), 5-HT and phenylephrine (PE). CA was obtained from transplant patients and donor hearts (for which no recipient was available). CA (4mm rings), denuded of endothelium, was set up for isometric tension recordings and cumulative concentration response curves constructed to ET-1 (0.1-300nM), A-II (10pM-100nM), 5-HT (1nM-10μM) and PE (1nM-3μM). Experiments were terminated by addition of 100mM KCl to give the maximum contractile response and agonist responses expressed as a % of this. Values of potency ($pD_2$) and maximum response ($E_{max}$ %KCl) are given as mean±sem, n-values are the number of patients from whom CA was obtained. There was no difference in the responses to KCl in CA used to test the four agonists (ANOVA $P=0.36$). All arteries responded to ET-1 ($n=165$) with a $pD_2$ of 8.03±0.04 and $E_{max}$ of 74.4±1.4%. Only 78% of vessels ($n=28$ responders of 37 tested) responded to A-II ($pD_2$=8.78±0.19; $E_{max}$= 31.7±4.5%), 95% ($n=40/42$) responded to 5-HT ($pD_2$=6.62±0.09; $E_{max}$=38.7±4.4%) and 78% ($n=28/36$) responded to PE ($pD_2$=7.70±0.15; $E_{max}$=28.0±3.8%). The potency of ET-1 in atherosclerotic CA ($pD_2$=7.98±0.06, $n=55$) was comparable to that in healthy donor CA ($pD_2$=7.96±0.12, $n=7$) but the maximum response to ET-1 was significantly greater in diseased (75.9±2.1%) compared to donor CA (56.6±4.2%, $P<0.005$, Student's *t*-test, 2-tailed). These data show that ET-1 contracted all CA tested, in contrast to the greater variability in response to, for example, A-II. This may help to explain why not all patients respond to currently used drugs. In disease, the loss of dilator NO tone in atherosclerosis will be further compounded by the unexpected enhanced sensitivity of atherosclerotic CA to ET-1.

P-037

**Arterial stiffness, physical activity, and endothelin converting enzyme and endothelin receptor gene polymorphisms in elderly humans**

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The increases in arterial stiffness are associated with aging and several pathological states, including systolic hypertension and arteriosclerosis. Regular exercise improves aging-induced increase in arterial stiffness and has a protective effect against these diseases. ET-1 implicates in regulation of vascular tonus and atherosclerosis. The aim was to investigate the relation among arterial stiffness, daily physical activity, and ET converting enzyme-1 (ECE-1) and -2 (ECE-2) and ET receptor-A (ET-A) and -B (ET-B) gene polymorphisms. **Methods and Results:** 163 healthy older humans (65±7 yr old) participated in a cross-sectional study. Genotypes of 2013(+289)A/G in intron 17 and 735(+248)C/T in intron 6 of ECE-1, 669(+17)T/C in intron 5 of ECE-2, 958A/G in exon of ET-A, and 831A/G in exon of ET-B were determined by TaqMan PCR method. Arterial stiffness evaluated by measurement of arterial pulse wave velocity (PWV). Daily physical activity measured using a uniaxial accelerometer and subjects divided into inactive and active groups at a median value (186 kcal/day) of mean energy expenditure per day in all subjects. Age and blood pressure did not differ between active and inactive subjects. PWV in active group is significantly higher than in inactive group. PWVs in genotypes of AA at 2013(+289)A/G and CC at 735(+248)C/T of ECE-1, TT at 669(+17)T/C of ECE-2, AA at 958A/G of ET-A, and AA at 831A/G of ET-B were significantly lower in active group than in inactive group ($p < 0.05$, respectively). On the other hand, there were no significant differences in PWVs in genotypes of G allele at 2013(+289)A/G and T allele at 735(+248)C/T of ECE-1, C allele at 669(+17)T/C of ECE-2, G allele at 958A/G of ET-A, and G allele at 831A/G of ET-B between active and inactive groups. **Conclusion:** These results revealed that polymorphism of ECE, ET-A, and ET-B influences the relation between arterial stiffness and physical activity in elderly humans.

P-038

**The effect of acute ischaemia and reperfusion on ET-1 and its receptors in patients with chronic lower limb ischaemia**

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Elevated plasma and tissue ET-1 in critical limb ischaemia (CLI) patients has been described. Here, the effect of a period of acute ischaemia and subsequent reperfusion on circulating ET-1 and tissue ET-1/ET-1 receptors on skeletal muscle biopsies in patients with/without CLI was studied. Peripheral blood and muscle biopsies were obtained from two groups of patients. The control group were undergoing total knee replacement (TKR, n=15). Samples were taken at the start of surgery, after 1 hour of tourniquet application (ischaemia), and 15 minutes after tourniquet release (reperfusion). Similarly, samples were taken from CLI patients undergoing femoro-distal bypass surgery (n=10), at the start of the procedure, after a period of vascular clamping (ischaemia) and following clamp release (reperfusion). Plasma ET-1 was determined by ELISA. Tissue ET-1 was assessed by counting ET-1 immunostaining cells per unit area and ETA/ETB receptors were identified by in vitro autoradiography and binding determined by densitometry. There was increased circulating ET-1 in CLI patients compared with TKR controls ($p<0.05$) but no further change in either group occurred following acute ischaemia or reperfusion. There was a significant increase in ETA/ETB receptor binding in CLI compared with TKR muscle sections that was not affected by acute ischaemia or reperfusion. Tissue ET-1 was higher ($p<0.05$) in CLI compared with TKR controls, much of which was associated with macrophages and microvessels. This increased in both cases during acute ischaemia and reperfusion. We have shown that both plasma ET-1 and tissue ET-1/ET receptors are increased in CLI patients. Increased muscle ET-1 levels occurred following acute ischaemia and reperfusion in patients with and without underlying chronic ischaemia. In CLI patients, where ET-1 is already upregulated, this further increase may exacerbate existing pathological processes and contribute to ischaemia-reperfusion injury. ET-1 antagonists may therefore be useful adjuncts to such surgical

procedures to reduce ischaemia-reperfusion damage.

P-039

**ETA blockade prevents polydipsia in the face of volume expansion and hypertension in salt-fed Wistar-Kyoto rats**

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Inbred Wistar-Kyoto (WKY) rats exibit mild hypertension when chronically fed a high-sodium diet which is prevented by concurrent administration of the ETA-selective antagonist, ABT-627. We also observed that ETA blockade reduced the ad lib water intake in salt-fed rats by 18% while having no impact on chow or salt intake. We hypothesized that this reduced drinking behavior may play a role in preventing intravascular volume expansion and hypertension in salt-fed rats and undertook a series of experiment to examine the volume statue of these rats under various conditions. Comparing rats fed a sodium-deficient diet for 3 weeks with rats fed a high-sodium diet for 3 weeks, we found that high dietary sodium leads to an increase in mean arterial pressure, MAP, of ~10mmHg. This is accompanied by a reduction in blood urea nitrogen (BUN, 15±0.3 for sodium-deficient diet vs. 12±4 for the high-sodium diet, $p<0.0001$) and plasma creatinine (Cr, 0.48±0.02 for sodium-deficient diet vs 0.45±0.01 for high-sodium diet, $p<0.0001$) and an increase in renal creatinine clearance (Crcl, 4.8±0.2 for sodium-deficient diet vs. 6.1±0.2 for high-sodium diet, $p<0.0001$). This suggests that hypertension in salt-fed WKY rats is accompanied by an increase in vascular volume. Comparing rats fed a high-sodium diet for three weeks with and without concurrent chronic ETA blockade, we found that ETA blockade prevented salt-induced increases in arterial blood pressure and reduced the amount of body weight gained while on the high-sodium diet. However, it does not alter salt-induced reductions in BUN or Cr, or the salt-induced increases in Crcl, indicating that vascular volume is increased. We were unable to detect changes in water balance over the course of the 3 weeks of treatment. Conclusions: ETA activation modulates spontaneous drinking behavior in WKY rats given a high-sodium diet. ETA mediated polydipsia may contribute to vascular volume expansion and hypertension in these rats. This work was supported by NIH R01 HL64720.

P-040

**Blood pressure responses of endothelin-11-31 within the rostral ventrolateral medulla through conversion to endothelin-11-21**

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Endothelin-11-31 (ET-11-31), a novel member of the endothelin family comprising 31 amino acids and derived from the selective hydrolysis of big ET-1 by chymase, directly activates endothelin receptors or converts to ET-11-21 by ET converting enzyme (ECE). The cardiovascular effects of central ET-11-31 are not identified. The present study was designed to investigate the cardiovascular actions of ET-11-31 within the rostral ventrolateral medulla (RVLM) in anesthetized rats. Bilateral injection of ET-11-31 (0.5, 1, and 2 pmol for each side) into the RVLM produced an initial pressor and/or a long-lasting hypotensive action but did not affect HR. Unilateral microinjection of 2 and 4 pmol of ET-11-31 into the RVLM only produced a significant ($P<0.05$) transient increase in BP by an average of 13 and 12 mmHg, respectively, while unilateral microinjection of 8 pmol of ET-11-31 produced a sustained fall in BP (from 92 ±6 to 69 ± 8 mmHg, $P<0.05$). The transient pressor effect of unilaterally injecting ET-11-31 (4 pmol) into the RVLM was completely abolished by pretreatment with either ETA receptor antagonist BQ123 (83 ± 2 vs. 84 ± 5 mmHg, $P>0.05$) or ECE inhibitor phosphoramidon (99 ± 5 vs. 99 ± 7 mmHg, $P>0.05$) but not ETB receptor antagonist IRL1038 (89 ± 6 vs. 96 ± 7 mmHg, $P<0.05$). In conclusion, the current results suggest that the cardiovascular effects of intra-RVLM ET-11-31 might be the result of conversion of ET-11-31 to ET-11-21 through activation of ETA receptors.

P-041

**In Vivo PPARα Activation Attenuates ET-1-Induced Endothelial Dysfunction and High Blood Pressure in Salt-Sensitive Hypertension**

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BACKGROUND: Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors/ligand-dependent transcription factors. PPARα agonist fenofibrate has been shown to abrogate increased prepro-endothelin-1 expression in deoxycorticosterone acetate (DOCA)-salt hypertension. We reported previously that ET-1 induces endothelial dysfunction via NADPH oxidase-derived superoxide in this model. The present study tested the hypothesis that chronic PPARα activation reverses ET-1-induced, NADPH oxidase/superoxide-mediated endothelial dysfunction in DOCA-salt hypertensive rats. METHODS: DOCA-salt or sham-operated rats were treated with fenofibrate (150 mg/kg/day in food) for 4 weeks beginning with DOCA-salt regimen. Average systolic blood pressure, oxidative stress markers and endothelial function were determined in the carotid arteries thereafter. RESULTS: Arterial ET-1 levels were significantly increased in DOCA-salt compared to Sham rats (0.91±0.09 vs. 0.37±0.01 pmol/g, n=5, $p<0.01$), which were suppressed by fenofibrate (0.59±0.10 pmol/g, n=6). Treatment with fenofibrate also significantly reduced arterial NADPH oxidase activity (44.2±4.2 vs. 21.6±1.7 nmol/min/mg) and superoxide levels (0.62±0.04 vs. 0.37±0.04 nmol/min/mg, DOCA vs. DOCA+PPARα, both n=5-10, $p<0.05$), resulting in decreased VCAM-1 expression and serum lipid peroxidation as well as increased eNOS cofactor tetrahydrobiopterin (BH4) levels and endothelial nitric oxide synthase (eNOS) activity. Concomitantly, fenofibrate treatment of DOCA-salt rats significantly improved endothelium-dependent nitric oxide (NO)-mediated relaxation to acetylcholine (41.4±5.8 vs. 55.7±4.5%, DOCA vs. DOCA+PPARα, n=3-8, $p<0.05$) and blunted systolic blood pressure (186±8 vs. 139±6 mmHg, DOCA vs. DOCA+PPARα, n=11-16, $p<0.01$). CONCLUSION: These findings indicate that chronic in vivo PPARα activation prevents progression of hypertension and attenuates ET-1-induced endothelial dysfunction in mineralocorticoid hypertension.

P-042

**Sex differences and the role of superoxide in salt-induced hypertension in ETB receptor deficient rats**

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Evidence for endothelin involvement in the control of fluid volume balance and arterial pressure has been derived in part from the observations that rats lacking the ETB receptor develop hypertension when placed on a high salt diet. The present study was designed to determine the influence of superoxide on salt-induced hypertension in male and female ETB deficient (sl/sl) and wild-type control (+/+) rats. After 12 days on a high salt (8% NaCl) diet, female sl/sl rats had a significantly elevated arterial pressure (182±3 mmHg, tail cuff), compared to female +/+ rats (137±2 mmHg). The response to high salt was lower in sl/sl males (159±2 mmHg) yet significantly greater than male +/+ controls (135±3 mmHg). Separate groups of male and female sl/sl and +/+ rats were given tempol (1 mM in drinking water) during high salt treatment. Arterial pressures were 140±4 mmHg in male and 144±4 mmHg in female sl/sl rats treated with tempol, values that were similar to controls on a normal salt diet. After 15 days, however, both male and female sl/sl rats escaped from the blood pressure lowering effects of tempol. In female sl/sl rats on high salt, arterial pressure was 167±6 and 167±6 mmHg in tempol-treated and untreated groups, respectively, on day 15. In male sl/sl rats, arterial pressure was 148±1 and 155±1 mmHg in tempol-treated and untreated groups, respectively. On day 15, no differences among groups were observed in plasma TBARS concentrations or urinary excretion of TBARS with or without tempol. These results suggest that the female sex is more dependent on the ETB receptor for maintenance of pressure natriuresis compared to males. In addition, these results indicate that superoxide contributes to the early phase of salt-induced hypertension in this model, however, it appears that increased superoxide generation does not account for the greater salt sensitivity observed in female ETB receptor deficient rats.

P-043

**Plasma endothelin-1 concentration and arterial stiffness in strength- and endurance-trained athletes**

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**Purpose**: Endurance exercise training induces a decrease in arterial stiffness, whereas strength exercise training causes an increase in arterial stiffness. Endothelin-1 (ET-1), which is produced by vascular endothelial cells, has potent vasoconstrictor and proliferative activity in vascular smooth muscle cells. We hypothesized that endogenous ET-1 participates in alteration of arterial stiffness by different exercise training types (i.e., endurance exercise training and strength exercise training). The purpose of the present study was to investigate plasma ET-1 concentration and arterial stiffness between strength- and endurance-trained athletes. **Methods and Results**: Subjects were male strength-trained athletes (discus, hammer or javelin throwers; 21.8 ± 0.5 years), male endurance-trained athletes (long or middle distance runners; 20.3 ± 1.0 years), and sedentary healthy men (20.3 ± 1.1 years). Maximum hand grip strength was markedly higher in thrower compared with runner and sedentary men (55.4 ± 2.8 vs. 41.4 ± 2.7 vs. 41.0 ± 2.2 kg, $P < 0.05$). Maximum oxygen uptake was markedly higher in runner than in thrower and sedentary men (60.3 ± 1.3 vs. 41.9 ± 1.5 vs. 43.3 ± 2.4 ml/kg/min, $P < 0.05$). Arterial pulse wave velocity (PWV), which is an index of arterial stiffness, was significantly higher in thrower than in runner and sedentary men (690 ± 64 vs. 526 ± 17 vs. 599 ± 16 cm/sec, $P < 0.05$). In runner, PWV was significantly lower in comparison to that in sedentary men ($P <0.05$). Thus, arterial stiffness was higher in thrower than in runner and sedentary men, and lower in runner than in sedentary men. Plasma ET-1 concentration was significantly higher in thrower compared with runner and sedentary men (1.53 ± 0.29 vs. 1.18 ± 0.17 vs. 1.27 ± 0.12 pg/ml, $P < 0.05$). Plasma ET-1 concentration tended to be lower in runner than sedentary men. **Conclusion**: These results suggest that the difference in plasma ET-1 level may participate in a mechanism underlying different adaptation of arterial stiffness between strength- and endurance-trained athletes.

P-044

**Superoxide dismutase mimetic reduces the pressor response to acute environmental stress in Dahl salt-sensitive rats**

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Elevated superoxide has been shown to produce an increase in vasoconstrictor sensitivity. We therefore tested the hypothesis that tempol, a superoxide dismutase mimetic, inhibits the pressor response to acute environmental stress in Dahl salt-sensitive (DS) rats, an established model of ET-1-dependent hypertension. Stress was induced by restraint and administration of air jet pulses (3 minutes) in rats maintained on a normal salt diet before and after 3-day treatment with tempol (1mM) in the drinking water. Mean arterial pressure (MAP) and heart rate (HR) were monitored by telemetry. Tempol had no effect on 24-hour MAP, but caused a significant decrease in baseline HR (411±3 vs. 397±3 beats/min, $p<0.05$). Plasma ET-1 was not changed by tempol treatment, nor did tempol have an effect on baseline plasma norepinephrine (NE) and epinephrine (Epi). The total pressor response (area under the curve) to acute stress was significantly reduced (22.3.6±3.2 vs. 13.6±3.1 mmHg x 3 min, $p<0.05$) in tempol-treated animals. The stress-induced increase in plasma NE was unchanged, whereas the rise in Epi was attenuated by tempol (548±49 vs. 385±46 pg/ml, untreated vs. tempol, $p<0.05$). In anesthetized DS rats, tempol had no effect on the pressor response to exogenous phenylephrine. In summary, tempol suppressed the pressor response to acute stress and attenuated the rise in plasma Epi in DS rats. These findings suggest that superoxide contributes to the pressor response to acute environmental stress. [Supported by AHA-0030370Z, HL-64776, HL-69999]

P-045

**Endothelin-1 Mediates Inward Arterial Remodeling in Response to Decreased Blood Flow**

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Arterial remodeling in response to altered blood flow/shear forces is important in vascular development, during physiologic adaptation in the mature circulation, and in the progression of vascular disorders that compromise blood flow. In response to decreased blood flow, the vessels adapt by inward remodeling. Since endothelial endothelin-1 (ET-1) expression increases when shear stress is reduced in vitro, we hypothesized that persistent constrictor properties of this agonist participate in inward arterial remodeling after blood flow reduction. To test our hypothesis, we ligated the left common carotid artery in adult male New Zealand White rabbits, thus reducing left common carotid artery blood flow by 80%. Arteries were examined at 3 days (acute vasoconstriction) and at 14 days (chronic remodeling, structurally entrenched) following the ligation surgery. To test the role of ET-1 in the narrowing of arteries, we administered an ET-1 receptor antagonist, TAK-044 (30 mg/day). TAK-044 significantly suppressed acute and chronic vessel narrowing. Since down-regulation of nitric oxide (NO) and up-regulation of matrix metalloproteinases (MMPs), could also play a role in the process of inward remodeling, we also assessed the role of NO and MMPs by administering the NO synthase inhibitor, L-NAME (0.1g/L/day) and the non-specific inhibitor of MMP, doxycycline (0.2 g/L/day), respectively. L-NAME failed to influence the acute vasoconstriction, but significantly inhibited the chronic vascular narrowing, whereas, doxycycline significantly reduced acute and chronic inward remodeling. MMP-2 can process big endothelin to produce a novel vasoconstrictor, ET-1[1-32], rather than ET-1[1-21], and our data, supported by assessment of protein levels of ET-1[1-21], endothelial NO synthase and MMP-2 is consistent with a role for ET-1[1-32] pathway in initiating inward remodeling after flow reduction. We infer that ET-1[1-32], activated by MMP-2, is an important regulator of inward arterial remodeling.

P-046

**ECE-1 Isoforms in Human Atherosclerotic Pathologies: Correlation of Expression with Disease Progression**

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Endothelin-converting enzyme-1 (ECE-1) is a critical enzyme in the production of the potent vasoconstrictor peptide endothelin (ET-1). It has previously been shown that the levels of both ET-1 and ECE-1 are raised in atherosclerosis, but the possible relevance of the isoforms of ECE-1 in these changes has not yet been investigated. The aim of this study was to examine the expression of the ECE-1a and ECE-1c isoforms in human atherosclerotic pathologies. Immunohistochemical analysis was carried out on sections from atherosclerotic and non-atherosclerotic vascular tissue using a combination of ECE-1 isoform-specific antibodies, anti-$\alpha$ actin antibodies to identify smooth muscle cells (SMC) and anti-CD68 antibodies to identify macrophages. ECE-1 isoform expression was also examined in cultured SMC and macrophages isolated from human blood. Results indicates differences in isoform expression in atherosclerotic lesions, with distinct patterns of staining for ECE-1a and ECE-1c. ECE-1c immunoreactivity was seen in macrophages, and also correlated with actin staining. ECE-1a was also localised to macrophages and SMC. Results of this study suggest that local changes influence the expression patterns of the ECE-1 isoforms within individual cell types. Correlation of these isoform expression patterns with the stage of atherosclerosis could provide novel indicators of disease progression.

P-047

**Beneficial effects of endothelin ETA receptor blockade on right heart remodeling and blood pressure in established long-term heart failure after myocardial infarction**

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Early blockade of the endothelin system in experimental congestive heart failure after myocardial infarction improves survival by cardiac remodeling. The effects of endothelin receptor blockade in established experimental heart failure are unknown. This study investigated the effects of endothelin ETA receptor blockade in rats with established congestive heart failure. Sprague-Dawley rats were subjected to ligation of the left anterior descending coronary artery. After six months, surviving animals were randomized to treatment with the endothelin ETA receptor antagonist darusentan (50 mg/kg/d) or placebo for 6 weeks. Sham-operated animals served as controls. Body weight, systolic blood pressure, and heart rate, lung, right atrial and ventricular weight, and myocardial, renal, and pulmonary ET-1 levels were determined. Systolic blood pressure was significantly lower in rats with CHF and decreased further during the study period ($p<0.05$). CHF animals had higher organ weights of lungs, RV, and RA (all $p<0.05$). ET-1 protein levels were increased only in lungs but not in the myocardium or kidneys. Darusentan treatment prevented the fall in blood pressure seen in untreated CHF rats at the end of the study, and reduced the elevated organ weights of lung, RV, and RA. These data suggest that ET-1 contributes to hemodynamic regulation in rats with established heart failure independently of tissue ET-1 peptide expression or nitrate content. This independence implies a yet unknown mechanism underlying the beneficial effects of chronic ET receptor blockade on cardiovascular hemodynamics after myocardial infarction affecting the pulmonary circulation and myocardium of the right heart.

P-048

**Modification of ET-1 Induced Pulmonary Vasoconstriction in Congestive Heart Failure**

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Introduction: Endothelin (ET) levels are elevated in congestive heart failure (CHF) and correlate especially well with the severity of pulmonary hypertension (PH), suggesting that this peptide could contribute to the pathophysiology of venous PH. Alterations of pulmonary vasoreactivity to ET in CHF and the respective roles of the ETA and ETB receptors has never been evaluated. Methods: Myocardial infarction (MI) was induced by ligation of the left anterior descending coronary artery of male Wistar rats. Three weeks following the intervention, small pulmonary vessels (150 to 200 um diameter) were mounted on a microvascular myograph and bathed in Krebs solution at a resting tension of 80 to 100 mg. For each vessel, endothelium-dependant vasodilatation to Ach and maximal vasoconstriction to 127 mM KCl were determined. Cumulative concentration-response curves to ET-1 and S6c were performed. Response to ET was also assessed in the presence of ETa antagonist (10 nM, A-147627.1), ETb antagonist (1uM, A-192621.1) and the combination of both. Heterodimerization of receptors in these vessels was evaluated by immunoprecipitation of the ETb, followed by western blotting for the expression of the ETa receptor. Results: The maximal vasoconstriction and sensitivity induced by ET-1 were similar in sham and MI with Emax values of 88 +/- 3.9% and 81.5 +/- 3.1% respectively. The response to S6c was similarly less in both sham (67.3 +/- 5.7%) and MI groups (59.0 +/- 5.2%). The ETa and ETb antagonists alone had no significant effect on Emax or EC50 values. However, the combination of both antagonists significantly reduced ET-1 induced vasoconstriction (54.0 +/- 5.7%, $P<0.001$)) but did not modify the EC50 values. In vitro studies demonstrated Co-immunoprecipitation of the ETa and ETb receptors. Conclusion: In CHF there is no modification of ET-induced pulmonary vasoconstriction, despite previous demonstrations of down-regulation of ETb receptors in CHF lungs. The use of dual antagonism is necessary for optimal blockade of vasoconstriction, possibly because the ETa and ETb receptors can form functional heterodimers.

P-049

**Dual ET receptor antagonist attenuates cardiac hypertrophy early post-infarction in the rat**

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The ET system is activated early post-infarction (MI) and in heart failure (HF). ET receptor antagonists (ETRA) elicit beneficial effects in these conditions. Yet in post-MI models of HF, early and prolonged use of ETRA also has adverse effects. We tested the hypothesis that short-term treatment will only have beneficial effects. Rats received a dual ETRA LU420627 (LU, 50 mg/kg/day p.o.) for 3 days before coronary ligation or sham operation, and for 7 days thereafter. This protocol produced 4 groups: Sham, Sham/LU, MI, MI/LU. Seven days post MI, both MI and MI/LU exhibited similar increases in RVSP, LVEDP, lung weight, and a poor survival (40% in both). MI size was equivalent in both MI groups (38 +-5 vs 43 +-3%, NS). On gel zymography, LV matrix metalloproteinases-2 and -9 were increased in both MI groups, and they were very high in the infarct scar. The active form of MMP-2 (62 kD) was present only in MI and MI/LU hearts. RV hypertrophy, very marked in MI (0.78 +-.03 vs 0.66+- .03 mg/g in Sham, $P<0.01$) was completely abolished in MI/LU (0.65+-.02 mg/g) while LV hypertrophy was marginally improved. On histomorphometry of mid-LV sections, scar thickness was equivalent in both MI groups, and LV cavity area exhibited the same degree of dilation (0.33+-.05 and 0.33+-.02 vs 0.19+-.02 cm2 in Sham). Plasma ET level was elevated in MI (+115% vs Sham, $P<0.02$), and it was normalized in MI/LU. Pulmonary ET, also increased in MI (+70% vs Sham, $P<0.001$), was mildly (-20%) but significantly suppressed in MI/LU. Conclusions: Short-term dual ETRA antagonism prevents RV hypertrophy early post-MI without improving survival rate, cardiac hemodynamics and pulmonary hypertension. This effect is suggestive of a blockade of the direct hypertrophic effect of ET on ventricular myocardium, independent of that of pulmonary hypertension. Within the 7-day time-frame post MI, dual ETRA blockade did not exhibit any adverse effects on scar healing or ventricular dilation.

P-050

**Time course alterations of myocardial endothelin-1 production during the formation of exercise training-induced cardiac hypertrophy**

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**Purpose:** Endothelin (ET)-1 is produced by endothelial cell and cardiac myocyte. ET-1 has positive inotropic and chronotropic effects in the heart and causes myocardial cell hypertrophy. We hypothesized that myocardial ET-1 is involved in the formation of exercise-induced cardiac hypertrophy, because ET-1 induces a powerful myocardial cell hypertrophy. We investigated an alteration of myocardial ET-1 gene expression and production in the heart of rats during a formative process of exercise training-induced cardiac hypertrophy. **Methods and Results:** We used the hearts of exercise-trained rats for 4-week (4WT) or 8-week (8WT) and sedentary control rats for 4-week (4WC) or 8-week (8WC). Exercise-trained rats performed treadmill running for 5 days/week (60 min/day). Left ventricular mass index and wall thickness and stroke volume index, measured by using echocardiography, in the 8WT were significantly greater than that in the 8WC group, although there were no differences between 4WC and 4WT in these parameters. These results indicated that the 8-week trained rats developed cardiac hypertrophy, whereas the 4-week trained rats did not yet induce it. Myocardial ET-1 gene expression (1.3±0.1 vs. 0.9±0.1 arbitrary units, $p < 0.05$) and tissue ET-1 concentration in the heart (64.3±8.7 vs. 46.7±3.6 pg/g tissue, $p < 0.05$) were significantly higher in the 8WT than in the 8WC, whereas these did not differ between 4WC and 4WT. Furthermore, LV mass index was significantly correlated with myocardial ET-1 concentration in 8WC and 8WT ($r=0.52$, $P < 0.05$). **Conclusion:** The present study suggests that an alternation of myocardial ET-1 production corresponds with formative process of exercise training-induced cardiac hypertrophy. Therefore, the exercise training-induced change in myocardial ET-1 production may participate in a mechanism of exercise training-induced cardiac adaptation.

P-051

**Impaired response to ETB receptor stimulation in heart failure. Functional evidence of endocardial endothelial dysfunction?**

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It was recently shown that the inotropic effect of selective ETB receptor stimulation depends on the functional integrity of endocardial endothelium (EE), being negative when it is intact and positive when it is damaged. These results have been attributed to the existence of two subtypes of ETB receptors in the heart: ETB1, which are located on EE and decrease inotropy, and ETB2, which are located on myocardial cells and increase inotropy. In the present study we investigated the functional integrity of EE in a heart failure (HF) model (toxic cardiomyopathy due to doxorubicin), by evaluating the contractile response to ETB1 stimulation. New Zealand white rabbits were treated with doxorubicin (DOX, 2mg/kg, iv/ 8 weeks) or with saline. The contractile effects of increasing doses of a selective agonist of endothelial ETB receptors, IRL1620 (10e-9 to 10e-6M), were studied in papillary muscles (Krebs-Ringer: 1.8mM CaCl2, 35$^{o}$C) from control rabbits (n=10) and from rabbits treated with DOX (n=7). Isotonic and isometric twitches were recorded and analyzed. Reported parameters include: active tension (AT) and maximum velocities of tension rise (dT/dtmax) and tension decline (dT/dtmin). Only significant results (mean±SEM, $p<0.05$) are given, expressed as % change from baseline. In the control group, IRL1620 induced dose dependent negative inotropic and lusitropic effects decreasing at 10e-6M: 26±3% AT, 17±2% dT/dtmax and 17±6% dT/dtmin. In the DOX group, these effects were significantly reduced. At the same concentration IRL1620 decreased: 7±3% AT and 8±3% dT/dtmax, without significantly affecting dT/dtmin. This study showed an impaired response to endothelial ETB receptors stimulation, suggesting the presence of EE dysfunction in the DOX HF model and reinforcing the importance of ETB1 receptors as markers of endothelial function.

P-052

**Obligatory role of the endocardial endothelium in the increase of myocardial distensibility induced by endothelin-1**

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Endothelin-1 (ET-1) increases myocardial distensibility in conditions of cardiac overload, through ETA receptor and $Na^{+}/H^{+}$ exchanger activation. This study investigated how the endocardial endothelium (EE) influences the effects of ET-1 on diastolic distensibility. ET-1 (0.1, 1, 10 nM) was tested in rabbit papillary muscles (Krebs-Ringer; 1.8 mM CaCl2, 35°C): (i) in presence of intact EE (n=10); (ii) after damaging EE (0.5% Triton X-100, n=11); and (iii) in presence of RES701 (selective endothelial ETB receptor antagonist, 1 μM, n=6). Only significant results (mean±SEM, $p<0.05$) are given, expressed as % change from baseline. In papillary muscles with intact EE, ET-1 induced dose dependent positive inotropic and lusitropic effects: Active tension (AT) increased 11.9±5.6%, 38.6±9.4% and 77.8±17.0%; dT/dtmax increased 10.7±4.7%, 33.1±6.0% and 81.7±10.1%; and dT/dtmin increased 10.2±5.5%, 35.4±7.7% and 76.6±17.1% (at 0.1, 1 and 10 nM, respectively). These effects were maintained when ET-1 was given after damaging EE (AT increased 14.6±4.3%, 43.1±10.5% and 70.4±12.4%; dT/dtmax increased 11.3±3.4%, 44.7±9.2% and 92.7±13.8%; and dT/dtmin increased 7.3±2.6%, 34.7±13.7% and 56.1±14.2%), but were significantly reduced in the presence of RES701: AT increased 0.1±0.2%, 1.0±0.5% and 29.7±6.4%, dT/dtmax increased 1.0±0.7%; 1.7±0.8% and 37.4±7.0%, and dT/dtmin increased 1.0±0.5%, 1.6±0.8% and 28.9±8.7%. ET-1 reduced resting tension (increased diastolic distensibility) by 3.1±1.0%, 5.4±1.4% and 9.0±2.4% (at 0.1, 1 and 10 nM, respectively) in muscles with intact EE. This effect was abolished after damaging the EE or in presence of RES701. In conclusion, ET-1 induced increase in myocardial distensibility, previously shown to be mediated by ETA receptor stimulation, requires an intact EE and active endothelial ETB receptors. These findings suggest a novel cross talk between myocardial ETA and endothelial ETB receptors that might improve our understanding about the role of ET-1, namely on diastolic function, which has been greatly overlooked in most studies.

P-053

**Intrapericardial angiotensin II stimulates endothelin-1 and atrial natriuretic peptide formation of the in situ dog heart**

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Angiotensin II (AT-II), endothelin-1 (ET-1) and atrial natriuretic peptide (ANP) play a role in cardiovascular regulatory processes under physiological and pathophysiological conditions. All of these agents are present in the pericardial fluid and alteration of their pericardial concentrations may reflect changes in their myocardial interstitial levels. It has been demonstrated that AT-II increases the release of ET-1 and ANP from isolated cardiomyocytes. The local myocardial effect of AT-II on cardiac ET-1 and ANP production, and on cardiovascular function was studied by intrapericardial (i.p.) administration of AT-II (0,125-1 µg/kg) to the *in situ* dog heart (n=8). Big-endothelin (big-ET), ET-1 and ANP (1-28) concentrations were measured by ELISA in pericardial infusate samples and in peripheral blood before and after AT-II treatment for 15 minutes. Systemic blood pressure (BP), heart rate (HR) and ventricular contractility (dP/dt) were also recorded. In our results the pericardial big-ET (but not ET-1) concentration was increased to a maximum value of 139±28 vs. 74±12 pg/mL (control) ($p<0.02$) to i.p. AT-II, with parallel elevations of the pericardial ANP levels (36.8±7.2 vs. 24.4±3.6 ng/mL, $p<0.05$). The i.p. administration of AT-II elicited non-significant change in HR or moderate changes in BP ($BP_{max}$: +19±3 mmHg, $p<0.01$, $dP/dt_{max}$: +12±4%, $p<0.02$). The plasma levels of big-ET, ET-1 and ANP did not change significantly. The results suggest that AT-II promotes production of big-ET and ANP in the heart. However, no detectable conversion of big-ET-1 to ET-1 was observed within 15 minutes. The myocardial formation of big-ET-1 and ANP occurred, at least in part, independently from the changes of cardiovascular function.

P-054

**A Long-Acting Calcium Channel Blocker Ameliorates Diabetic Cardiac Remodeling Accompanied by Normalization of the Upregulated Endothelin System in Diabetic Rats**

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We investigated if benidipine, a long-acting calcium channel blocker (CCB), can normalize cardiac expression profiles of the endothelin (ET)-1 system in insulin-resistant diabetes at its subdepressor dose. Potential mechanisms for the regulation of ET-1 gene by CCB may be considered on the basis of the existence of two putative binding sites identified in the promoter sequence of this gene. The ET-1 system is deemed to play a role in the progression of cardiac remodeling, but whether this system can be affected by CCBs which prove useful in preventing the remodeling process is unknown. Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a model of human type 2 diabetes, were treated for 12 weeks with vehicle or benidipine (3 mg/kg/day). Blood pressure was significantly higher in OLETF rats compared to control rats and was unchanged by benidipine treatment. OLETF rats exhibited a significant increase in ET-1 in plasma and LV tissues compared with non-diabetic controls. Expression of preproET-1 and endothelin converting enzyme mRNA in LV tissues was also significantly higher in OLETF rats. Moreover, LV expression of $ET_A$ and $ET_B$ receptors in OLETF rats was increased more than in controls at both gene and protein levels. The two mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinase and p38MAPK, both of which are activated by ET-1, were more abundantly expressed in OLETF rat LV tissues. All these alterations were reversed to non-diabetic levels when OLETF rats were treated with at the subdepressor dose. TGF-ß1 was strongly upregulated in OLETF heart and was reversed by benidipine treatment. Furthermore, benidipine therapy resulted in hindering cardiomyocyte hypertrophy and cardiac perivascular fibrosis in OLETF rats. The beneficial actions of benidipine at the subdepressor dose on cardiac remodeling in insulin-resistant diabetes may involve normalization of the upregulated ET-1 system. Thus, the present data add this novel effect to the list of the potential mechanisms underlying the benefits of CCBs in reversing the remodeling process.

P-055

**Endothelin-1 stimulates the expressions of VEGF, its receptors and eNOS in neonatal cardiomyocytes without enhancing the HIF-1α expression**

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Recently, gene transfer of vascular endothelial growth factor (VEGF) displays a mitogenic effect on adult cardiomyocytes and it is suggested that VEGF may have a therapeutic role in diseases characterized by myocardial cell loss. The cardiomyocytic origin of VEGF acts as a contributing factor in cardiac allograft arteriosclerosis. Increased VEGF expression is seen in cardiomyocytes after acute myocardial ischemia. Expression of VEGF in cardiomyocytes is enhanced by several factors. Endothelin-1 (ET-1) stimulates VEGF expression in a variety of cells, like endothelial cell, vascular smooth muscle, osteoblast, tumor cells and so on. ET-1 is a potent cardiomyocytic hypertrophic factor in both vivo and vitro. However, no study yet has investigated whether ET-1 stimulates cardiac VEGF either in vivo or in vitro. In the present study we used neonatal cardiomyocytes and then treated with ET-1 to examine whether ET-1 would enhance the expression of VEGF, its receptors and eNOS in the ET-1-induced hypertrophied cardiomyocytes. Ventricular cardiac myocytes were isolated from 2-day-old Sprague-Dawley rats, cultured in DMEM/Ham F12 medium supplemented with 0.1% fatty acid-free BSA for 3 days and at day 4 treated with ET-1 (0.1 nM). 24 hours after ET-1 treatment, different experiments were carried out. VEGF expression was upregulated in ET-1-induced hypertrophied cardiomyocyte by 45%. The expressions of the two principal receptors of VEGF, Flt-1 and Flk-1, were also increased (2.2 fold and 1.6 fold, respectively) in ET-1-treated cardiomyocytes. In parallel to VEGF, eNOS was upregulated in hypertrophied cardiomyocytes by 55%. Previous studies show that ET-1 induces VEGF by increasing hypoxia-inducible factor-1α (HIF-1α). However, in the current investigation, in cardiomyocyte no significant induction of HIF-1α was seen after ET-1 administration. Thus, the present study demonstrates that ET-1 stimulates the expression of VEGF, its receptors (Flt-1 and Flk-1) and eNOS in neonatal cardiomyocytes without altering HIF-1α level.

P-056

**Humoral and Hemodynamic Responses after Left Ventricular Assist Device Implantation and Heart Transplantation**

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Background: Left ventricular assist device (LVAD) implantation and heart transplantation (HTx) are established therapeutic approaches in the treatment of end-stage heart failure. The postoperative humoral responses to the two treatments have not yet been directly compared. Methods: All patients were treated with inhaled nitric oxide (iNO) on weaning from cardiopulmonary bypass due to pulmonary hypertension. We investigated ANP, BNP, cGMP, ET-1, big ET and hemodynamic parameters following LVAD implantation (15 patients, age 51±8 years) or HTx (10 patients, age 53±6 years) preoperatively, on cardiopulmonary bypass and postoperatively up to 72h after cessation of iNO. Results: Preoperatively CI, PA pressures, PCWP, CVP and MAP were similar for both groups. Likewise ANP, BNP, cGMP, ET-1 and big ET were comparable prior to surgery. Seventy-two hours after weaning from iNO the administered epinephrine dose was higher in the HTx group (p=0.003) whereas CVP (p=0.04) and PVR (p=0.03) were lower. The following humoral parameters differed markedly:, ANP (pg/ml) (preop: LVAD 98±124 , HTx: 197±199 (p=0.14) ; 72h post iNO: LVAD 110±106 , HTx: > 640±0 (p=0.003) and cGMP (pg/ml) (preop: LVAD 4.4±5.8 , HTx: 5.1±3.1 (p=0.35) ; 72h post iNO: LVAD 8.0±10.8 , HTx: >26.2±25.8 (p=0.02). Conclusions: Although the hemodynamic effects of both LVAD implantation and HTx in the treatment of end-stage heart failure are comparable, except for the effects on CVP and PVR, the humoral responses with respect to ANP and cGMP were strikingly different. These effects are independent of volume status, iNO and endothelins.

P-057

**Eicosapentaenoic acid, a major component of fish oil, prevents the progression of endothelin-1-induced cardiomyocytic remodeling in vitro**

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It was reported that the cardiovascular benefit of fish oil including eicosapentaenoic acid (EPA) in humans and experimental animals. Endothelin (ET)-1 is well-known factor for cardiac hypertrophy. While many studies link EPA to cardiac protection, the effect of EPA on cardiac hypertrophy has yet to be clarified. The present study investigated whether ET-1-induced cardiomyocytic change could be prevented by the concomitant administration of EPA. Cardiomyocytes were accumulated from neonatal rat heart. At day 4 of culture, the cardiomyocytes were divided into three groups: control, ET-1 (0.1nM) treated group and ET-1 with concomitant EPA (10μM) treated group. 24 hours after the treatment, cardiomyocytes of 3 groups were evaluated. To determine whether there is any change in cardiomyocytic size, cell images were taken and cardiomyocytic area was calculated with NIH image software which showed a 44% increase in cardiomyocytic surface area after ET-1 treatment and this ET-1 induced cardiomyocytic hypertrophy was markedly prevented in EPA co-administered group. To assess the total protein synthesis in ET-1-induced cardiomyocytic hypertrophy and the suppressive effect of EPA, $^{14}$C-leucine incorporation test was done. It showed a significant decrease of $^{14}$C leucine uptake in EPA administered group compared to ET-1 alone treated group. ET-1 treated cardiomyocytes showed a 2.3-fold and 2.1-fold increase in ANP and BNP mRNA expression levels respectively, the classical markers of cardiomyocytic hypertrophy and these increases were remarkably suppressed with the concomitant administration of EPA. Moreover, the increased mRNA levels of c-fos and c-jun were observed in ET-1 treated cardiomyocytes whereas the concomitant administration of EPA could regress these upregulations. In conclusion, the present study showed that ET-1 can induce significant hypertrophic changes in cardiomyocytes with the upregulations of important hypertrophic markers, and that this remodeling was effectively prevented by the co-administration of EPA, suggesting that EPA may be beneficial in cardiac hypertrophy associated with ET-1.

P-058

**Regression of endothelin-1-induced cardiomyocytic hypertrophy by eicosapentaenoic acid, a major component of fish oil, is independent of TGF-ß1, FGF-2 and ACE in vitro**

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Eicosapentaenoic acid (EPA), a polyunsaturated fatty acid found in fish oil, therapeutically has been categorized as a hypolipidemic, antiplatelet agent and cardioprotectant. Currently, growth promoting factors such as angiotensin (Ang)-II, endothelin (ET)-1, transforming Growth Factor (TGF)-ß1, insulin-like growth factor-1 and others have been identified as direct triggers of a hypertrophic response at the level of the cardiomyocyte. There is increasing evidence that fibroblast growth factor (FGF)-2, an endogenous and multifunctional protein is also a "player" in this context. The present study has been designed to investigate whether ET-1 administration in neonatal cardiomyocytes would upregulate the expressions of several hypertrophic factors like TGF-ß1, FGF-2 and Angiotensin Converting Enzyme (ACE) and also whether concomitant administration of EPA in ET-1-treated cardiomyocytes would prevent these growth factors upregulations. Cardiomyocytes were accumulated from neonatal heart of 2-day-old Sprague-Dawley rats and then cultured in D-MEM/Ham F12 medium supplemented with 0.1% fatty acid-free BSA. At day 4 of culture, the cardiomyocytes were divided into three groups: control, ET-1 (0.1nM) treated group and ET-1 with concomitant EPA (10 μM) treated group. 24 hours after the treatment, cardiomyocytes of three groups were evaluated. ET-1 treatment caused remarkable cardiomyocytic hypertrophy, and concomitant administration of EPA in ET-1 treated cardiomyocytes could stop the progression of hypertrophic remodeling. Expressions of TGF-ß1, FGF-2 and ACE were increased in ET-1 treated cardiomyocytes by 1.8-fold, 2.1-fold and 3.2 fold, respectively and co-administration of EPA could not block these hypertrophic factors upregulations, although EPA could block the cardiomyocytic structural remodeling. Thus, from the present study we conclude that ET-1 induces the upregulations of TGF-ß1, FGF-2 and ACE in neonatal cardiomyocytes and concomitant administration of EPA prevents the ET-1-induced cardiomyocytic hypertrophy without altering the upregulations of TGF-ß1, FGF-2 and ACE.

P-059

**Effect of Type-1-Diabetes on the Regulation of Insulin and Endothelin-1 Receptor(s) in Rat Hearts**

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Objectives: This project assesses the treatment role with insulin and/or angiotensin II receptor subtype-1 (AT1-R) antagonist (Losartan) on insulin receptor and endothelin-1 receptor subtypes (ETA and ETB) regulation in rat hearts suffering from insulin-dependent-diabetes-mellitus (IDDM). Design: Animals were divided into six groups: groups one, three, and five were controls consisting of normal, diabetic (Streptozotocin-treated), and diabetic supplemented with insulin respectively; while groups two, four, and six were the controls treated with Losartan. One month after enrollment, rats were sacrificed, and heart tissue samples were snapped frozen for indirect immuno-staining and Western Blotting. Results: Insulin receptor density was observed to be up-regulated in the cardiomyocytes of diabetic hearts, however down-regulated with insulin treatment alone. Co-treatment with insulin and Losartan resulted in drastic increase in insulin-receptor density in the diabetic group. In addition, the expression of ETA in cardiomyocytes was up-regulated and was consistently maintained within the various treatment modalities. However, ETB expression was significantly reduced in the diabetic group treated with both insulin and Losartan. These results were further confirmed by determining the number of insulin, ETA, and ETB receptors as the percentage of fluorescent dots per surface area of oil immersed microscopic field using a specific computer program. Conclusions: The changes in the expression of the insulin, the ETA, and the ETB receptors at the various sites of the myocardium and the effect of both insulin treatment and blockade of the AT1-R can explain the new benefits related to cardiac protection which was shown in the halting of myocardial remodeling in IDDM rats.

P-060

**Enhanced cardiac fibrosis of endothelin-1 (ET-1) transgenic mice is independent of the inducible nitric oxide synthase (iNOS)**

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Background: Etndothelin-1 is involved in cardiac fibrosis and cardiac inflammation leading to heart failure and death of ET-1 transgenic mice. ET-1 and NO form a control cycle with a negative feedback mechanism, which explain our previous observations of enhanced expression of the inducible nitric oxide synthase (iNOS) in ET-1 transgenic animals. In line with these observations, we generated ET-1 transgenic mice lacking the iNOS gene to prove the concept that cardiac fibrosis in ET-1 transgenic mice is dependent on iNOS. Methods: We generated ET-1 transgenic mice (ET-1+/+) with an additional knockout for the iNOS gene (iNOS -/-) (Et-1+/+; iNOS-/-). After catheterization of the left cardiac ventricle of the mice, pressure volume loops were recorded, and animals were sacrificed. The organ weights were determined and blood samples were taken. Global cardiac matrix protein synthesis was analysed after Sirius red or hematoxylin-eosin staining. Results: The systolic blood pressures of ET-1 transgenic mice missing the iNOS gene were markedly increased compared to mice with iNOS expression, but was similar to the systolic blood pressures of wild type animals. (ET-1+/+), as well as (Et-1+/+; iNOS-/-) mice developed a significant interstitial fibrosis of the cardiac muscle (see table 1, stiffness constant b and by dP/dt min), and this was independent from blood pressure values. The perivascular fibrosis was diminished in (Et-1+/+; iNOS-/-) mice, when compared to (ET-1+/+) mice. In addition, (ET-1+/+)mice show elevated systolic and diastolic ventricular pressure values in the heart. Conclusion: Endothelin-1 enhanced cardiac fibrosis is independent of iNOS gene expression. The elevated intracardiac pressure values might be the result of cardiac malformation or function. Further studies are necessary to further elucidate these interactions.

P-061

**Short term effect of rate control in patients with tachyarrhythmias after catheter ablation on serum endothelin levels**

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Radiofrequency catheter ablation (ABL) or modification of atrio-ventricular junction is an effective therapeutic approach to drug refractory supraventricular tachyarrhythmias (ST). Higher endothelin (ET) levels were observed during STs. The aim of this study was to observe the short term effect of rate control on serum ET levels. 22 patients (pts) (12 male, age:64.4±13.2 yrs) had atrial (A) fibrillation, A flutter, A paroxysmal tachycardia and refractory sinus tachycardia in 11,7,3 and 1 cases, respectively; underlying diseases: coronary artery disease (n=8), dilative cardiomyopathy (n=5) and hypertension (n=9), EF: 41.8±11.2%, NYHA:I,II and III in 3,11 and 8 cases, respectively; all grp had catheter ABL with the same protocol (35±10.3 min). In grp I pacemaker (PM) was implanted 2 months before (n=9), in grp II implantation was during ABL (n=7) and in grp III no PM was implanted (n=6). Rate was 100-170/min in every case before ABL, 70-80/min in PM and 70-90/min in noPM grp after ABL. A test grp (n=13, 7male, age: 66.15±6.7yrs) with sinus rhythm got PM without ABL. Blood samples were collected from cubital vein immediately before (control), 5 minutes and 24 hours (24h) after ABL. ET-1 levels were evaluated with Western blot analysis. Results: comparing the control, 5 min and 24h samples, ET-1 levels decreased significantly after SVT ABL in grp I and III. Serum bigET levels did not change significantly in either grps. ET levels remained unchanged after PM implantation in test grp. Conclusion: rapid decrease of ET neogenesis after catheter ABL suggests that high ventricular rate can be a craggy, fast trigger of ET production, although reaction on implantation procedure was more sensitive in ST than sinus rhythm. (mean±SEM. *p<0.05. 5min, 24h vs. ET control in all grps). Results are in fmol/ml

| | grp I | grp II | grp III | test grp |
|---|---|---|---|---|
| ET control | 0.66±0.13 | 0.93±0.32 | 0.68±0.12 | 0.50±0.17 |
| ET 5min | 0.50±0.12* | 0.77±0.12 | 0.61±0.14 | 0.58±0.24 |
| ET 24h | 0.29±0.14* | 0.61±0.15 | 0.34±0.12* | 0.68±0.33 |
| bigET control | 0.80±0.20 | 1.34±0.49 | 1.12±0.64 | 0.90±0.20 |
| bigET 5min | 0.78±0.28 | 1.28±0.60 | 1.02±0.56 | 0.93±0.24 |
| bigET 24h | 0.67±0.26 | 1.81±0.49 | 0.78±0.74 | 0.95±0.30 |

P-062

**ETB receptors located on vascular smooth muscle cells of cardiomyopathic hamsters are importantly involved in the clearance of circulating endothelin**

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In the hamster, we recently reported that vascular smooth muscle cell-$ET_B$ receptors are involved in ET-1 plasma clearance (Honore *et al.* 2005; *Am J Physiol, Epub*), and suggested that those receptors must be spared from any pharmacological antagonism in pathological conditions involving increased ET-1 production. In the present study, the putative protective role displayed by $ET_B$ receptors in conditions of heart failure was assessed. We evaluated the effects of chronic $ET_A$ and/or $ET_B$ receptor blockade, induced by atrasentan (5 mg/kg/d) and/or A-192621 (24 mg/kg/d), respectively, in cardiomyopathic Bio 14.6 hamsters treated from 52 to 61 weeks of age. Bio 14.6 hamsters had a reduced survival rate as compared to their healthy littermates, and this variable was not affected by pharmacological treatments. Basal blood pressure was not modified by the selective $ET_A$ antagonist. Conversely, selective $ET_B$ and surprisingly non-selective $ET_A$/$ET_B$ blockade induced systemic blood pressure increases. Interestingly, these increases were correlated with enhanced ET plasma levels observed after selective $ET_B$ and non-selective $ET_A$/$ET_B$ receptor blockade. With regards to cardiac parameters, selective or non-selective antagonism of $ET_A$ receptors reduced the left ventricular end diastolic pressure but did not improve the cardiac index of contractility (LV +dP/dt). Notably, only the selective $ET_A$ antagonist reduced ET-1 levels in the cardiac left ventricle. On the other hand, nitrate/nitrite plasma levels were decreased in Bio 14.6 hamsters and not altered by treatments with antagonists. Our results suggest that despite no improvement in the survival rate of hamsters with advanced congestive heart failure, selective $ET_A$ antagonism provides beneficial cardiac effects. Importantly, blockade of vascular smooth muscle cell-$ET_B$ receptors triggers deleterious effects which might be related to their role in ET-1 clearance from the circulation.

P-063

**Elevated endogenous endothelin triggered with ventricular fibrillation and shock application may have role in occurrence of ventricular arrhythmias**

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Our earlier investigations presented direct arrhythmogenic effect of ET-1, shown up during ventricular tachycardias(VT) at low dose intracoronary exogenous ET-1 infusion. Elevated serum levels of ET-1 and bigET were measured after triggered and spontaneous malignant VAs in humans. The aim of our study was to investigate dynamics of ET-1 and bigET production and to observe the developement of VAs after triggered ventricular fibrillation(VF) and shock applications. After implantable cardioverter defibrillator implantation no VF inductions were in group (grp) I (n=5) while VF inductions and endocardial defibrillations(25J) were 10 and 20 times in grp II (n=7) and III (n=6). Peripheral(P) venous blood samples were taken simultaneously with arterial(aortic root, AR) samples before implantation and 0,30,60,120min after the last shock delivery. ECG and haemodynamical parameters were recorded continuously. Results: both peripheral ET-1 and bigET levels were elevated significantly in grp II and III after 120min compared to control period. Only 20x25J grp had significant ET elevation in AR after 120min, although bigET levels in both grp II and III were elevated significantly. Comparing P and AR levels, bigET values were significantly higher in P samples after 120min, but not in control period. Number of ventricular premature beats and non sustained VTs were higher in grp II and III significantly compared to grp I (chi-square: 3,98; p<0,05).Sustained VTs occurred in one dog in grp III. Compared to control grp, endogenous ET levels induced by VF induction and shock application elevated significantly. In these grps the ratio of VA occurrence was significantly higher. (mean±SEM. *p<0.05 120min vs. control in all grps; #p<0.05 P vs. AR in all grps). Results are in fmol/ml.

| | ET control | ET 120 min | BigET control | BigET 120min |
|---|---|---|---|---|
| P grp I | 1.04±0.30 | 0.90±0.26 | 1.93±0.7 | 2.11±0.90 |
| AR grp I | 0.68±0.28 | 0.67±0.30 | 1.60±0.51 | 1.58±0.56 |
| P grp II | 1.01±0.41 | 1.62±0.49* | 2.19±0.57 | 4.23±0.68* # |
| AR grp II | 0.66±0.19 | 0.83±0.21 | 2.01±0.87 | 2.65±0.83* |
| P grp III | 1.01±0.29 | 1.75±0.67* | 1.47±0.76 | 3.33±1.54* # |
| AR grp III | 0.62±0.31 | 1.05±0.43* | 1.62±1.04 | 2.97±1.08* |

P-064

**Urban pollutants Interact toxicologically to regulate lung ET system genes and plasma endothelin**

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Periodic elevation of ambient particulate matter and ozone levels is linked to acute cardiac morbidity and mortality. Increased plasma levels of ET-1, a prognostic indicator of cardiac mortality, have been detected in both animal models and humans after exposure to air pollutants. The lungs are the primary source of circulating ET-1, but the direct effects of individual air pollutants and their interaction in modulating the pulmonary ET system are unknown. Fischer-344 rats were exposed by nose-only inhalation to particles (0, 5, 50 mg/m3 EHC-93), ozone (0, 0.4, 0.8 ppm), or combinations of particles and ozone for 4 h. Changes in gene expression were measured using real-time RT-PCR, and ET peptides were measured by HPLC immediately after exposure and following 24 h recovery in clean air. Both pollutants increased preproET-1 and ECE-1 mRNA in the lungs shortly after exposure, consistent with the concomitant increase in plasma ET-1 peptide. While plasma ET-3 was also increased immediately after pollutant inhalation, lung preproET-3 mRNA decreased. PreproET-1 mRNA remained elevated 24 h after exposure to particles but not after ozone, in line with previously documented changes of the peptide in plasma. Both pollutants transiently increased ET-B receptor mRNA, while ozone decreased ET-A receptor mRNA levels. Exposure to the combination of particles plus ozone increased preproET-1 mRNA but not plasma ET-1. This coincided with an increase in the lungs of matrix metalloproteinase-2, an enzyme that cleaves bigET-1. Our results show that ozone and urban particulate matter independently regulate expression of lung ET system genes, but in combination show evidence of toxicological interaction with respect to plasma ET-1. Because of the known pathophysiological consequences of elevated ET-1 in individuals with cardiovascular disease, our data provide direct evidence of a plausible mechanism for the acute, adverse health impacts of urban pollutants.

P-065

**Etiology-Specific Endothelin-1 Clearance in Human Precapillary Pulmonary Hypertension**

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Introduction: Endothelin-1 is a mediator of the vascular remodelling seen in human pulmonary hypertension, and it is normally cleared via endothelial ETB receptors. Increased levels of endothelin-1 are found in precapillary PH, partly from increased synthesis. The effects of endothelial dysfunction and vascular remodelling on endothelin-1 clearance in human precapillary pulmonary hypertension are unknown. We hypothesized that a severely remodeled vascular bed would have reduced ET-1 clearance. Patients and Methods: Fourty-four patients with pulmonary arterial hypertension (PAH; 19 idiopathic; 14 from connective tissue disease), and 11 patients with chronic thromboembolic PH were studied. Using indicator-dilution methods, the first-pass extraction of radiolabelled endothelin-1 through the pulmonary circulation, and PS, an index of functional microvascular surface available for endothelin-1 clearance were determined. Results: Mean extraction for idiopathic PAH and thromboembolic PH groups was normal, but it was slightly reduced in PAH from connective tissue disease. 68% of all patients studied had normal extraction. The mean PS product was reduced significantly for all 3 etiologies as compared to normal, but 58% of idiopathic PAH and 43% of connective tissue disease-related PAH had normal PS products. Conclusion: Receptor-mediated endothelin-1 extraction and functional vascular surface area for clearance varies between etiologies of precapillary PH and, unexpectedly, extraction is preserved in many patients.

P-066

**Transforming growth factor ß(1) induction of endothelin-1 expression in lung normal and scleroderma lung fibroblasts: insights into the molecular basis of pulmonary fibrosis**

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Purpose The pro-fibrotic proteins transforming growth factor-beta (TGF-ß) and endothelin-1 (ET-1) are found in elevated amounts in bronchoalveolar lavage (BAL) of individuals with scleroderma (SSc). However, whether these proteins cooperate to produce the SSc phenotype is unknown. The ability of TGFß to induce ET-1 in lung fibroblasts has been investigated. Methods Pulmonary fibroblasts (n=5) were obtained from healthy individuals and scleroderma patients with lung fibrosis (FASSc). Cells were used between passage 2-5. ET-1 protein, in the presence or absence of exogenous TGFß, was detected by ELISA. Promoter deletion and mutation analysis were used to identify the cis-acting sequences and trans-acting factors responsible for the TGFß-induction of the ET-1 promoter. Results Application of TGF to normal lung fibroblasts induced ET-1 protein and promoter activity. Mutation and deletion analysis of the ET-1 promoter suggested that an Ap-1 (activator protein-1) site, but not a Smad recognition motif, was at least partially responsible for the TGFß induction of ET-1. Similarly, overexpression of dominant negative c-jun, but not the inhibitory Smad Smad7, significantly blocked the TGFß-induction of the ET-1 promoter ($p<0.05$). ET-1 expression is elevated in FASSc fibroblasts (42.57+7.03) compared to normal lung fibroblasts (11.09+1.62) ($p<0.05$). Moreover, ET-1 promoter activity is elevated in FASSc fibroblasts compared to normal cells, in a fashion that is independent of the Smad response element, but dependent at least on part on elevated Ap-1 activity. Conclusions These results suggest that activation of the Ap-1 pathway may contribute to the pathogenesis of lung fibrosis and that ET-1 may be a mediator of the pro-fibrotic effect of TGFß in vivo.

P-067

**Role of the Endothelium and of ETA and ETA Receptors on ET-1 Induced Vasoreactivity of Isolated Rat Pulmonary Resistance Arteries: Importance of Receptors Heterodimerization**

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Introduction: The respective roles of the ETa and ETb receptors and of the endothelium on ET-induced vasoreactivity of pulmonary resistance arteries is still incompletely understood. Endothelial ETb receptors induce vasodilatation by releasing nitric oxide and prostanoids, but can also induce vasoconstriction by the release of thromboxane A2. Methods: Rat pulmonary vessels (150 to 200 microM diameter) were mounted on an isometric myograph. For each vessel, endothelium-dependant vasodilatation to Ach and maximal vasoconstriction to 127 mM KCl were determined. Cumulative concentration-response curves to ET-1 and S6c were performed with or without endothelium. The same protocols were performed in the presence of meclofenamic acid (MA)(100 uM), lipoxygenase inhibitor (LOX)(10 uM, A-85761.0), ETA antagonist (10 nM, A-147627.1), antagonist (1uM, A-192621.1), or in the presence of both. Heterodimerization of receptors was evaluated by immunoprecipitation of the , followed by western blotting for the expression of the ETa receptor. Results: The maximal vasoconstriction and sensitivity induced by ET (Emax 91+/-4%, EC50 11.0+/-2.2 nM) and S6c (Emax 85+/-4%, EC50 5.0+/-1.6 nM) were not different. Removal of the endothelium did not significantly modify the maximal response and EC50 values to ET and to S6c. The LOX inhibitor and MA also had no significant effect. Both the ETa antagonist (Emax 73.3 +/- 5.9) and the antagonist (Emax 79.0 +/- 2.4) significantly but very mildly reduced ET-1 induced vasoconstriction. The combination of both however greatly reduced the maximal response (Emax 57.7 +/- 3.0 $p<0.001$) and the EC50 values (EC50 0.36 +/- 0.13 uM, $p=0.0150$). In vitro studies demonstrated co-immunoprecipitation of the ETa and receptor. Conclusion: In the normal rat, ET-induced pulmonary vasoconstriction of small arteries is mediated by the ETa and ETb receptors with no significant modulator role of the endothelium. The use of dual antagonism is necessary for optimal blockade of vasoconstriction, possibly because the ETa and ETb receptors can form functional heterodimers.

P-068

**Dual Role of the ETB Receptor and Importance of Prostanoids on ET-1 Response in Isolated Rat Lungs**

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Introduction: The endothelin (ET) system contributes to the pathophysiology of pulmonary hypertension (PHT). While ET-induced pulmonary vasoconstriction is mediated by both the ETA and ETB receptors on smooth muscle, activation of the endothelial ETB receptor can mediate the release of the vasodilator nitric oxide and the potent vasoconstrictor thromboxane A2. Methods To elucidate the respective roles of the ETA and ETB receptors and prostanoids on the pulmonary vasoconstriction response induced by ET-1 in isolated rat lungs. Lungs were perfused at a constant flow rate (10 ml/min) and subjected to cumulative concentration-response curves with ET-1 (10 nmol/L to 0.3 umol/L) and sarafotoxin 6c (S6c, 10 nmol/L to 0.3 umol/L) with and without meclofenamic acid (0.1mM), lipoxygenase inhibitor (10 um), ETA antagonist (10nm, 100 nm, 1um), ETB antagonist (1 um, 100 um) and in the presence of a non-selective antagonist (Bosentan, 0.1 mM). Results: Both ET-1 (36.5 +/- 6.4 mmHg delta pressure, mean +/- SEM) and S6c (35.8 +/- 7.6 mmHg) induced important and similar vasoconstrictions with the formation of severe pulmonary oedema. Meclofenamic acid partially antagonized ET-mediated vasoconstriction ($p=0.001$) whereas the LOX inhibitor and the ETB antagonist had no significant effect. The ETA antagonist (17.8 +/- 8.0 mmHg, $p=0.05$) significantly reduced the ET-1 induced vasoconstriction whereas Bosentan (6.1 +/- 1.7 mmHg, $p=0.001$) almost completely suppressed the response to ET and the development of pulmonary oedema. In preconstricted lungs with ETA receptor blockade, ETB stimulation with S6c induced mild vasodilation at low concentrations, but severe vasoconstriction at higher concentrations. Conclusion: In isolated rat lungs, ET-1 induced pulmonary vasoconstriction is modulated by prostanoids. The mild vasodilator role of the ETB receptor, evident at low agonist concentration, is masked at higher concentrations. Although the majority of ET-1 induced vasoconstriction is mediated by the ETA receptor, non-selective blockade provided superior efficacy.

P-069

**Endothelin-1 influences the efficacy of inhaled nitric oxide in experimental acute lung injury**

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Beneficial effects of inhaled nitric oxide (iNO) on arterial oxygenation in acute lung injury (ALI) suggest the presence of vasoconstriction in ventilated lung regions. The underlying pathophysiological mechanisms are still unclear; however, an increased pulmonary release of endothelin-1 (ET-1) has been demonstrated in ALI. In this study, we investigated a possible interaction between ET-1 and iNO in experimental ALI. Sixteen piglets were anesthetized and mechanically ventilated ($FiO_2$ 1.0). After induction of ALI by surfactant depletion, animals were randomly assigned to either inhale 30 ppm NO (iNO group, n=8), or to receive no further intervention (Controls, n=8). Parameters of hemodynamics and gas exchange, and ET-1 plasma levels (BI20052 ELISA, Biomedica, Vienna, Austria) were measured during a 4 h period. In all animals, induction of ALI significantly decreased $PaO_2$ from 569±15 (pre-lavage) to 58±3 mmHg, and increased intrapulmonary shunt from 14±1 to 50±2 %. Inhalation of NO increased $PaO_2$ at 4 h after onset of ALI to 265±51 mmHg, and decreased Qs/Qt to 21±3 % (Controls: 50±4 mmHg and 55±4 %; $p<0.01$ vs. iNO). Pre-lavage ET-1 plasma levels were comparable between groups (iNO: 0.74±0.03, Controls: 0.71±0.03 fmol/ml, n.s.). After an initial increase at 1 h following onset of ALI (iNO: 1.01±0.06, Controls: 1.08±0.1 fmol/ml;n.s.), values were significantly different at 3 h (iNO: 0.93±0.06, Controls: 1.25±0.09 fmol/ml; $p<0.05$). $PaO_2$ changes induced by iNO (calculated as difference from $PaO_2$ at the onset of ALI in 32 single determinations) revealed a moderate and significant correlation with ET-1 plasma levels ($R=0.548$, $p=0.001$). Our data suggest that endogenous ET-1 production influences the efficacy of iNO in ALI. Furthermore, iNO reduced ET-1 plasma levels, possibly indicating anti-inflammatory properties of iNO in the early phase of ALI.

P-070

**Differential changes of pulmonary ET-1 and eNOS expression in rats with a chronic left ventricular pressure overload**

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The expressions of endothelin-1 (ET-1) and endothelial nitric oxide synthase (eNOS) were assessed in the lung of adult Wistar rats undergoing an ascending aortic banding (AOB) to increase left ventricular afterload for one day, 2 weeks, 4 weeks and 12 weeks. The AOB resulted in significant medial hypertrophy of pulmonary arterioles artery at 4 and 12 weeks, and increased pulmonary arterial pressures at 1 day (banded, 45± 1.8 mmHg; sham, 20 ± 1.3 mmHg), 4 weeks (banded, 31 ±1.3 mmHg; sham, 21 ±1.8 mmHg) and 12 weeks (banded, 36 ± 1.7 mmHg; sham, 20 ± 1.0 mmHg). The competitive reverse transcription-polymerase demonstrated significant increases in pulmonary preproET-1 mRNA at 1 day ( banded, 2.11 ± 0.11; sham, 1.0 ± 0.07), 4 weeks (banded 1.9 ± 0.1, sham, 1.0± 0.1), and 12 weeks (banded, 2.35 ± 0.14, sham, 1.0 ± 0.06), and in pulmonary eNOS mRNA at 1 day(banded 2.9 ± .26, sham, 1.0 ±0.08) and 12 weeks (banded 1.86 ± 0.18, sham, 1.0 ± 0.07). In addition, the western blot analysis showed increases in pulmonary eNOS by 320 % and 190%, at 1 day and 12 weeks. But there were only increased lung cGMPs at 1 day (AOB, 5.2 ± 0.33 pmol/g protein; sham, 3.1 ± 0.16 pmol/g protein). However, tissue ET-1 contents were increased at 1 day (banded 309 ± 11 ng/g protein, sham, 203 ±7 ng/g protein), 4 week (banded 232 ± 11 ng/g protein, sham, 201 ± 5 ng/g protein) and 12 weeks (banded 256 ± 14 ng/g protein, sham, 202 ±8 ng/g protein). These results indicate that the gene expressions of ET-1 and eNOS are up-regulated during the early stage of left ventricular afterload at 1day, returned to be normal at 2 weeks. In addition, the up-regulated ET-1 expression developed again, prior to eNOS, at 4 weeks in banded rats. In conclusion, the development of pulmonary hypertension and medial hypertrophy of pulmonary arterioles is closely related to the up-regulation of ET-1 expression in the AOB model.

P-071

**The effects of Sildenafil on the lung expression of ET-1, eNOS and cGMP in pulmonary hypertension secondary to heart failure in aorta-banded rats**

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Sildenafil, an oral phosphodiesterase type-5 inhibitor, has vasodilatory effects. We investigated whether the sildenafil attenuated pulmonary hypertension, and how altered the expression of endothelin-1 (ET-1), endothelial nitric oxide synthase (eNOS) and cGMP in Wistar rats with left ventricular dysfunction caused by an ascending aortic banding. The banded rats were randomized to receive saline from Days 1-28 ($AOB_{28}$, n=8), sildenafil (50 mg/Kg/Day) from Day 14-28 ($AOB_{28}/Sil_{14-28}$, n=8) or from Day 1- 28 days ($AOB_{28}/Sil_{1-28}$, n=8). There was significant development of pulmonary hypertension with pulmonary vascular remodeling 4 weeks after banding. Although there were significant attenuations of thickened medial layer of pulmonary arterioles in both sildernafil groups (% wall thickness: sham, 22 ± 1.1 (mean ± SE) %; $AOB_{28}$, 40 ± 2.2 %; $AOB_{28}/Sil_{14-28}$, 33 ± 1.8 % ; $AOB_{28}/Sil_{1-28}$, 26 ± 1.4 %), the inhibition of the increased mean pulmonary arterial pressure was significantly noted only in the $AOB_{28}/Sil_{1-28}$ group (sham, 20 ± 1.0 mmHg; $AOB_{28}$, 33 ± 2.0 mmHg; $AOB_{28}/Sil_{1-28}$,, 26 ± 1.1 mmHg). PreproET-1 mRNA and eNOS mRNA were measured by competitive reverse transcription-polymerase chain reaction, eNOS was measured by western blotting, and ET-1, Big ET-1 and cGMP were checked by radioimmunoassay. Subsequently, the sildenafil did not alter the contents of pulmonary ET-1, PreproET-1 mRNA, Big ET-1 and eNOS in banded rats. But it increased pulmonary cGMP in both groups(sham, 4.1 ± 0.22 pmol/g protein; $AOB_{28}$, 3.1 ± 0.1pmol/g protein; $AOB_{28}/Sil_{14-28}$, 3.9 ± 0.16 pmol/g protein; $AOB_{28}/Sil_{1-28}$, 4.6 ± 0.15 pmol/g protein). These results suggest that early co-apply with sildenafil for one month inhibits both the rise in pulmonary arterial pressure and the pulmonary vascular remodeling in pulmonary hypertension secondary to failure, and the its antiproliferative effects maybe result from eNOS-independent-cGMP pathway.

P-072

**The effects of debanding on the lung expression of ET-1, eNOS and cGMP in pulmonary hypertension secondary to heart failure in aorta-banded rats**

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Our previous work observed that there was significant development of pulmonary hypertension secondary to heart failure in aortic banded (AOB) rats. To evaluate the possible the mechanism involved, the present study investigated whether debanding (DeB) attenuated pulmonary hypertension, and how altered the expression of endothelin-1 (ET-1) and endothelial nitric oxide synthase (eNOS) in Wistar rats with left ventricular dysfunction caused by an ascending aortic banding. 28 days after banding, the banded rats were randomized to receive debanding, or simple thoracotomy without debanding. All the rats had been fed continuously for further 21 days, and divided into three groups (sham(n=7), $AOB_{1-49}$(n=7) , $AOB_{1-28}/DeB_{29-49}$(n=7)).The attenuation of thickened medial layer of pulmonary arterioles, and the inhibition of the increased mean pulmonary arterial pressure (sham, 19 ± 1.3 mmHg; $AOB_{1-49}$, 32 ± 2.7 mmHg; $AOB_{1-28}/DeB_{29-49}$,, 20 ± 1.3 mmHg) were significantly noted in the $AOB_{1-28}/DeB_{29-49}$ group. PreproET-1 mRNA and eNOS mRNA were measured by competitive reverse transcription-polymerase chain reaction, eNOS was measured by western blotting, and ET-1 and cGMP were checked by radioimmunoassay. Subsequently, the debanding did not alter the contents of pulmonary eNOS, eNOS mRNA and cGMP in banded rats. But it decreased pulmonary preproET-1 mRNA by 200 %, pulmonary ET-1 contents (sham, 210 ± 12 pg/g protein, $AOB_{1-49}$, 370 ± 49 pg/g protein, $AOB_{1-28}/DeB_{29-49}$, 206 ± 1.9 pg/g protein), and plasma ET-1 levels (sham, 10.1 ± 1.5 pg/ml, $AOB_{1-49}$, 15.4 ± 2.0 pg/ml, $AOB_{1-28}/DeB_{29-49}$, 10.3 ± 0.9 pg/ml protein). These findings suggest that debanding inhibits both the rise in pulmonary arterial pressure and the pulmonary vascular remodeling in pulmonary hypertension secondary to failure, and is directly involved in attenuating up-regulated ET-1 gene expression in the heart failure model.

P-073

**Sitaxsentan Therapy in Pulmonary Arterial Hypertension Results in Significantly Fewer Liver Function Abnormalities than Bosentan**

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PURPOSE: The first approved endothelin receptor antagonist (ETRA) for the treatment of pulmonary arterial hypertension (PAH) is bosentan (BOS), an oral, twice-daily, non-selective ETA/ETB ETRA. While a significant advance for PAH, BOS therapy has been complicated by abnormal liver function tests [>3X upper limit of normal (abLFT)] in a controlled trial setting (12% at the labeled dose) and in clinical practice, a finding that has been subsequently seen at varying rates with all ETRAs studied in PAH. Sitaxsentan (SITAX), is an oral, once-daily, highly selective (>6500:1) ETA ETRA in development for PAH which demonstrated lower abLFT rates with 100mg QD in STRIDE-1. These lower abLFT rates were confirmed in STRIDE-2: 6.5% of patients randomized to placebo, 3.2% for SITAX 100mg, 4.9% for SITAX 50mg, and 11.5% for BOS. Here, we report on the long-term LFT rates observed for patients (pts) treated for up to a year with BOS, used according to the product label, or SITAX. METHODS: STRIDE-2 was an 18 week, multi-center, placebo-controlled study that randomized 246 patients (pts) 1:1:1:1 to PBO, SITAX 100 mg, SITAX 50 mg or open label, efficacy-rater blinded, BOS followed by an extension with pts receiving either BOS or SITAX 100 mg. During the extension, pts on PBO in STRIDE-2 were randomized to SITAX 100mg or BOS; pts on SITAX 50mg received SITAX 100mg and pts on SITAX 100mg or BOS were continued on those treatments. RESULTS: Kaplan-Meier estimate of time to abLFT at 1 year of exposure are 4.0% for SITAX 100mg and 18.7% for BOS (p = 0.0086). CONCLUSIONS: Long-term treatment with SITAX 100mg QD results in significantly less liver function abnormalities than bosentan. CLINICAL IMPLICATIONS: Sitaxsentan 100mg QD has been shown to be safe and effective in the treatment of PAH, with a lower rate of liver function abnormalities than bosentan.

P-074

**Endothelin-1 and nitric oxide alter differently in lungs in different time-points in endotoxemia**

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Expression of endothelin (ET)-1, a potent vasoconstrictor, is altered in various diseases. Some evidences suggest that circulating ET-1 is elevated in sepsis. However, no study has reported the time dependent alterations of different components of ET system in the lung of sepsis. The present study investigated whether ET system (ET-1, $ET_A$ and $ET_B$ receptors) has a potential role in the acute lung injury of sepsis in a time-dependent manner. Male Wistar rats at 8 weeks of age were administered with either saline or lipopolysaccharide (LPS) at different time points (1, 3, 6 and 10 hours). The rats were killed with ether and lungs were either frozen or preserved in formalin. Blood gas analysis was done immediately after opening the chest. The features of acute lung injury were observed in 1 hour after LPS administration, which gradually became severe with time. Systolic and diastolic pressures were drastically lower just 1 hour after LPS administration. A time-dependent increase of ET-1 expression was observed in LPS administered lungs compared to control lungs and ET-1 level was peaked at 6 hours (3-fold) after induction of endotoxemia by LPS while the expression of preproET-1 mRNA was peaked in lung tissue at 1 hour after LPS administration. Immunoblot analysis and Real-Time PCR experiments demonstrated a time-dependent gradual increase of $ET_A$ receptor after LPS administration while opposite result was found in $ET_B$ receptor expression. $ET_B$ receptor with vasodilating property, was remarkably downregulated in septic lung in a time-related manner. Immunohistochemical studies revealed differential expressions of ET-1 and its receptors in pulmonary tissues after induction of endotoxemia. Nitric oxide levels as Nitrate/Nitrite were increased in septic lung in a time-dependent manner although the expression of eNOS was downregulated with time. Thus, gradual increase in iNOS expression might cause the non-functional increased NO production in lung tissue in sepsis. We conclude that time-dependent increase of ET-1, $ET_A$ receptor, non-functional NO together with the downregulation of $ET_B$ receptor and eNOS may play a role in the pathogenesis of acute lung injury in endotoxemia.

P-075

**A Major Role of Endothelial Cells-derived Endothelin-1 in Preventing Hypoxia-induced Pulmonary Hypertension in Mice: A Conditional Knockout Study**

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Chronic hypoxia-induced pulmonary hypertension (PH) is associated with increased serum and pulmonary expression of endothelin-1 (ET-1). However, in lung with PH, ET-1 is produced not only by pulmonary vascular endothelial cells, but also by vascular smooth muscle cells, airway epithelial cells and alveolar type II pneumocytes. To directly address the causative role of endothelium-derived ET-1 in the pathogenesis of pulmonary hypertension, we inactivated the ET-1 gene selectively in endothelial cells by using the Tie-2 promoter in mice and exposed them under hypoxic condition for 3 weeks. Twelve-week-old WT (n=11) and KO (n=14) mice were studied. KO mice exhibited 60% reduction in pulmonary ET-1 levels than did WT mice, and undetectable levels of ET-1 in plasma. Following 3-weeks of hypoxic exposure, the KO mice had lower RV systolic pressure (31±0.78 in WT vs. 20±1.2 mmHg in KO; $P<0.01$), RV/BW ratio (1.83±0.21 in WT vs. 1.26±0.10 in KO; $P<0.05$), and RV/(LV+S) ratio (0.54±0.04 in WT vs. 0.35±0.03 in KO; $P<0.005$) than the WT mice, but they are not completely prevented from the hypoxia-induced pulmonary hypertension. Hypoxia increased ~4-folds of the serum levels of ET-1 only in WT mice ($P<0.005$ vs. normoxia). On the contrary, pulmonary ET-1 levels rose ~2-folds in both WT and KO mice ($P<0.05$ vs. normoxia). These data demonstrated that although ET-1 produced by endothelial cells plays a major role to the pathogenesis of hypoxia-induced pulmonary hypertension, ET-1 produced by other cell types in lungs also contributes to the development of pulmonary hypertension.

P-076

**Primary Structure of Cat Preproendothelin-2 and Increased Renal Expression in Naturally Occurring Renal Failure**

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While there is accumulating evidence showing that endothelin is involved in the pathogenesis of various renal disorders in human medicine, little known about the contribution of endothelin to renal disease in veterinary medicine. In this study we focus on an important veterinary disease, ferine chronic renal failure, and we explore, through analysis of mRNA expression in the affected kidney, the possible involvement of a member of endothelin family, endothelin-2 (ET-2), in renal disorders. We also perform comparative analysis of the primary preproprotein structure among mammals based on the cloned cat preproendothelin-2 (PPET-2) cDNA sequence. Fundamental protein features, commonly observed in endothelin family preproproteins, including domain organization of mature peptide, big form and like peptide regions as well as processing target sites for dibasic endopeptidase and endothelin converting enzyme were all found in cat PPET-2. Homology analysis showed that the similarity of the cat PPET-2 amino acid sequence with those from human, mouse, rat, rabbit, dog, ferret, cow and horse was 73.0, 68.6, 69.1, 76.4, 81.2, 83.1, 76.3 and 79.2 %, respectively. mRNA expression analysis by reverse transcription-polymerase chain reaction demonstrated a marked increase in the affected kidney. These findings suggest a pathophysiological involvement of ET-2 in ferine chronic renal failure. Because chronic renal failure in older cats can be a serious clinical problem in veterinary medicine, a new pharmatherapeutic approach to inhibit the progression of renal disease, focusing on the overexpression of the endothelin in kidney, is anticipated.

P-077

**Possible role of endothelin system members in the bovine corpus luteum after induced luteal regression**

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There are evidences that endothelin-1 (ET-1) is involved in the function and regression of the corpus luteum (CL) in cows. Previous studies have shown that ET-1 directly inhibits progesterone (P) secretion in luteal cells via ETR-A. The aim of the study was to investigate real-time changes in luteal tissue of endothelin system members in mRNA expression, tissue concentration and tissue localization after prostaglandin F2-alpha (PG) induced luteal regression in cow. Corpora lutea (Days 8-12) were collected by transvaginal ovariectomy before and 2, 4, 12, 24, 48 and 64 h (n=5/time point) after PG injection. In addition, we examined the effect of intraluteal injections of ETR-A antagonist (ANT) on PG induced luteolysis. The Endothelin converting enzyme (ECE-1) mRNA expression showed a tendency of an increase after the PG injection, which was significant after 12 h and declined thereafter significantly. The ET-1 mRNA expression tended to increase after 2 h which was significant after 4, 12, and 24 h followed by a significant decrease thereafter. The ET-1 peptide concentration tended to increase after 2, 4, and 12 h and became significant after 24 and 48 h with a decline thereafter. The mRNA for both receptors were upregulated for ETR-A after 4 h and for ETR-B after 2 h. The expression of both receptors decreased after 48 h. Localization of ET-1 in tissue revealed very weak staining before PG application followed by a clear increase of staining predominantly in large luteal cells, but also in endothelial cells at the time of the structural luteolysis. In addition, ANT treatment delayed the reductions of the CL volume (6 h for CONT vs. 48 h for ANT) and the blood flow area within the CL (8 h for CONT vs. 48 h for ANT). In conclusion, the results suggest that ET family members are regulated during induced luteal regression. ET-1 may act as vasoconstrictor during functional luteolysis, but also as an apoptosis inducer during functional/structural luteolysis in cows.

P-078

**Effect of ET-1 on aquaporin 2 (AQP2) expression and its relationship with NO system**

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The aim of this study was to investigate if ET-1 has a direct action on renal AQP2 water channel and its relationship with NO system at collecting tubules (CT) level. Male Wistar rats were divided in three groups: A: ET-1 (5 ng/Kg/min), B: ET-1 (50 ng/Kg/min) and C: control (0.09 ml/min saline), n=5. Saline and/or ET-1 were administered via jugular veins for 60 minutes after a 45 min period of stabilization. For immunohistochemistry kidneys were excised, fixed, embedded in paraffin and cut on a microtome in 4 µ sections. A rabbit polyclonal antibody against AQP2 was used followed by biotinylated donkey anti-rabbit IgG, and the reaction was revealed with streptavidin diaminobencidine. Labeled cells per ten fields were counted by light microscopy at 1000x magnification in cortex (Co), outer medulla (OM) and inner medulla (IM). For histochemistry, kidneys were excised, fixed, cryoprotected, frozen and cut in 14 µ sections. NOS activity was evaluated by histochemical reaction of NADPH-diaphorase measuring optical density (OD) in CT through an image analyzer. Number of labeled cells increased in OM in group B (12.85 ± 2.03)*** vs C (6.62 ± 0.71) and in IM in A (8.85 ± 0.95)** and B (14.62 ± 1.26)***# vs C (3.34 ± 0.60). There were no significant differences in number of labeled cells in Co. OD increased in CT of OM (outer stripe) in A (0.219±0.008)* and B (0.235±0.004)***# vs C (0.186±0.007); in OM (inner stripe) in A (0.161±0.002)* and B (0.167±0.006)** vs C (0.144±0.003) and in IM in A (0.141±0.002)*** and B (0.142±0.003)*** vs C (0.116±0.002). Statistical analysis was performed by ANOVA followed by Tuckey test. * p<0.05; **p<0.01; ***p<0,001vs C; #p<0,05vs A. These results show that ET-1 increases AQP2 expression both in OM and IM in a dose dependent manner. Bearing in mind the antidiuretic properties of ET-1 and ET-1-mediated increase in NOS activity at CT level, it is possible that besides the increase in AQP2 expression, this peptide could promote AQP2 translocation to apical membrane via NO pathway.

P-079

**Lifestyle modification reduces plasma endothelin-1 concentration in obese men**

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Obesity is associated with endothelial dysfunction that may contribute to the development of atherosclerosis. Endothelin-1 (ET-1), which is produced by vascular endothelial cells, has potent vasoconstrictor and proliferative activity in vascular smooth muscle cells and, therefore, has been implicated in regulation of vascular tonus and progression of atherosclerosis, suggesting that ET-1 may be important in the endothelial dysfunction. We studied whether diet- with/without exercise-induced weight loss (i.e., lifestyle modification) affects plasma ET-1 concentration in obese individuals. We measured plasma ET-1 concentration in 12 obese men (age: 50 ± 2 years old, body mass index: 27.5 ± 0.4 kg/m$^2$) before and after 3-month diet- with/without exercise-induced weight reduction program (i.e., lifestyle modification program). Caloric restriction with/without regular exercise reduced body weight from 79 ± 2 to 70 ± 2 kg (*p<0.0001*) and resulted in 11.2 ± 0.6% reduction in body mass index (24.4 ± 0.3 kg/m$^2$, *p<0.0001*). After the weight reduction program, systolic and diastolic blood pressure significantly decreased (131 ± 5 vs. 118 ± 4 mmHg, *p<0.01* and 88 ± 3 vs. 78 ± 2 mmHg, *p<0.001*, respectively). The maximal oxygen consumption ($VO_{2max}$) significantly increased after the weight loss program (32.5 ± 1.4 vs. 36.8 ± 1.8 ml/kg/min, *p<0.01*). The plasma level of ET-1 significantly decreased after the program (4.8 ± 0.3 vs. 3.9 ± 0.2 pg/ml, *p<0.001*). The relationship between percentage weight reduction and percentage plasma ET-1 concentration reduction was linear (r = 0.72, *p<0.01*). We conclude that weight loss by low-calorie diet with/without regular exercise (i.e., lifestyle modification) reduces plasma ET-1 concentration in obese individuals. This reduction may contribute to the improvement of obesity-induced endothelial dysfunction.

P-080

**Paradoxical downregulation of renal ECE-1 and ECE-2 in early autoimmune diabetes: Reversal by chronic ETA-receptor blockade**

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Endothelin (ET)-1 and ET converting enzymes (ECE) are increased in diabetes and play an important role in the development of diabetes-associated renal injury. The pathways by which the high-glucose environment in diabetes mediates increased levels of ET have not been completely clarified but appear to involve ECEs, which convert inactive big ET-1 to active ET-1 peptide. Although it has been shown that blockade of ETA-receptor reverses proteinuria in patients with diabetic nephropathy, it is unknown, to what extent transcriptional regulation of renal ECE-1 and ECE-2 are affected during early stages of autoimmune diabetes mellitus and whether ETA-receptors are involved in the regulation. Female non-obese-diabetic (NOD) mice were used at a pre-diabetic stage at the age of 16 weeks and animals was treated with the ETA-receptor antagonist BSF461314 or placebo for six weeks. Transcriptional regulation of renal ECE-1 und ECE-2 was determined by determined by real time-PCR, and renal morphology was assessed using standard histological techniques. Expression of ECE-1 and ECE-2 was present in control and diabetic NOD mice. Unexpectedly, expression of ECE-1 was reduced in diabetic NOD mice by approximately 50% compared to controls ($p<0.01$). The reduction was even stronger for ECE-2, which was decreased by almost 10-fold ($p<0.01$). After blockade of ETA-receptors with BSF461314, the reduction of ECEs gene expression was no longer present in diabetic NOD mice ($p<0.05$ versus untreated) while treatment had no effect in control animals (n.s.). Analysis of renal histology revealed no pathological findings. In conclusion, at the onset of diabetes paradoxical down-regulation of renal ECE-1 and ECE-2 occurs in early type 1 diabetes and might involve effects mediated like by plasma glucose and renal ETA-receptors. The observed decrease of ECEs could be related to negative gene regulation by endogenous ET-1. This notion is supported by the observation that the ETA-receptor antagonist BSF461314 restored gene expression. These observations may be important to the pathogenesis of type 1 diabetes and its associated diseases.

P-081

**The influence of three endothelin-1 (ET-1) polymorphisms on the progression of IGA nephropathy (IGAN)**

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Background: The clinical course of chronic renal diseases and their progression to end stage renal failure is highly variable. Different candidate gene polymorphisms, affecting mainly the onset/development of arterial hypertension, have been advocated as possible modulators of the progression. Endothelin-1 (ET-1) has been suggested to be a major disease promoting factor in renal disease. We investigated a possible association of three single-nucleotide polymorphisms of ET-1 K198N, T-1370G and 3A/4A with the progression of IGAN towards end stage renal disease, as well as the clinical and histological manifestations of IGAN. Methods: We examined a group of 173 patients (pts.) with histologically proven IGAN (99 pts. with normal renal function, 74 pts. with ESRF), as a control group we used 200 genetically unrelated healthy subjects. DNA samples from collected blood were genotyped for three single-nucleotide polymorphisms of ET-1 K198N, T-1370G and 3A/4A by means of polymerase chain reaction (PCR) with defined primers, electrophoresis on 2 % agarose gel and UV light visualization. We compared the frequencies of different genotypes between the IGAN groups with normal renal function and ESRF. Results: The ET-1 genotype distribution showed no differences among the groups of IGAN with normal renal function ( 1. K198N: 64,6 % KK, 31,3 % KN, 4,0 % NN;2. TT: 69,1 % TT, 27,8 % TG, 3,1 % GG; 3. 3A/4A: 41,5 % 3A/3A, 47,6 % 3A/4A, 10,9 % 4A/4A ), IGAN with ESRF ( 1. K198N: 64,9 %KK, 33,8 %KN, 1,4 % NN;2. TT: 76,1 % TT, 22,5 % TG, 1,4 % GG, 3. 3A/4A: 65,9 3A/3A, 17,0 % 3A/4A, 17,0 % 4A/4A ) and control group (1. K198N: 62,5 % KK, 34,5 % KN, 3 % NN, 2. TT: 76 % TT, 22,5 % TG, 1,5 % GG, 3. 3A/4A: 51,5 % 3A/3A, 45 % 3A/4A, 3,5 % 4A/4A ). The distribution of ET-1 genotypes did not differ among IGAN with normal renal function, IGAN with ESRF and control group. Conclusion: We excluded the effect of K198N, T-1370G and 3A/4A polymorphisms of ET-1 gene on the progression of IGAN to ESRF. Supported by the grant project NK 7733-3, VZ MSMT 00216 208 06

P-082

**Mechanisms of endothelin A receptor antagonist protection in kidneys of streptozotocin-induced diabetic rats**

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Endothelin (ET) receptor antagonists are beneficial in the treatment of diabetic nephropathy. Because ET stimulates reactive oxygen species production and induces cyclooxygenase-2, we hypothesized that the renoprotective effects of $ET_A$ receptor antagonists in diabetic rats are due to reduced oxidative stress and prostanoid production. Diabetes was induced by streptozotocin, and sufficient insulin was provided to maintain moderate hyperglycemia for 10 weeks (HG). Some HG rats also received the $ET_A$ antagonist ABT-627 (HG+ABT; 5 mg;$kg^{-1}$;$day^{-1}$). Sham rats received vehicle treatments. HG kidneys exhibited increased glomerular collagen staining that was attenuated by ABT. Urinary microalbumin was also increased in HG rats (Table, p=0.08, n=8-10/group) and was reduced by ABT. Indices of oxidative stress, urinary excretion of thiobarbituric acid reactive substances (TBARS) and H2O2 and plasma TBARS, were significantly greater in HG rats than sham rats, but these effects were not prevented by ABT (Table: * p< 0.05 vs sham, # p<0.05 vs HG). Urinary excretion of prostaglandin E metabolites (PGEM) was increased in HG rats and was reduced by ABT. Urinary excretion of 6-keto-prostaglandin F1$\alpha$ and thromboxane B2 was increased in HG rats and unchanged by ABT treatment. As the $ET_A$ receptor antagonist, ABT-627, attenuated sclerotic injury in diabetic rats without reducing oxidative stress, we conclude that the beneficial effects of this agent in diabetic nephropathy are not likely the result of reduced oxidative stress. We propose that the renoprotective effects of $ET_A$ receptor blockade may be mediated, at least in part, by a reduction in prostaglandin E synthesis.

| | Microalbumin excretion mg/day | TBARS excretion µmol/day | H2O2 excretion nmol/day | Plasma TBARS nmol/ml | PGEM excretion ng/day |
|---|---|---|---|---|---|
| Sham | 9 ± 2 | 0.24 ± 0.02 | 13 ± 5 | 8.9 ± 0.5 | 14 ± 1 |
| HG | 20 ± 7 | 40 ± 6 * | 308 ± 61 * | 31 ± 3 * | 143 ± 14 * |
| HG+ABT | 11 ± 3 | 42 ± 8 * | 206 ± 43 * | 34 ±2 * | 87 ± 10 *# |

P-083

**The effects of different doses of atorvastatin on plasma ET-1 levels in type 2 diabetic subjects**

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The effects of different doses of hydroxymethylglutaryl coenzyme A reductase inhibitors (statins) on plasma ET-1 levels in type 2 diabetic patients with dyslipidemia are not known. Methods: We investigated the effects of 3 different daily doses (10mg, 20 mg, 40 mg) of atorvastatin, a hydroxymethylglutaryl coenzyme A reductase inhibitor, on plasma ET-1 and high sensitive C-reactive protein(CRP) levels in type 2 diabetic subjects. Twenty nine type 2 diabetic subjects with dyslipidemia were enrolled and randomly assigned to receive atorvastatin orally at 10 mg(GroupA, n=10), 20 mg(Group B, n=10), or 40 mg(Group C, n=9) daily for 12 weeks. Blood samples were taken for analysis of lipid profiles as well as ET-1 and CRP before and 12 weeks after atorvastatin therapy. Plasma ET-1 and CRP concentrations were measured by an ELISA method. Results: All diabetic subjects had higher plasma ET-1 concentrations (Group A:1.02±0.12 pg/ml, mean±SEM; Group B:1.17±0.17 pg/ml; Group C: 0.87±0.15 pg/ml, all $p< 0.001$ vs control) than that of age and sex-matched normal control subjects (0.63±0.04 pg/ml). Levels of plasma lipids (total cholesterol, LDL-cholesterol and triglyceride) were significantly decreased ($p<0.001$, $p<0.001$, $p<0.01$, respectively) after treatment with atorvastatin. Plasma ET-1 levels showed a borderline significant decrease by 22% in diabetic patients treated with 10 mg atorvastatin ($p=0.05$), and by 30% in patients treated with 20 mg atorvastatin ($p=0.06$). Paradoxically, plasma ET-1 levels increased by 2% in diabetic patients treated with 40 mg atorvastatin. Similarly, though insignificantly, plasma concentrations of CRP also tended to decrease by 12% and 48%, and paradoxically increased by 18% in diabetic patients treated with atorvastatin 10mg, 20 mg, and 40 mg, respectively. Conclusions: The experimental biphasic lipid-independent statin effects which may be potentially harmful could also be seen in humans. The present study suggested that the optimal dose of atorvastatin therapy in humans may be 20 mg daily.

P-084

**Hyperthyroidism is associated with higher plasma ET-1 concentrations and insulin resistance**

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To determine the change of plasma ET-1 concentrations and insulin resistance index after therapy of hyperthyroidism. We studied 20 hyperthyroid patients (15 females and 5 males, age 34.0 ±2.8 years), and 31 euthyroid goiter subjects as control (27 female, 4 male, age 37.0±2.4years). All hyperthyroid patients were treated with antithyroid drugs. The patients received evaluations before and after normalization of thyroid function. The evaluations included BMI, body fat mass, and measurement of circulating concentrations of thyroid hormones, glucose, insulin, and ET-1. Hyperthyroid subjects had higher plasma ET-1 concentrations than controls (0.93±0.06 pg/ml vs 0.63± 0.04 pg/ml, $p< 0.001$). No significant differences of serum glucose and insulin concentrations and insulin resistance index estimated by HOMA-R were noted. Plasma ET-1 concentrations decreased after correction of hyperthyroidism compared with those of pretreatment (0.69±0.04 pg/ml vs 0.93±0.06 pg/ml, $p = 0.006$). Serum glucose concentrations decreased after correction of hyperthyroidism (4.68±0.08 mmol/l vs 5.01 ±0.08 mmol/l, $p = 0.005$). Both body weight adjusted insulin concentrations and HOMA-R index were decreased after correction of hyperthyroidism compared with those of pretreatment (0.107±0.012 vs 0.152±0.019, $p = 0.026$; 0.023±0.003 vs 0.035±0.005 , $p = 0.019$, respectively). Pearson's correlation revealed that plasma ET-1 level positively correlated with serum T3 ($r = 0.556$, $p < 0.001$) and FT4 ($r = 0.474$, $p < 0.001$) concentrations. Serum insulin concentrations and HOMA-R index positively correlated with BMI ( $r = 0.411$, $p < 0.001$; $r = 0.398$, $p < 0.01$, respectively) and percentage of body fat ($r = 0.311$, $p < 0.01$; $r = 0.296$, $p < 0.05$, respectively). HOMA-R index also positively correlated with serum T3 ($r = 0.244$, $p < 0.05$) and FT4 ($r = 0.271$, $p < 0.05$) concentrations. Neither insulin concentrations nor HOMA-R index correlated with ET-1 levels. Correction of hyperthyroidism is associated with a decrease of plasma ET-1 levels as well as insulin resistance.

P-085

**CGS 35601, a single molecule triple vasopeptidase inhibitor, modulates the hemodynamic and metabolic profiles in chronically instrumented, conscious and unrestrained type II Zucker diabetic fatty rats**

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CGS 35601 is a potent triple inhibitor of angiotensin converting enzyme, neutral endopeptidase, and endothelin converting enzyme-1. The aim of the present study was to assess the pharmacological and toxicological profiles of this vasopeptidase inhibitor (VPI) in Zucker diabetic fatty (Zdf) rats, a gene-prone model of type II diabetes. Males Zdf (14 weeks, n=19-23) were implanted with a left carotid catheter and placed individually in a metabolic cage for 30 days. The hemodynamic, hematological and biochemical profiles were assessed daily. After a 7-day stabilization period, the rats were divided into 2 groups: Gr. 1 (n=9-13) received increasing doses of CGS 35601 (0.1, 1 and 5 mg/kg/d x 6 d each, i.a.) followed by a 5 d wash out period; Gr. 2 (n=10) control-vehicle received saline (250 µl/h). CGS 35601 reduced mean arterial blood pressure (MABP) by 5.6% (102±5 to 96±4 mmHg) at 0.1 mg/kg/d. At 1 and 5 mg/kg/d, MABP was reduced to 89±6 and 80±4 mmHg, respectively. Plasma concentrations of angiotensin II and endothelin-1 decreased steadily, while atrial natriuretic peptide and bradykinin concentrations increased over the same range. Interestingly, CGS 35601 induced a dose-dependant decrease in cholesterol and HDL: from 3.35±0.15 and 1.75±0.14 mmol/L to 2.6±0.1 and 1.46±0.11 mmol/L (at 5 mg/kg/d), respectively. However, CGS 35601 did not improve oral glucose tolerance tests. CGS 35601 presented an excellent short-term safety profile determined though > 40 parameters measured in both plasma and urine reflecting cardiac, renal and hepatic functions. This novel molecule and class of VPI may be of interest for treating type II diabetes-related complications such as hypertension. Support: FICQ, FMCQ, FRSQ (BB-Scholar), IPS Pharma, Novartis.

P-086

**Bosentan effect on alpha-smooth muscle actin expression and NADPH diaphorase activity in diabetic rat kidney**

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We aimed to investigate the effect of bosentan on alpha-smooth muscle actin (α-SMA) expression and NADPH diaphorase activity in experimental diabetic nephropathy. Male Wistar rats were made diabetic by injection of 70mg/kg streptozotocin. Controls were injected with the citrate buffer. The animals were divided in three groups: 1:control, 2: diabetic, 3:diabetic bosentan as food admix (100mg/kg/day), n=6, for 10 weeks. Immunohistochemical studies were performed on fixed, paraffin embedded 4 µm sections of kidney. Sections were incubated with mouse monoclonal antibody anti α-SMA followed by biotinylated horse anti mouse IgG. The reaction was revealed with streptavidin-diaminobencidine. Images were obtained with light microscope at 400x and immunoreactive cortical α-SMA area per ten fields was measured using a computerized image analyzer. For histochemistry kidneys were fixed, cryoprotected, frozen and cut in 14 µm sections. Reaction of NADPH-diaphorase was used to identify Nitric Oxide Synthase (NOS) activity in the cortex, measuring optical density (OD) by a computerized image analyzer. Immunoreactive cortical α-SMA area increased in group 2 (13.98 ± 2.83)** vs 1 (3.89 ± 0.87) and diminished in group 3 (4.47 ± 0.65) ** vs 2 (13.98 ± 2.83). There were no significant differences in immunoreactive cortical α-SMA area in group 1 vs 3. OD increased in cortical proximal tubule in group 2 (0.27 ± 0.026) *** vs 1 (0.22 ± 0.026) and diminished in group 3 (0.24 ± 0025) *** vs 2 (0.27 ± 0.026). Statistical analysis was performed by ANOVA followed by Bonferroni test **p<0.01; ***p<0,001. Our data show that ET is probably implicated in the development of fibrosis in renal cortex of diabetic rats. Inhibition of its action by bosentan leads to a reduction in α-SMA expression and NOS activity. We suggest a possible relationship between α-SMA expression and NO system.

P-087

**Preventive effect of flavangenol on ischemia/reperfusion-induced acute renal failure in rats, possibly through the suppression of NF-κB-induced ET-1 production**

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Flavangenol, an extract from French maritime pine bark (PBE), is a complex mixture of bioflavonoids with oligomeric proanthocyanidin as main constituents. It has been reported that nutritional supplementation of PBE produces a variety of potentially protective effects against chronic age-related diseases such as atherosclerosis, hypertension, and diabetes. In the present study, we examined the effects of flavangenol on nuclear factor-kappa B (NF-κB) activation and the subsequent NF-kB-induced ET-1 gene expression in cultured vascular endothelial cells. Flavangenol dose-dependently suppressed tumor necrosis factor (TNF)-α-induced NF-κB activation. Quantitative real-time PCR showed that flavangenol markedly decreased TNF-α-induced prepro ET-1 mRNA expression. We next examined whether flavangenol have a preventive effect on ischemia/reperfusion-induced acute renal failure (ARF) that results from aberrant ET-1 production. Ischemic ARF was induced by occlusion of the left renal artery and vein for 45 min followed by reperfusion, 2 weeks after contralateral nephrectomy. Renal functional parameters such as blood urea nitrogen, plasma creatinine, creatinine clearance, urine flow, urinary osmolality and fractional excretion of sodium were measured. Renal function in ARF rats significantly decreased at 1d after reperfusion. Pre-ischemic treatment with flavangenol (3-30 mg/kg, i.v.) attenuated the ischemia/reperfusion-induced renal dysfunction. Histopathological examination of the kidney of ARF rats revealed sever renal damages, such as tubular necrosis, proteinaceous casts in tubuli and medullary congestion, which were also significantly suppressed by the administration of flavangenol. Taken together with our present results, it is most likely that inhibitory effects of flavangenol on NF-kB activation and the subsequent ET-1 gene expression is closely related to the amelioration of ischemia/reperfusion-induced renal damage, and that flavangenol supplementation is useful as a prophylactic treatment in the development of the ET-1-related several diseases.

P-088

**ETA and ETB receptors differentially modulate afferent and efferent arteriolar responses to endothelin**

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The segment specific actions of ET peptides and agonists have not been thoroughly investigated in the renal microcirculation. The current studies were performed to assess the relative contribution of ETA and ETB receptors to the renal pre- and post-glomerular arteriolar responses to ET-1. Experiments determined the effect of selective ETA (A-127722; 30nM) and ETB (A-192621; 30nM) receptor blockade, on arteriolar responses to ET-1 concentrations of 0.001 to 10nM in rat kidneys using the isolated juxtamedullary nephron technique. Renal perfusion pressure was set at 110mmHg. Baseline afferent arteriolar diameter was similar in all groups and averaged 17.8±0.6μm (n=14). In control experiments (n=6), ET-1 produced significant concentration-dependent decreases in arteriolar diameter, with 10nM ET-1 decreasing diameter by 85±1%. Selective blockade of ETA receptors (n=6) prevented ET-1-mediated vasoconstriction, except at higher concentrations, 1 and 10nM. Similarly, the vasoconstrictor profile was right shifted during selective ETB receptor blockade (n=4). Combined ETA and ETB receptor blockade (n=4) completely abolished afferent arteriolar diameter responses to ET-1. ETB selective agonists (S6c and IRL-1620) produced unique responses. S6c produced a concentration dependent vasoconstriction of afferent arterioles. In contrast, S6c produced a concentration dependent dilation of efferent arterioles that could be blocked with an ETB receptor antagonist. IRL-1620, another ETB agonist, was less effective at altering afferent or efferent diameter and produced a small reduction in pre- and post-glomerular arteriolar. These data demonstrate that both ETA and ETB receptors participate in ET-1-mediated vasoconstriction of afferent arterioles. ETB receptor stimulation provides a significant vasodilatory influence on the efferent arteriole. Furthermore, since selective ETA and ETB receptor antagonists abolished pre-glomerular vasoconstrictor responses at lower ET-1 concentrations, these data support a possible interaction between ETA and ETB receptors in the control of afferent arteriolar diameter.

P-089

**Endothelin-1 stimulates NO and cyclic GMP production in inner medullary collecting duct via NOS1, but NO does not inhibit vasopressin-stimulated cyclic AMP accumulation**

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Endothelin-1 (ET-1) inhibition of vasopressin (AVP)-stimulated cyclic AMP (cAMP) accumulation in the collecting duct has been hypothesized, but never shown, to be mediated, at least in part, by nitric oxide (NO). To examine this issue, we determined the effect of ET-1 on NO production by acutely isolated rat inner medullary collecting duct (IMCD) cell suspensions, and then studied whether this was involved in mediating ET-1 effects on AVP-stimulated cAMP accumulation. ET-1 stimulation for 30 minutes caused a dose-dependent (first evident at 100 pM ET-1), albeit modest (~130%) increase in IMCD NO production as determined by DAF-FM fluorescence. This effect was blocked by ETB receptor (BQ788), but not ETA receptor (BQ123), antagonism. Incubation with non-specific NOS inhibitors (L-NAME or L-NMMA) or NOS-1 inhibitors (SMTC or VNIO) inhibited the ET-1 response, while NOS-2 or NOS-3 inhibitors (L-NAA or 1400W) were without effect. ET-1 (30 minute exposure) also increased cGMP accumulation. ET-1 (10 nM) caused a 35% reduction in AVP-stimulated cAMP levels (in the presence of IBMX), however this response was not affected by L-NAME, L-NMMA, SMTC or VNIO, suggesting that NO did not mediate the ET-1 response. Addition of L-arginine (2-20 mM) or tempol (to reduce superoxide-dependent conversion of NO to peroxynitrate) did not affect the response. To determine if NO can inhibit AVP-induced cAMP accumulation, IMCD cells were exposed to NO donors (SNP, TNG, or spermine NONOate) at concentrations that markedly stimulated DAF-FM fluorescence and increased cGMP levels in IMCD cells. None of these NO donors (varying concentrations and exposure for 10-60 minutes) altered AVP-stimulated cAMP accumulation in the IMCD cell suspensions. In conclusion, ET-1 stimulates IMCD NO production through activation of the ETB receptor and NOS-1. However, neither ET-1-mediated NO production nor NO donors inhibit AVP-stimulated cAMP accumulation (in the presence of phosphodiesterase inhibition) indicating that, under these conditions, NO does not regulate IMCD cAMP levels.

P-090

**Effect of endothelin-A receptor blockade on nutritive skin capillary circulation in patients with type 2 diabetes and microalbuminuria**

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Background: Most complications of diabetes have their basis in disturbed microvascular function. Endothelin-1 (ET-1) is increased in patients with type 2 diabetes and may contribute to impaired microvascular function. Aim: To investigate the effect of selective endothelin-A receptor (ETA) blockade (BQ 123) on skin microcirculation in patients with type 2 diabetes and microalbuminuria. Material and methods: Ten patients and eight non diabetic controls, matched for sex and age, were investigated. A percutaneous catheter was inserted into the left brachial artery for infusions. The nutritive capillary circulation was investigated by videophotometric capillaroscopy, measuring capillary blood cell velocity (CBV; mm/s) in nailfold capillaries of the left hand and the total microcirculation of the same skin area was assessed by laser Doppler fluxmetry (LDF). Skin microcirculation, at rest and following a 1 min arterial occlusion, was investigated before and after 15 min infusion of saline, and after 15, 30, 45 and 60 min infusion of BQ123 (10 nmol/min). Five of the patients were also investigated on a second occasion with the same protocol but with 60 min of saline infusion. Results: In patients, CBV increased significantly after 30 min (0.58±0.11; p=0.02), 45 min (0.71±0.17; p=0.04) and 60 min (0.75±0.17; p=0.01) BQ123 infusion, as compared to baseline. CBV was unchanged in controls 0.54±0.20, 0.55±0.66 and 0.54±0.20, respectively. Peak CBV during postocclusive reactive hyperaemia and skin temperature also increased significantly in patients, but not in controls during BQ 123 infusion. LDF was not significantly changed during infusion of BQ123 in patients or controls, as compared to baseline. CBV, LDF and skin temperature did not change significantly during 15 and 60 min of saline infusion. Conclusion: ETA-receptor blockade markedly improves nutritive skin capillary circulation in patients with type 2 diabetes and microangiopathy. ET-1 may be involved in the pathogenesis of diabetic microangiopathy and targeting the ET-1 system might be beneficial in treating complications related to diabetic microangiopathy.

P-091

**Differential regulation of micro vs. macrovascular dysfunction by endothelin-1 in type-2 diabetes**

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Vascular dysfunction characterized by a hyperreactivity to vasoconstrictors and/or impaired vascular relaxation contributes to increased incidence of cardiovascular disease in diabetes. Endothelin-1 (ET-1) is chronically elevated in diabetes. However, the role of ET-1 on resistance vs larger vessel function in mild diabetes remains unknown. Accordingly, this study investigated vascular function of third order mesenteric arteries and basilar artery in control Wistar and Goto-Kakizaki (GK) rats, a model of mild Type 2 diabetes. 6-weeks after the onset of diabetes, contractile responses to ET-1 (0.1-100 nM) and relaxation responses to acetylcholine (ACh, 1 nM-10 μM) in ET-1 (100 nM) treated vessels or in vessels preconstricted (baseline + 60%) with serotonin (5-HT, 10nM-10μM) were assessed by myograph studies in the presence or absence of a NOS inhibitor (L-NNA). Blood pressure (MAP) monitored by telemetry was significantly higher in GK rats (121 ± 1 vs 104 ± 2mmHg*). Maximum contractile response to ET-1 was augmented in mesenteric vessels (161 ± 19% in GK and 81 ± 6 % in control, n=3-7*) but not in the basilar artery (134 ± 28% in GK vs 90 ± 6% in control, n=4). However, vascular relaxation was impaired in the basilar artery (22 ± 4% in GK and 53 ± 7% in control, n=4/group*) but not in mesenteric arteries of GK rats. Inhibition of NOS decreased the relaxation response of basilar artery to 15 ± 8 and 42 ± 5% in GK and control rats*, respectively, whereas in resistance vessels, corresponding values were 64 ± 6% and 87 ± 3%* (vs 110 ± 2% and 112 ± 3% without NOS blockade) indicating the involvement of different vasorelaxation-promoting pathways in these vascular beds. These findings provide evidence that the ET system is activated even under mild hyperglycemia and contributes to the hyper-reactivity of resistance vessels, which may play an important role in elevated blood pressure in Type-2 diabetes. (* $p<0.05$, GK vs Control)

P-092

**Effect of feeding behavior on endothelin-2 expression in mouse intestines**

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The function, regulation and gene expression of the endothelin-2 (ET-2) system in intestine is not well understood. We investigated the dependence on feeding schedule of the regulation of ET-2 gene expression in tongue, stomach, duodenum, jejunum, colon and pancreas. Mice were fed freely, fasted for 48 hours, and re-fed for 0.5 and 2.0 hours after fasting. Gene expression was analyzed by real-time RT-PCR. ET-2 gene expression was highest in jejunum compared with other tissues examined in the fasting mice. Re-feeding increased gene expression in duodenum and colon, and did not change gene expression in jejunum. To identify the difference in regulation of ET-2 gene expression in jejunum and colon induced by re-feeding, we examined the effects of intragastric and intravenous injection of glucose solution. The intragastric and intravenous administration of glucose to 48-hour fasted mice resulted in decreased ET-2 gene expression in jejunum and colon. Our results suggest that an increase in ET-2 expression in colon induced by re-feeding does not depend on any biological function of glucose.

P-093

**Effect of FK409, a nitric oxide donor, on ischemia/reperfusion-induced renal injury and endothelin-1 production in rats**

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The contribution of nitric oxide (NO) to ischemic acute renal failure (ARF) is controversial. There are some reports indicating that NO is a deleterious factor on renal injury after ischemia. On the other hand, we have demonstrated that pre-ischemic treatment with FK409, a spontaneous NO donor, protects against ischemic ARF in rats and that pre-ischemic treatment with nonselective NO synthase inhibitors aggravate the ischemic ARF. These findings suggest that NO exerts protective effects on ischemic ARF. In the present study, we examined the effect of post-ischemic treatment with FK409 on ischemic ARF. Ischemic ARF was induced by clamping of the left renal artery and vein for 45 min followed by reperfusion, 2 weeks after contralateral nephrectomy. At 24 h after reperfusion, renal function in untreated ARF rats markedly decreased and histopathological examination of the kidney of untreated ARF rats revealed severe renal damages. In addition, increases in renal endothelin-1 (ET-1) content were evident in ischemic ARF rats at 2, 6 and 24 h after reperfusion, respectively. Pre-ischemic treatment with FK409 (1, 3 mg/kg, i.v.) at 5 min before ischemia improved the ischemia/reperfusion-induced renal dysfunction, histological damage and increases in ET-1 content. In contrast, post-ischemic treatment with FK409 (3, 10 mg/kg, i.v.) at 6 h after reperfusion aggravated the renal injury but did not affect the increased ET-1 content at 24 h after reperfusion. These results suggest that pre-ischemic treatment with FK409 exerts protective effects on ischemic ARF, probably through the suppression of renal ET-1 overproduction, whereas post-ischemic treatment with the NO donor worsens the ischemia/reperfusion-induced renal injury, through mechanisms unrelated to ET-1 production.

P-094

**Alterations of NO, eNOS and endothelin-1 in whole kidney of streptozotocin-induced early diabetes and effects of endothelin antagonism**

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Diabetic vascular complications are important factors which influence the prognosis of diabetic patients. The development and progression of vascular endothelial cell damage is the basis of diabetic microangiopathy, which suggests that ET-1 may participate in the development and progression of diabetic microangiopathy. Type I diabetes was induced by intraperitoneal injection of streptozotocin (65mg/kg) in Sprague-Dawley rats while control (Con) rats received only citrate buffer. After 1 week, the streptozotocin-administered rats were randomly divided into two groups, dual ET receptor antagonist administered group (DMTx, SB209670, 1 mg/rat/day, by osmotic mini-pump for 2 weeks) while the diabetic rats received (vehicle) was DM group. The random blood glucose level was 405±103 mg/dl in DM animals and were unchanged by ET antagonism. Body weight was decreased in DM rats than in Con rats. Whole kidney weights were higher in DM rats compared to Con rats. Vascular endothelial growth factor (VEGF) and phosphorylated Akt (pAkt) levels in whole kidneys were unchanged in three groups. eNOS level was greatly higher in DM kidney and was partially reversed by endothelin antagonism (Con:DM:DMTx: 32±8 : 77±23 : 52±13, pg/mg respectively). However, nitric oxide levels (NOx) were significantly reduced in DM kidney and was greatly recovered after endothelin antagonism (Con:DM:DMTx: 350±23 : 309±45 : 397±45, μM/mg respectively). Renal ET-1 level was higher in DM rats and endothelin antagonism could ameliorate this upregulation (Con:DM:DMTx: 3.2±1.2 : 4.4±1.4 : 3.7±0.6, pg/mg respectively). Previous reports show that VEGF expression is increased in glomerular podocytes, distal tubules and collecting ducts after 3 weeks and 32 weeks of streptozotocin - diabetes in rats, the apparent discrepancy in the present study may be due to the use of whole kidney rather than specific cell type in kidney and the duration of diabetes. The present findings demonstrate that endothelin antagonism reverses the downregulated NO levels in kidney together with the increased ET-1 level, and therefore prevents partly the progression of diabetes-induced renal injury.

P-095

**Changes of NO and ET-1 in plasma and cardiac tissues in streptozotocin-induced early diabetic rats: effects of selective and dual ET receptor antagonists**

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Nitric oxide (NO) and endothelin-1 (ET-1) are two important vasoregulatory molecules and are affected in some organs of several diabetic models. In the present study, male Sprague-Dawley rats (weight, 450±26 g) were administered citrate saline vehicle or streptozotocin (65 mg/kg IP). Diabetes was confirmed by hyperglycemia and after 1 week of diabetes, animals were separated into those receiving endothelin-A/B (ET-A/B) dual receptor antagonist (SB209670, 1 mg/day/rat), endothelin-A (ET-A) receptor antagonist (TA-0201, 1 mg/kg) or saline (vehicle) for 2 weeks by osmotic mini pump and then sacrificed. Glucose levels in DM rats significantly increased (405±103 mg/dL) than in non-DM rats (120±8 mg/dL). ET-1 levels in plasma were [non-DM : DM : TA-0201 : SB209670 (2.4±0.7 : 2.1±0.5 : 2.5±0.5 : 7.4±1.7, pg/ml, respectively)]. Cardiac ET-1 level was 30% higher in DM, completely reversed to non-DM level by ET-A/B dual antagonist and marginally affected by ET-A antagonist. Plasma NO level was almost unchanged in DM rats compared to non-DM rats irrespective of ET antagonism, but cardiac NO level was 27% increased in DM rats and further increased by both types (ET-A/B and ET-A) of ET-antagonist treatments. Cardiac iNOS levels were [non-DM : DM : TA-0201 : SB209670 (7.1±2.4 : 12±4.4 : 9.3±2.0 : 9.0±3.4, U/mg, respectively)]. Cardiac eNOS levels were [non-DM : DM : TA-0201 : SB209670 (108±14 : 144±29 : 72±16 : 145±25, pg/mg, respectively)]. Thus, in early diabetes, ET-1 is altered in plasma and heart in a dissimilar way, and also different ET antagonists affect both local and systemic ET-1 in various manners. While plasma NO level is unchanged in DM, cardiac NO level is upregulated in DM, which may be resulted from the over expressions of both eNOS and iNOS, and both types (ET-A/B and ET-A) of ET blockers further upregulate cardiac NO level. These data suggest that ET antagonism causes favorable change in the tissue levels of both ET-1 and NO in the diabetic heart caused by streptozotocin, although the plasma levels of ET-1 and NO do not reflect the cardiac tissue levels in this DM model.

P-096

**Clinical implication of endothelin antagonism in diabetic erectile dysfunction: Changes in VEGF and NO in type I diabetic penis and beneficial or detrimental effects of endothelin antagonism**

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Erectile dysfunction (ED) affects about 50% of male diabetic patients possibly due to the vascular and neuropathic complications. Vascular endothelial growth factor (VEGF) has been extensively documented for its pathogenic significance in different complications of diabetes and we have already reported that VEGF signaling is greatly diminished in penis in a rat model of type II diabetes. The present study used a three weeks duration of streptozotocin (STZ)-induced diabetic (DM) rat model to assess the VEGF expression with NO system in penile tissue and concomitantly the effects of endothelin antagonism has been studied on these changes. Male Sprague-Dawley rats (weight, 450±26 g) were administered citrate saline vehicle or STZ (65 mg/kg IP). Diabetes was confirmed by hyperglycemia and after 1 week of diabetes, animals were separated into those receiving endothelin-A/B (ET-A/B) dual receptor antagonist (SB209670, 1 mg/day/rat), endothelin-A (ET-A) receptor antagonist (TA-0201, 1 mg/kg) or saline for 2 weeks by osmotic mini pump and then sacrificed. Glucose levels in DM rats significantly increased (405±103 mg/dL) than in non-DM rats (120±8 mg/dL) and local ET-1 level in DM penis was higher by 20%. A 30% decrease in VEGF expression in penile tissue was seen in DM rats, ET-A antagonist did not improve this downregulation but ET-A/B dual antagonist showed a tendency to further decrease the VEGF levels in DM penis. Phosphorylated Akt (pAkt) was reduced in DM penis by 20% and was unchanged by both types of ET blockers. Penile NO and eNOS level was decreased in DM rats, whereas greatly improved by ET-A receptor antagonist while unchanged or decreased by ET-A/B dual antagonist. iNOS was not significantly changed in penile tissues among non-DM, DM and ET-A antagonist treated groups. Thus, we conclude that although VEGF and pAkt were downregulated in type 1 DM penis, both types of ET antagonisms could not recover these downregulations, but ET-A antagonist was potentially effective in reversing the decreased NO and eNOS levels in DM penis than the ET-A/B dual antagonist.

P-097

**Endothelin expression is upregulated by exogenous platelet-activating factor in the kidney of the pregnant rat**

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Platelet-activating factor (PAF) infusion produces fetal growth restriction (FGR) in the rat. The mechanism for PAF-induced FGR has not been identified. Increased levels of both endothelin-1 (ET-1) and PAF have been shown to be important in the pathophysiology of another model of FGR in the rat. The kidney is known to be an important site for endogenous ET-1 synthesis. Whether PAF could up-regulate the expression of ET-1 has not been determined *in vivo*. **Objective:** To quantify preproET-1 expression in the kidneys of the pregnant rat in response to exogenous PAF. **Methods:** Two groups of pregnant Sprague-Dawley rats (n=5 each) were implanted with venous catheters on gestational day 14 (term = 22 days) and received carbamyl-PAF (2.5 µg/kg/h) or saline IV via osmotic pumps on days 14-21. The rats were euthanized on gestational day 21; their kidneys were removed and frozen for total RNA extraction and analysis of preproET-1 mRNA expression by real-time quantitative PCR. **Results:** After seven days of infusion, the ratio of preproET-1 mRNA copy number normalized to 18 S rRNA was significantly higher in the kidneys of PAF-treated rats, as compared with the saline treated animals. Means and standard errors for the ratio of preproET-1 mRNA/18 S rRNA (X $10^3$) were 0.46 ± 0.07 and 0.89 ± 0.15 for the saline and PAF treatment groups, respectively ($p<0.05$ using an unpaired student *t* test). **Conclusions:** Endothelin-1 transcription is up-regulated by PAF in the kidneys of the pregnant rat. ET-1-mediated vasoconstriction may reduce uterine and placental perfusion in this model, as has been shown for other models of FGR. ET-1-mediated reduced placental perfusion, then, may be the cause of the FGR observed in response to exogenous PAF. Supported by NIH grants HD01484 and HD046968.

P-098

**Endothelins signal through multiple pathways via ETA and ETB receptors in rat adrenal medulla**

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We investigated the effect of endothelins (ETs) on receptor-mediated phosphoinositide turnover and cGMP formation in whole adrenal medulla. ET-1, 2 and 3 increased phosphoinositide (PI) turnover by 30% in whole adrenal medulla prelabeled with [3H]-myoinositol. ET-induced InsP1 accumulation was inhibited by BQ 123, a selective antagonist of the ETA receptor, while BQ 788, a selective antagonist of the ETB receptor, was ineffective. The selective agonist at the endothelin receptor, IRL 1620, was ineffective in inducing changes in inositide metabolism. On the other hand, all three isoforms of ETs, at equimolar doses, increased cGMP levels to a similar degree. IRL-1620, a selective ETB receptor agonist, also increased cGMP formation mimicking the effects of ETs, but the increase was higher than those produced by ETs. L-arginine analogue, N-nitro-L-arginine (L-NAME), an inhibitor of NO-synthase; and two inhibitors of soluble guanylyl cyclase: methylene blue and ODQ, significantly inhibited the increase in cGMP production induced by ETs or IRL 1620. Likewise, the selective ETB receptor antagonist, BQ 788, significantly inhibited ET-1 or ET-3-induced cGMP generation. Our data indicate that stimulation of PI turnover and NO-induced cGMP generation constitute both signaling pathways of ETs in rat adrenal medulla. The former action is mediated through ETA receptor activation, while the latter through ETB receptor. These results suggest that endothelins could play a role in the regulation of adrenal medulla function.

P-099

**The combined inhibition of COX-2 and both endothelin receptors leads to an improvement of survival in murine lupus nephritis**

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Background: Approaches to the treatment of lupus nephritis include immunosuppressants associated with anti-inflammatory drugs, mainly steroids, which are known to cause major side effects. Our study evaluated the therapeutic effect of Bosentan, an unselective endothelin-receptor antagonist and Celecoxib, a selective COX-2 inhibitor. We tested these medications individually or in combination in NZB/WF1 hybrid mice with established lupus nephritis. Method: We randomized 36 NZB/WF1 hybrid mice with an age of 180 days and severe lupus nephritis in 4 groups and treated them with the following pharmaceuticals orally: vehicle, Bosentan 180 mg/kg, Celecoxib 40 mg/kg, or Bosentan + Celecoxib. We monitored the mice over a time period of 57 days. We measured intra-arterial blood pressures, analyzed blood samples and histologically evaluated kidney sections of these mice. Results: The animals treated with a combination of both drugs showed a prolonged survival compared to the untreated group. Laboratory values like serum creatinine, total serum protein, C-reactive protein or DNA antibodies as well as urinary values like glomerular filtration rate or proteinuria were not significantly different between the groups. Furthermore, the histological data regarding the development of glomerulosclerosis, interstitial or perivascular fibrosis or extra- or intracapillary proliferation were not significantly different. Conclusion: The combination of bosentan with celecoxib is a useful tool for treating lupus nephritis, as shown by the to prolonged survival rate of treated mice. This cannot be explained by a significantly improved renal function of these mice, as described above. Further studies, including larger groups of mice will be designed to clarify the mechanisms for this success.

P-100

**The effect of targeted disruption of endothelial cell endothelin B receptors on gene expression of the renal endothelin system**

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Expression studies have demonstrated reduced $ET_A$ expression in the renal tissue of rescued $ET_B$ knockout (KO) rodents[1], thought to be secondary to the higher plasma ET-1 concentration associated with $ET_B$ deficiency[2]. By crossing floxed $ET_B$ mice (FF/--) with Tie2-Cre mice[3], we have generated an endothelial cell (EC)-specific $ET_B$ KO mouse (FF/Tie2-Cre). We have examined the effect of selective KO of EC $ET_B$ receptors on the expression of components of the ET system within the kidney. RNA was isolated from kidneys harvested from female mice (aged 2-4 months; 25-35 g) using an acid guanidinium thiocyanate-phenol-chloroform extraction technique[4]. Semi-quantitative PCR was carried out to determine $ET_A$, $ET_B$ and ECE-1 expression relative to the number of GAPDH transcripts detected in each sample. $ET_B$ and ECE-1 expression was significantly down-regulated in the FF/Tie2-Cre females (0.47 ± 0.08 $ET_B$/GAPDH ratio; 0.43 ± 0.05 ECE-1/GAPDH ratio) compared to FF/-- controls (1.22 ± 0.2 $ET_B$/GAPDH ratio; 0.86 ± 0.01 ECE-1/GAPDH ratio; n = 3; $p< 0.05$). In contrast to our results obtained using quantitative autoradiography in male mice, expression of $ET_A$ was also significantly reduced in the FF/Tie2-Cre female mice (0.26 ± 0.002 $ET_A$/GAPDH) relative to controls (0.42 ± 0.04 $ET_A$/GAPDH ratio; n = 3; $p< 0.05$). EC specific KO of $ET_B$ would appear to result in compensatory down regulation of gene expression of other components of the ET system in female mice. Further work is needed to determine whether this pattern of gene expression is similarly altered in male EC $ET_B$ specific KO mice.

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P-101

**Endothelin-1 mediated production of EDB+ fibronectin in diabetic retinopathy**

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Fibronectin (FN), a widely distributed extracellular matrix protein with various cellular functions, may be alternatively spliced to produce several isoforms. Among the spliced variants, extra domain-B (EDB+) FN is absent in mature adult tissues and is exclusively expressed during embyogenesis, tissue repair, and angiogenesis. The present study was aimed at elucidating the mechanisms of EDB+ FN expression in diabetic retinopathy. Human Umbilical Vein Endothelial Cells (HUVECs) and Human Microvascular Endothelial Cells (HMECs) exposed to 25mM glucose showed increased total FN and EDB+ FN mRNA and protein expression, which were prevented by endothelin (ET) receptor antagonist bosentan and transforming growth factor-ß(TGFß)neutralizing antibody. Similar upregulation of EDB+ FN was seen in the retina of streptozotocin-induced diabetic rats, in association with increased ET-1 and ET-3 mRNA. Such upregulation was also prevented by bosentan treatment. We further investigated vitreous samples from patients undergoing vitrectomy for proliferative diabetic retinopathy. Here we found similar increase in EDB+ FN, total FN, TGFß and ET-1 mRNA. We generated an antibody against the EDB segment of FN and demonstrated an increase in serum EDB+ FN levels in patients with diabetic retinopathy and nephropathy. To investigate functional significance of EDB+ FN, we used siRNA to block its expression. Exposure of transfected cells to 25mM glucose prevented glucose-induced VEGF mRNA expression and endothelial proliferation and vascular morphogenesis, whereas glucose-induced increased ET-1 mRNA expression remained unchanged. Data from these experiments demonstrate that glucose-induced and ET-mediated expression of EDB+ FN may be an important mechanism leading to angiogenesis in proliferative diabetic retinopathy. Supported by a grant from Canadian Diabetes Association.

P-102

**Effects of endothelin-1 on human trabecular meshwork cell contraction**

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Trabecular meshwork (TM) cells are now considered to play an active role in the aqueous outflow mechanism, since they exhibit smooth muscle-like contractile properties Endothelin-1 (ET-1), a potent vasoconstrictor peptide, has been proposed to play a role in the local regulation of aqueous outflow and IOP control. We propose an in vitro culture model as a method in the study of ET-1 induced human trabecular meshwork (HTM) cell contractility. Experiments were performed on semi confluent HTM cells (primary cultures established from normotensive human donor eyes) at the 2nd passage with ET-1 and PBS as control. The trabecular meshwork area was isolated under the dissecting microscope at 50X with a microscalpel, grasped with forceps and removed, taking care to exclude the Schlemm canal portion. The HTM cells were cultured in 6-well containing wells, at 37°C in a 5% CO2 atmosphere in the same medium as above; confluence was reached after 3 weeks. HTM cells from the established three primary lines were characterized with respect to their growth characteristics, morphology and cytoskeletal proteins: the immunocytochemical procedures for detection of smooth muscle actin were performed. HTM cell viability was checked with trypan blue staining. The contractile status of the cells was evaluated by a morphometric analysis of cell area, assuming that HTM cells in culture are able to reduce their area as a consequence of cytoskeletal contraction rather than regulatory volume decrease. After incubation with 10 µM ET-1 for 5 minutes we observed a reduction of HTM cell area as respect to PBS treated cells: 2425.76 ± 876.08 µm2 vs. 3125.87 ± 987.56 µm2 ($p<0.001$)and cells exhibited a retraction in shape and indented profiles. Administration of ET-1 at progressively lower doses produced a correspondent lower reduction of HTM cell area suggesting a dose-response ET-1 effect Our data would indicate that ET-1 induced HTM cell area reduction statistically significant vs. control and can directly influence the aqueous outflow.

P-103

**Immunoreactivity of endothelin B receptor in human glaucoma optic nerve**

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Purpose: In glaucoma, there is a systemically and locally increased level of endothelin-1 (ET1), a strong vasomotor peptide. Since ET1 may also act as a potential pathogen in central nervous system to target neurons via a pathological pathway mediated by type B endothelin receptor (ETbR) on astrocytes, the purpose of the study was to 1) quantitatively compare the ETbR immunoreactivity (IR) in the optic nerves in groups of human normal and glaucoma subjects; 2) to assess the relationship of the ETbR and the reactive glial cells in the optic nerves. Methods: Twenty-six post-mortem donor eyes from 16 glaucoma patients (82.7 ±9.3 y) with varied duration and severity, and 10 age-matched normal (82.2 ±8.9 y) were processed for paraffin sections. Longitudinal sections across the anterior optic nerves were labeled immunohistochemically with corresponding antibodies to reveal the ETbR and glial cells. The ETbR IR was semi-quantified with an image analysis system and compared statistically between glaucomatous and normal optic nerves. Results: Nine out of the sixteen glaucomatous optic nerves showed different levels of ETbR IR within the axonal bundles compared to one out of the 10 normal age-matched controls (chi2 Test, P = 0.02). The ETbR IR appeared mostly as tiny dots, but some of them were star-shaped. The ETbR IR was predominantly located on the astrocytic processes demonstrated by co-staining of GFAP and by detailed morphological comparison between the IR and axonal morphology. Conclusion: The high occurrence and quantitatively increase of ETbR IR in the processes of astrocytes of human glaucomatous optic nerves suggests that the glial-endothelin system is involved during the period of pathological processes of glaucomatous optic neuropathy.

P-104

**Endothelin-1 via endothelin B ($ET_B$) receptor activation contributes to apoptosis of rat retinal ganglion cells**

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**Introduction**: Increasing evidence points to the involvement of endothelin-1 (ET-1) in the pathophysiology of glaucoma. The purpose of this study was to determine if ET-1 treatment could directly contribute to cell death of retinal ganglion cells both in culture and in vivo in rats. **Methods**: Brown Norway rats, intravitreally injected with 2 nmole ET-1, were sacrificed 48 h post-injection and retinas were processed for TUNEL assay to detect apoptosis. Wild type and $ET_B$-deficient transgenic rats were intravitreally injected with 2 nmole ET-1 and retinal sections were analyzed for apoptotic changes. Intraocular pressure of wild-type and $ET_B$-deficient rats was elevated by the Morrison's method and optic nerve head sections were analyzed for expression of glial fibrillary acidic protein. Virally transformed rat retinal ganglion cells (RGC-5 cells) were treated with 100 nM ET-1 for 96 h either with or without pretreatment with ET receptor antagonists and apoptotic changes were monitored using TUNEL assay. $ET_B$ expression was studied by immunoblot analysis in RGC-5 cells treated with 1, 10 and 100 nM ET-1 for 24 hr. **Results**: Intravitreal ET-1 treatment produced an appreciable increase in apoptotic cell death of retinal ganglion cells, compared to vehicle-injected control eyes. Wild-type rats with IOP elevation showed increased immunostaining for GFAP suggestive of glial activation, which was markedly lower in $ET_B$-deficient rats. ET-1 mediated apoptosis of retinal ganglion cells was attenuated in $ET_B$-deficient transgenic rats. ET-1 (100 nM) treatment for 4 days produced a modest increase in number of cells undergoing cell death which was appreciably blocked by pretreatment with the $ET_B$ receptor antagonist. ET-1 treatment for 24 hr produced a concentration-dependent increase in $ET_B$ receptor expression in RGC-5 cells. **Conclusions**: Elevations in ocular endothelin concentrations (as seen in primary open angle glaucoma) acting through $ET_B$ receptors, could contribute to glial activation and apoptosis of retinal ganglion cells. These studies suggest the possibility of developing endothelin receptor antagonists as potential neuroprotective agents in glaucoma.

P-105

**$ET_A$ receptors are downregulated in the brain of $ET_B$ globally deficient mice but $ET_A$ density is unaltered in the endothelial cell-specific ETB knock-out mouse**

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$ET_B$ receptors predominate in the brain and are expressed by neurons, astrocytes and endothelial cells, with a small proportion $ET_A$ receptors present on neurones and smooth muscle cells of intracerebral vessels. We have previously shown that in the brain of homozygous (-/-) $ET_B$ deficient mice, $ET_A$ receptor density is significantly downregulated in the brain by 45%. Since ET-1 does not cross the blood-brain barrier, these results cannot be explained by a compensatory downregulation in response to increased circulating levels of ET-1 and suggest an unexpected modulation of $ET_A$ receptors by $ET_B$ in the CNS. Floxed $ET_B$ mice (FF/--) were crossed with Tie2-Cre mice to generate an endothelial cell (EC)-specific $ET_B$ knockout mouse (FF/Tie2-Cre). Immunohistochemistry using selective antisera confirmed that $ET_B$ could not be detected in EC in the periphery but expression was unaltered in other cell types. Following euthanasia, sections were cut from the brains of 3 FF/Tie2-Cre and control male mice. Competition binding assays were carried out using a fixed concentration of [$^{125}$I]-ET-1 (0.1 nM,) and increasing concentrations of BQ-3020 (20 pM-100 μM). In brains from both control and FF/Tie2-Cre mice, BQ3020 competed for [$^{125}$I|]-ET-1 binding biphasically with a more abundant high affinity site corresponding to the $ET_B$ receptors and a low affinity site corresponding to $ET_A$ receptors. There was no significant difference in the affinity of $ET_B$ receptors: 53±7 nM in control and 55±6 nM in brains of FF/Tie2 mice. No change in the density of $ET_B$ receptors could be detected in the EC-specific knock-out (150±15 fmol/mg protein) versus control (146±14 fmol/mg protein) reflecting low numbers of EC in brain relative to other cell types. In contrast to $ET_B^{-/-}$ mice, $ET_A$ receptor was not reduced in EC-specific knock-outs (7±2 fmol/mg protein) versus control (7±1 fmol/mg protein). These results suggest EC $ET_B$ receptors can be excluded from modulating $ET_A$ receptor expression in the brain, which in $ET_B^{-/-}$ mice may be the result of interaction between $ET_B$ receptors expressed by other cell types such as neurones or glia.

P-106

**Regulation of ECE-1 expression in the brain structures of rat and human neuroblastoma cells in culture**

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In recent years endothelin-converting enzyme (ECE) has been suggested to play an important role in amyloid metabolism as one of the amyloid-degrading enzymes. In this connection the analysis of the levels of expression and distribution of ECE in the brain under normal and pathological conditions is an important area in neurodegeneration and pathogenesis of Alzheimer's disease. In our study we analyzed the levels of expression of ECE in the cortex and striatum of rats at different stages of postnatal development under normal conditions and after hypoxia or ischaemia. The data demonstrated that ECE-1 was significantly reduced in the cortex of adult rats after 15 minutes of global ischaemia and restored to control values after 2 hour reperfusion. Prenatal hypoxia (13th day of gestation, 7% oxygen, 3 hours) also resulted in a decrease in the levels of ECE expression in the striatum of rats at early stages of their postnatal life. These data suggest that hypoxia and ischemia might lead to a deficit of ECE which affects both endothelin and amyloid metabolism and makes these subjects prone to accumulation of amyloid deposits and development of Alzheimer's disease. In search of compounds which might up-regulate expression of ECE we examined the effects of some neuropeptides and their analogues on expression of ECE. We have found that somatostatin-14 had a significant up-regulating effect on ECE-1 at a protein level both in human neuroblastoma SK-N-SH and NB7 cells as had an opioid agonist DAGO. The phorbol ester PMA and a muscarinic agonist carbachol were found to reduce expression of ECE. Further study of the signaling mechanisms involved in regulation of ECE expression in neuronal cells might provide us with new therapeutic strategies for prevention or treatment of Alzheimer's disease in elderly patients and those who suffer from stroke or cerebrovascular disorders.

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**Endothelin-1-induced spreading depression in the rat is associated with a cortical microarea of neuronal damage**

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Spreading depression (SD) is assumed to be the pathophysiological correlate of the migrainous aura. Prolonged SDs are observed in animal models of ischemic stroke, intracerebral and subarachnoid hemorrhage as well as brain trauma. The use of subdural strip electrodes recently revealed prolonged SD events in the human brain in the latter conditions. In animal models, prolonged SDs are known to induce neuronal damage. Endothelin-1 (ET-1) has been implicated in all these clinical conditions and was shown to be the most potent inductor of SD in rats in vivo currently known. ET-1 is both a potent vasoconstrictor and a neuroglial modulator. It remained unclear whether primarily vascular or neuroglial targets mediated ET-1-induced SD. Here, we demonstrate that ET-1-induced SDs were associated with a microarea of neuronal damage in the rat brain cortex which may add another piece of evidence that ET-1-induced vasoconstriction/microischemia is the trigger of ET-1-induced SD. Neuropathological changes were studied 24 hours after the experiment (cresyl violet, hematoxylin-eosin, vanadium acid fuchsin-toluidine blue [VAF], assays for transferase dUTP nick-end labeling [TUNEL], glial fibrillary acidic protein [GFAP] and heat shock protein 70 [HSP 70]). Under halothane, ET-1-induced SDs are inhibited so that only 45% of animals generated SD in response to ET-1 at 1 µM. This led us to investigate whether selective histological changes were associated with ET-1-induced SDs under otherwise identical experimental conditions. In 4 of 9 animals in which ET-1 induced one to three SDs, necrotic, hyperchromatic, acidophilic neurons with perineuronal halo were observed. The damaged neurons were few in number and confined to a small area at the cortical surface where ET-1 had been superfused. Surrounding neurons were morphologically intact, but generally showed strong HSP 70 immunoreactivity. ET-1 did not induce neuronal necrosis in 5 of 9 animals that had not generated SD. In summary, ET-1-induced SD is associated with a microarea of neuronal damage at its origin.

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**Elucidating the role of endothelin-2 in inherited photoreceptor degenerations**

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Inherited photoreceptor degenerations (IPDs) are one of the most common causes of blindness and are characterized by the degeneration of the photoreceptor cells of the retina. The most common inherited photoreceptor degeneration in humans is retinitis pigmentosa (RP), which affects 9 million Americans and has a large medical and economic impact. Clarke et al., 2000, showed that mutant neurons in most, if not all photoreceptor degenerations are at a constant risk of death. Moreover, mutant neurons can function virtually normally for years and even decades in humans, as in the case of the central core of vision that is preserved in RP patients until late in disease progression. The mutant steady state (MSS) hypothesis was proposed to account for these observations, and states that the constant risk of death is conferred by slight alterations in the activities of a small number of mutant response genes (MuRGs), proteins (MuRPs) or metabolites (MuRMs). Microarray and real-time PCR analyses have been carried out in IPD mouse models to identify possible mutant response genes. The gene for endothelin-2 was found to be up-regulated 32-fold in the retinal degeneration slow (Rds) heterozygote model and 14-fold in the Rhodopsin P347S transgenic model (real-time PCR data). In situ hybridization results suggest that the 32-fold increase is originating from the photoreceptor cells. Endothelin-2 mRNA expression is also being examined using laser capture microdissection coupled with real-time PCR, and endothelin-2 protein expression by HPLC combined with radioimmunoassay. In vivo experiments using an endothelin-2 knockout mouse as well as an endothelin receptor antagonist will demonstrate whether endothelin-2 is playing a pathogenic or protective role in Rds mutant retinas. Depending on the function of endothelin-2, endothelin receptor agonists or antagonists could potentially be used as part of a therapeutic strategy for the treatment of photoreceptor degenerations.

P-109

**The pressor and vasopressin secretory responses Induced by endothelin 1 (ET-1) acting at the subfornical organ are mediated by an AMPA receptor within the paraventricular nucleus**

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Endothelin 1 (ET-1) acts at selected brain loci to elicit a pressor response and vasopressin (VP) secretion. Glutamatergic receptors of the NMDA subtype mediate ET-1 induced VP secretion in vitro, but the role of glutamatergic receptors in the pressor response and VP secretion in vivo has not been studied. We hypothesized that both the pressor response and VP secretion in response to ET-1 microinjection into subfornical organ (SFO) will be suppressed by NMDA receptor inhibition in the paraventricular nucleus (PVN). Sinoaortically-denervated male Long Evans rats were equipped with vascular catheters and intracerebral cannulae were placed stereotaxically into the SFO and the magnocellular region of the PVN bilaterally. Hemodynamic parameters were monitored continuously in conscious rats. Direct microinjection of 10 pmol ET-1 into the SFO resulted in a 23.0 ± 5.0 mmHg rise in MAP and a 12.9 ± 3.0 pg/ml increase in plasma VP ($p < 0.01$ vs CSF) that was blocked by selective ETA antagonism. Injection of artificial CSF into SFO 15 minutes after bilateral injection of the PVN with vehicle did not elicit either a pressor response or VP secretion. The pressor response to ET-1 was unchanged (23 ± 6.1 mmHg) despite prior injection of 5 µg dizocilpine into PVN bilaterally. VP secretion was attenuated, but this did not achieve significance. In contrast, bilateral PVN injection with CNQX prevented the pressor response -3.7 ± 3.9 mmHg and also inhibited VP secretion 0.16 ± 1.7 pg/ml ($p < 0.001$ vs ET-1). These findings are consistent with the interpretation that both the pressor response and VP secretion in response to ET-1 acting at the SFO are mediated by a AMPA/kainate glutamatergic receptor within the PVN. Participation of a diclozipine sensitive NMDA receptor within the PVN in the VP secretory response cannot be completely eliminated.

P-110

**Interactions between endothelin (ET-1) and its receptors in the control of the brain microcirculation following traumatic brain injury (TBI)**

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ET-1 (the most powerful vasoconstrictor of the endothelin isoforms) and its receptors (ETRA and ETRB) are expressed in the non-injured brain. We have shown an upregulation of ET-1 mRNA and alterations in the expression of ET-1 receptors in cerebral cortex and hippocampus up to 48 h post TBI. These alterations in ET-1, ETRA and ETRB were temporally associated with sustained cerebral hypoperfusion. Since ETRA and ETRB are the only known receptors that mediate ET-1 effects, we aimed to establish, spatial and temporal domains of these molecules after TBI in cortical and hippocampal cells. To our best knowledge, no previous attempt has been made to either reveal the interactions of these molecules at the cellular level or the biosynthetic equilibrium within the endothelin system following TBI. Using double immunoflurescence we report here that ET-1 immunopositive fibers impinge on endothelial cells positive for ETRA. ET-1 boutons impinge on ETRB labeled neurons and glial cells. Some endothelial cells are double labeled for ET-1 and ETRA . ELISA shows increased synthesis of ET-1 at 4 and 24 h post TBI. Western analysis indicates that ETRA is elevated three fold in hippocampus at 4h. In cortex, ETRA is slightly upregulated at 4h, and elevated two fold at 48 h. ETRB expression is elevated at all time points, reaching a peak at 24 h in cortex and hippocampus. The results suggest that endothelin can be delivered in the vicinity of various cellular elements post TBI. Furthermore, ET-1 may be an intracellular messenger since we detect ETRB on the nuclear membrane. In addition, the brain upregulates not only the powerful vasoconstrictor ET-1, but also the substrate (receptors) upon which the peptide acts. Upregulation of ETRB (according to some it has a vasodilator component) in hippocampus at 4h may indicate an attempt by the brain to counteract the increased synthesis of ET-1 and ETRA, two molecules involved in vasoconstriction. Finally, the results indicate that the microvessels in the cortex and hippocampus respond differently after TBI.

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P-111

**Subconjunctival injection of endothelin-1 to produce the model of retinal ischemia-reperfusion injury in rats**

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Purpose: The retinal ischemia-reperfusion model is used in the glaucoma research. Following 2 types are representative as a method for producing the ischemia-reperfusion model experimentally in the rat retina. (A) The intraocular pressure is remarkably raised by storing the needle in anterior chamber, and followed by heightening the bottle. The retinal blood flow is stopped by this method in fixed time. (B) Another is to ligate the blood vessel which flows into the retina by opening the orbit operatively. However, each method has some demerits. For example, in (A), the needle must be fixed in anterior chamber for one hour, and the technique is not stable. And, in (B), the invasion of the eyeball circumference increases which may affect the result expected. In this study, we injected endothelin-1 (ET-1) under the conjunctiva of the eyeball (subconjunctival injection), and evaluated whether the retinal ischemia-reperfusion model could be generated by this method simply and non-invasively. Methods: The Sprague-Dawley rat was used for this experiment. After general and local anesthesias, we injected ET-1 into the right eye and as a control vehicle (artificial tears) into the left eye. Chest was opened 30 minutes after the injection. Fluorescein isothiocyanate-dextran (FITC-dextran) (50mg/ml) was injected to the left ventricle. The extracted eyeball was fixed in 10% buffer formalin. Then, the removed retina was flat mounted on the slide. We compared perfusion condition of FITC-dextran to each retina in the right and left eye. Results: In the retina 30 minutes after the subconjunctival injection, there was the complete perfusion of FITC-dextran in the retinal main artery, vein and the capillary vessels in the control eye. However, perfusion could not be completely observed in the ET-1 injected eye. Conclusion: The method of subconjunctival injection of ET-1 would be a feasible technical option for producing animal retinal ischemia-reperfusion model.

P-112

**Reversal of upregulated VEGF and ICAM-1 levels by administration of endothelin type A receptor antagonist in type I diabetic retina**

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Diabetic retinopathy, a cause of blindness is often associated with the upregulation of VEGF in retina. Recently, leukostasis is claimed for the occlusion of retinal capillary vascularity, which ultimately progresses the diabetic retinopathy. In addition, ICAM-1, a representative factor for leukostasis, is increased in diabetic retinopathy. Endothelin-1 (ET-1), a potent vasoconstrictor peptide, is closely linked to the pathogenesis of diabetic retinopathy. Different therapeutic interventions concerning VEGF have already proposed to prevent the diabetic retinopathy. However, no study has reported whether ET-1 receptor antagonist could suppress the upregulated VEGF and ICAM-1 in diabetic retina. The present study investigated the effect of endothelin A receptor ($ET_A$) antagonist on the expressions of VEGF and ICAM-1 in rat diabetic retina. Type I diabetes was induced by intraperitoneal injection of streptozotocin (70 mg/kg) in Sprague-Dawley rats while control (Con) rats received only citrate buffer. After 1 week, the streptozotocin-administered rats were randomly divided into two groups, $ET_A$ antagonist administered group (DMTx, TA-0201 1mg/kg/day, by osmotic mini-pump for two weeks) while the diabetic rats received saline was DM group. After 2 weeks, the retina was removed from the eyeball. In DM group, the VEGF expression of retina was significantly increased (33.5 pg/ml) in comparison to that in the Con group (25.1 pg/ml) and this upregulation of VEGF was about 68% reversed in DMTx group (27.8 pg/ml). Moreover, we also showed the consistency between VEGF protein expression and mRNA level. Plasma ET-1 level was little decreased or unchanged suggesting that in type I diabetes ET-1 is accumulated in different risk organs. The expression of retinal ICAM-1 was increased in DM group (51.9 pg/ml) compared to Con group (45.0 pg/ml) and ET antagonism could completely block this increase (45.7 pg/ml). Thus, $ET_A$ antagonist might be proven useful in preventing the progression of diabetic retinopathy by suppressing the VEGF and ICAM-1 levels in rat retina.

P-113

**Prevention and reversal of vasospasm and ultrastructural changes in the basilar artery by continuous infusion CGS35066, a selective endothelin-converting enzyme-1 inhibitor, following experimental subarachnoid hemorrhage**

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Delayed cerebral vasospasm is a major complication after aneurysmal subarachnoid hemorrhage (SAH). Increasing evidence support the therapeutic value of blocking the production of ET-1 by endothelin-converting enzyme-1 (ECE-1) inhibitors for the treatment of this disease. The goal of this study was to evaluate the utility of a highly selective ECE-1 inhibitor in both the prevention and reversal protocols, by examining the effects of continuous intravenous infusion of CGS35066 on cerebral vasospasm following SAH. The present study also examined the preventive effects of this compound on ultrastructural changes in the basilar artery 7days after SAH. The preventive and reversal effects of CGS35066 on arterial narrowing after SAH were significantly attenuated in all treatment groups (1,3,and 10 mg/kg/day) in a dose-dependent manner. Histological studies of the basilar artery in CGS35066-treated group (10mg/kg/day) did not exhibit pathomorphological changes in the basilar artery as observed in the vehicle-treated group. These finding show that CGS35066 is a promising therapeutic agent for preventing and reversing cerebral vasospasm after SAH. These results also demonstrate that continuous intravenous infusion of an ECE-1 inhibitor could prevent the pathological changes in vascular walls after SAH.

P-114

**CGS26303, an endothelin-converting enzyme inhibitor, attenuates experimental subarachnoid hemorrhage-induced increases in circulating intercellular adhesion molecule and cerebral vasospasm**

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The purpose of present study was to examine whether the levels of intercellular adhesion molecules(ICAM), vascular cell adhesion molecule(VCAM) and E-selectin were altered after treatment with CGS26303 in this animal model of SAH. Rabbits were injected with 3 ml of autologous blood in the cisterna magna, and treatment with CGS26303(30 mg/kg i.v.) was inhibited 1 h later. The compound was subsequently administered at 12,24 and 36 h post-SAH. Blood samples were collected at 48 h post-SAH for deterring ICAM-1,VCAM-1 and E-selectin levels prior to sacrifice by perfusion-fixation. VCAM-1 levels showed no significant differences among all 4 groups. E-selectin levels were increased in the all animals subject to SAH(SAH only , SAH+vehicle and SAH+CGS26303 treatment) compared to healthy controls, but treatment with CGS26303 had no effect. However, ICAM-1 levels in the SAH only and SAH+vehicle groups were significant elevated ($p<0.001$), and treatment with CGS26303 reduced ICAM-1 to a level not differ indistinguishable from that of the control group. These results show that ICAM-1 may play a role and that a reduction of ICAM-1 levels after SAH may partly contribute to the anti-spastic effect of CGS26303.

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**Alterations of endothelin-1 and angiotensin II in the brain of endotoxaemia in the time dependent manner without change in nitric oxide**

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Endothelin (ET)-1 is recently being investigated in different cerebral pathologies. On the other hand, some reports suggest the involvement of ET-1 in sepsis. However, no study to date has reported the time dependent alterations of ET-1, angiotensin II (Ang II) and nitric oxide (NO) in the frontal cortex of septic brain. The aim is to reveal this question. Male Wistar rats at 8 weeks of age were administered with either saline or lipopolysaccharide (LPS, 15mg/kg) at different time points (1, 3, 6 and 10 hours). The rats were killed with ether and the brain tissues were harvested. Systolic and diastolic blood pressure was suddenly fallen at 1 hour after LPS administration and then gradually normalized. Morphological evaluation by HE staining showed no remarkable findings in the septic brain. Plasma ET-1 level peaked at 3 hour of sepsis by 25-fold. A time-dependent increase in frontocortical ET-1 level was observed after LPS administration while the ET-1 mRNA expression was peaked at 3 hour of sepsis, suggesting that endotoxaemia affects both the transcriptional and translational regulation of ET-1. A time-dependent gradual increase of $ET_A$ receptor (vasoconstrictive property) expression after LPS administration was observed while $ET_B$ receptor (vasodilatatory property) expression was decreased as sepsis progressed. Angiotensin II which also plays active role in various cerebrovascular diseases was also altered in endotoxaemic brain peaking at 6 hour (40% increase) compared to control brain. Interestingly, NO, the key vasodilator for brain was unchanged with its most important enzyme, eNOS. In conclusion, we report for the first time that ET-1 and Ang II, two important vasoconstrictive peptides, are altered in the brain of endotoxaemia and these alterations are time-dependent. No change in NO and eNOS levels suggests that NO-mediated compensatory adaptation may fail or may be unnecessary in the brain of endotoxaemia. The present study contributes to the understanding of the pathophysiological changes in three key vasoregulatory molecules (ET-1, Ang II, NO) in brain of endotoxaemia.

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**Alteration of the cerebrovascular endothelin (ET)(B)-receptor function after experimental subarachnoid hemorrhage (SAH) in the rat**

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Introduction: The substantial role of ET-1 in the development of cerebral vasospasm (CVS) after SAH is demonstrated by numerous experimental and recently also by clinical investigations. If the expression or the function of the ET(B)-receptor is altered in case of CVS, however, is still unclear. Aim of the present study was, therefore, to characterize the cerebroarterial ET(B)-receptor function during CVS. Methods: Experimental CVS was induced by the rat double hemorrhage model. Reduction of the cerebral blood flow (CBF) was proven by MR perfusion weighted imaging. Animals were sacrificed on the days 3 (d3) and 5 (d5). The basilar arteries (BA) were dissected, cut into ring segments, and prepared for measurement of isometric force in an organ bath. Concentration-effect curves (CECs) were constructed by cumulative application of ET-1, Acetylcholine (Ach), or Sarafotoxin S6c (S6c). Segments with (E+) and without (E-) endothelial function were used. CECs were compared by the maximum effect (Emax), the pD2 and the shift calculated on the pD2-level. Results: Relative regional CBF was reduced to 63% (d3) and to 32% (d5) after SAH compared to the controls. ET-1 induced a dose dependent contraction of segments with and without CVS. In E+ segments Emax for ET-1 was not significantly changed after SAH (control: 104±4% (mean±SEM), d3: 106±4%, d5: 104±3%). The CECs, however, were significantly shifted to the left versus the control by a factor of 2.4 (d3) and 3.6 (d5). Relaxation by S6c was significantly reduced after SAH (Emax: 73±11% (control), 21±13% (d3), 13±8% (d5)), whereas relaxation by Ach was not significantly changed (Emax: 45±7% (control), 56±6% (d3), 43±6% (d5)). Significant contraction by S6c was not observed in E+ and E- segments (control, d3, d5). Conclusion: The present data indicate the loss of the ET(B)-receptor mediated relaxation of cerebral arteries in case of CVS, which is independent of the endothelial NO synthase.

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**17ß-Estradiol inhibits endothelin-1 (ET-1) production and attenuates cerebral vasospasm following experimental subarachnoid hemorrhage (SAH)**

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This study was designed to evaluate the influence of 17ß-estrodiol (E2) on the production of ET-1 and cerebrovasospasm following an experimental SAH in a two-hemorrhage model in the rat. A 30-mm Silastic®tube filled with E2 in corn oil (0.3mg/ml) was subcutaneously implanted in male rats just before SAH induction. The degree of vasospasm was determined by averaging the cross sectional areas of basilar artery 7 days after the first SAH. Plasma samples collected before sacrifice were assayed for ET-1.Serum levels of E2 in the E2-treated rats were at physiological levels and were significantly higher than those in the healthy control and vehicle groups. The protective effect of E2 in attenuating vasospasm achieved statistical significant when compared with SAH only or SAH+vehicle groups ($P<0.01$).ET-1 concentrations were higher in SAH only and SAH+vehicle groups compared to the controls ($P<0.001$). Serum levels of ET-1 in SAH+E2 and E2 only groups were significantly lower than those in the SAH only and SAH+vehicle groups ($P<0.001$). There was no significant difference between the ET-1 levels in the healthy control and SAH+E2 groups. Significant correlation was found between the degrees of vasospasm and ET-1 levels($P<0.01$). The beneficial effect of E2 in attenuating SAH-induced vasospasm may be partially due to decreasing ET-1 production after SAH. Thus,E2-treatment holds therapeutic promise in the treatment of cerebral vasospasm following SAH and is meritorious of further investigation.

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**Correlation of morphologic measurements with motorsensory scores for evaluating cerebral vasospasm induced by subarachnoid hemorrhage in rabbits treated with CGS35066, a selective endothelin-converting enzyme inhibitor**

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The Purpose of the present study was to evaluate the relevance of measurements of lumen area(LA),basilar arterial vessel area(VA),and mean basilar artery vascular diameter(VD) in cerebral vasospasm following subarachnoid hemorrhage(SAH).Rabbits were subjected to experimental SAH and received intravenous injection of CGS35066,a selective endothelin-converting enzyme inhibitor, at doses of 1,3,and 10 mg/kg, b.i.d., in either prevention (treatments were initiated at 1 hr after SAH) or reversal paradigm(treatments were initiated at 24 hr post-SAH). After 48 hr, basilar arteries were harvested. The LA,VA and VD were measured using computer-assisted video microscopy. The motorsensory evaluation was recorded on days 0,1 and 2.Oue findings showed that the values of VA reflected the best on vasospastic response of basilar artery and correlated with motorsensory scores in rabbits subjected to SAH. The results provide in vivo evidence for the effectiveness of CGS35066 in the prevention and reversal of SAH-induced vasospasm as well as improvement of neurological function. The present study also suggests that, among the three methods examined, adoption of the measurement of VA is the most meaningful for evaluating therapeutic agents.

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**A hypoxic mimetic agent induces neurite outgrowth in rat pheochromocytoma PC-12 cells through regulation of endothelin-2/vasoactive intestinal contractor**

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We investigated whether endothelin-2 (ET-2)/vasoactive intestinal contractor (VIC) gene expression, up-regulated by hypoxia in cancer cells, was associated with cell proliferation, differentiation, and cell death in neuronal cells. RT-PCR analysis, morphological observations, and immunostaining revealed that a hypoxic mimetic agent at 200 μM increased expression of the ET-2/VIC gene, decreased expression of the ET-1 gene, and induced neurite outgrowth in PC-12 rat pheochromocytoma cells. These effects induced by the hypoxic agent at 200 μM were completely inhibited by the antioxidant N-acetyl cysteine at 20 mM. In addition, the hypoxic agent increased the level of intracellular reactive oxygen species (ROS) at an early stage. We found that addition of the hypoxic agent to the culture medium led to generation of ROS. These results suggest that the regulation of ET-2/VIC and ET-1, mediated by ROS, is involved in the differentiation of PC-12 cells induced by the hypoxic agent. Furthermore, the hypoxic agent at 200 μM also up-regulated expression of the IL-6 gene in PC-12 cells, suggesting that IL-6 is responsible for the differentiation induced by the hypoxic agent. On the other hand, when the cells were treated with 500 μM the hypoxic agent for 24 h, ET-2/VIC gene expression disappeared, IL-6 gene expression was down-regulated, and necrosis was subsequently induced in the PC-12 cells. Expression of ET-2/VIC and ET-1 may be associated with the regulation of neuronal differentiation or neuronal cell death in the nervous system.

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**Neuroprotective effect of CGS26303, an endothelin-converting enzyme inhibitor, on the ischemic-reperfusion spinal cord injury in rats**

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The purpose of present study was to examine whether the levels of neurological function deficit, neuronal loss of ventral horn and inflammation status of spinal cord were altered after pre-treatment with CGS26303 in this animal model of spinal cord ischemia. Rats were pretreated with saline (1ml) or CGS26303 (30mg/kg,i.v.) 30 min before, and subjected to a 15 min of spinal cord ischemic episode induced by temporal occlusion of the descending aorta with a 2F Fogarty arterial embolectomy catheter. Locomotor function by behavioral tests was performed in all animals before ischemia, and at Day 1 and 3 after ischemia. The spinal cords from different groups of rats were also collected after perfusion-fixation for histological analysis to delineate mechanism underlying the differential neurological functions. Motor deficit index (MDI) was increased in the all animals subject to spinal cord ischemia (spinal ischemia+saline and spinal ischemia+CGS26303 groups) compared to healthy controls. However, MDI levels in the spinal ischemia+saline groups were significant elevated ($p<0.001$), and pre-treatment with CGS26303 reduced neuron loss of ventral horn of spinal cord and inflammation status differ distinguishably from that of the saline-treated group. These results show that CGS26303 may possess a neuroprotective effect and that inflammatory status after spinal cord ischemia may partly contribute to the outcome of after spinal cord ischemia.

P-121

**Trypanosoma cruzi infection induces proliferation of vascular smooth muscle cells**

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Trypanosoma cruzi infection causes cardiomyopathy and vasculopathy which may be mediated, in part, by endothelin-1 (ET-1). In order to investigate the infection-associated vasculopathy, cultured human endothelial cells (ECs) and human smooth muscle cells (SMCs) were infected with the Tulahuen strain of T. cruzi, and the expression of several proliferation markers was examined. Both infected ECs and SMCs exhibited a significant increase in ERK1/2 activity, as well as a downregulation of caveolins. Infected SMCs revealed an increased expression of cyclin D1 and proliferating cell nuclear antigen (PCNA). SMCs were then incubated with supernatants of infected ECs. The pretreatment of these SMCs with ET-1 receptor blockers (BQ123 and BQ2030) reduced the activation of ERK1/2 and the increase in DNA synthesis. An in vivo confirmation was obtained by isolating carotid arteries from mice infected with T. cruzi. Infected arteries showed an increased expression of PCNA, cyclin D1 and its substrate, phospho-Rb, an increased activation of ERK1/2, a decreased expression of the cdk-inhibitor, p21 and caveolin peptides. In addition, the expression of the endothelin receptor A was elevated in arteries obtained from infected mice. Utilizing RT-PCR, infected arteries exhibited an increased abundance of prepro ET-1 mRNA. In conclusion, we demonstrated that T. cruzi infection stimulates SMC proliferation, possibly via the induction of ET-1, with concomitant activation of ERK1/2 and up-regulation of cyclin D1, as well as a decrease in caveolin levels.

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**Endothelin $ET_B$ receptor antagonist reduces mechanical allodynia in rats with trigeminal neuropathic pain**

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Trigeminal neuropathic pain, which is associated with marked orofacial mechanical allodynia, is frequently refractory to currently available drugs. As endothelins (ETs) can contribute to nociceptive changes in animal models of inflammatory, cancer and diabetic neuropathic pain, the present study evaluated the influence of $ET_A$ and $ET_B$ receptor antagonists on mechanical allodynia in a rat model of trigeminal neuropathic pain. Trigeminal neuropathic pain was induced by tying two loose silk 4-0 ligatures around the infraorbital nerve (ION) of male Wistar rats (180-200 g). On postoperative days 12 to 15 rats received a s.c. injection of morphine (2.5 mg/kg) or i.v. injections of dual $ET_A/ET_B$ (bosentan, 10 mg/kg), $ET_A$ (atrasentan, 10 mg/kg) or $ET_B$ (A-192621, 20 mg/kg) receptor antagonists and were submitted repeatedly to mechanical stimulation of the vibrissal pad with Von Frey filaments, to determine the minimal force required to evoke escape/attack reactions and/or face grooming. Injection of ET-1 (10 pmol) into the upper lip of naive rats caused long-lasting (up to 5 h) ipsilateral mechanical allodynia. ION injury caused bilateral mechanical allodynia which peaked on day 12 (from >10 g to 1.0 ± 0.3 and 1.5 ± 0.6 g on ipsi- and contralateral sides, respectively) and remained stable up to day 120. Morphine abolished mechanical allodynia for up to 90 min after injection, but bosentan and atrasentan failed to alter mechanical threshold. In sharp contrast, A-192621 caused a net 61 ± 15% reduction of mechanical threshold, lasting 2 h. Co-injection of atrasentan plus A-192621 did not modify ION injury-induced mechanical allodynia. Thus, $ET_B$ receptor-mediated mechanisms contribute to orofacial mechanical allodynia induced by ION injury, but somehow functional $ET_A$ receptors are required for expression of the anti-allodynic effect of $ET_B$ receptor blockade. Acknowledgements: Authors thank CNPq, CAPES and PRONEX (Brazil) for support and Abbott and Actelion for providing ET receptor antagonists.

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**Receptors mediating the pruritic action of endothelin-1 in mice**

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Subjects receiving intradermal endothelin-1 (ET-1) injection in the forearm describe a burning pruritus sensation, but in rodents it elicits nociceptive behavior when injected in the hind paw. As pain and pruritus in humans are distinct nociceptive sensory modalities, the current study evaluates the potential of ET-1 to elicit scratching behavior in mice. Male Swiss mice (30-35 g) received an intradermal (i.d.) injection of ET-1 (1 to 30 pmol), the mast cell degranulator compound 48/80 (C 48/80, 10 µg), histamine (100 nmol) or PBS into the scruff (i.e. back of the neck), were placed in individual containers and the number of scratching bouts (hind paw movements directed to injected site) was recorded over the first 40 min. ET-1 caused dose-dependent scratching bouts (i.e. pruritus) distributed evenly throughout the observation period (as seen with histamine), while responses to C 48/80 occurred mainly during the first 10 min following injection. The effect of ET-1 was maximal at 10 pmol (total 44.3 ± 5.4 bouts), a value similar to those seen in response to histamine (36.6 ± 4.4 bouts) and C 48/80 (39.5 ± 4.7 bouts). IRL-1620 (10 pmol) did not cause scratching behavior per se, but inhibited responses to histamine. Pruritus induced by ET-1 was markedly inhibited by $ET_A$ receptor antagonists BQ-123 (10 nmol, co-injected i.d.; net 87% inhibition) or atrasentan (10 mg/kg, i.p. 1 h before; net 83% inhibition) and $ET_B$ receptor antagonist A-192621 (20 mg/kg, i.p. 1 h before; net 64% inhibition), but was markedly enhanced by BQ-788 (3 nmol, co-injected i.d.; net 234% potentiation). None of the antagonist treatments affected responses to C 48/80 or responsiveness of PBS-treated mice. Thus, ET-1 displays potent pruritic actions in the mouse which are mediated to a substantial extent via activation of local $ET_A$ receptors. Although the findings with IRL-1620 and BQ-788 suggest that local $ET_B$ receptors exert an antipruritic role, this view is not supported by the pronounced inhibition of ET-1-induced scratching seen in animals given systemic injection of the $ET_B$ receptor antagonist A-192621. Further experiments are thus warranted to clarify the role of $ET_B$ receptors. Support: CNPq, PRONEX, FUNCITEC (Brazil).

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**Intracellular signaling mechanisms underlying endothelin-1-induced mechanical hyperalgesia in rats**

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Besides causing overt nociception (Gokin et al., J Neurosci, 21, 5358, 2001), intraplantar (i.pl.) endothelin-1 (ET-1) injection into the hind paw induces hyperalgesia to mechanical stimuli in rats (Da-Cunha et al., Eur J Pharmacol, 501, 87, 2004). The later effect is mediated by local $ET_B$ receptors, potentiated by rolipram and attenuated by inhibitors of protein kinase C (PKC; staurosporine and calphostin), but not PKA (H89). The present study further examines the intracellular signaling mechanisms underlying ET-1-induced mechanical hyperalgesia in the rat hind paw. ET-1 (30 pmol) or PBS was injected i.pl. into the right hind-paw of male Wistar rats (~ 250 g) and the threshold of responsiveness to mechanical stimulation was assessed repeatedly each hour up to 8 h and 24 h, using the dynamic plantar aesthesiometer test, which detects the minimal pressure required to evoke paw withdrawal. Different groups were treated, 15 min prior to ET-1 administration, with ipsilateral injection of selective inhibitors of either phospholipase C (PLC; U73122, 1 pmol), PKC (GF109203X, 1 nmol), $PLA_2$ (PACOCF3, 1 nmol), p38 MAPK (SB203580, 30 nmol) or ERK1/2 (PD98059, 30 nmol), to assess their influence on the hyperalgesic response. The mechanical hyperalgesia caused by ET-1 started 2 h after injection, peaked at 5 h (PBS 29 ± 0.5 g vs ET-1 17 ± 1.3 g) and lasted up to 8 h. The inhibitors of PKC, p38 MAPK and ERK1/2 caused long-lasting reductions of the mechanical hyperalgesia (inhibitions at 5 h of 100, 33 and 75 %, respectively). In contrast, the $PLA_2$ inhibitor reduced hyperalgesia only at 4 h (by 58 %)and the PLC inhibitor was inactive. These findings indicate that ET-1 triggers mechanical hyperalgesia in the hind paw through activation of signaling pathways involving PKC, p38 MAPK and ERK1/2, but not PLC. The limited effect of the $PLA_2$ inhibitor agrees well with the report that ET-1-induced mechanical hyperalgesia is insensitive to inhibition by dexamethasone or indomethacin (Da-Cunha et al.,2004). Financial Support: CNPq, CAPES, FUNCITEC and PRONEX.

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**Endothelin in a murine model of cerebral malaria**

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Cerebral Malaria is an important cause of morbidity and mortality in many parts of the world. It has been suggested that cerebral malaria is associated with reduced perfusion due to the blockage of blood vessels by parasitized erythrocytes; although no quantitative validation of this has been performed. We infected C57BL/6 mice with the ANKA strain of Plasmodium berghei and on day 6 of infection, we investigated alterations in brain function using arterial spin labeling MRI and proton MRS. MR images did not demonstrate signs of damage; however, there was a significant reduction in cerebral blood flow ($P < 0.012$) and the ratio of N-acetyl-aspartate (NAA) to creatine (Cr) ($P < 0.01$) relative to non-infected mice. The NAA/Cr ratios were significantly correlated with cerebral perfusion ($r = 0.87$) suggesting a relationship between impaired oxygen delivery and neuronal dysfunction. Pathological examination revealed accumulations of damaged axons providing a correlate for the decreased NAA/Cr ratio in infected mice. Concurrently, we analyzed the brains of these mice at day 5 post-infection along with their non-infected matched controls. Using both RT-PCR and Real-Time PCR, we were able to a detect a significant increase in the abundance of mRNAs for pre-pro ET-1, endothelin converting enzyme and endothelin receptor A 5 days post-infection compared to non-infected controls. These data indicate that in a murine model of cerebral malaria there is a reduction in cerebral blood flow associated with an increase in endothelin. This murine model underscores the utility of non-invasive studies of neurologic function during malarial infection which may assist in understanding the pathogenesis of cerebral malaria

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**Involvement of central endothelin receptors in neonatal morphine withdrawal**

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We have previously demonstrated the involvement of central endothelin (ET) receptors in neonatal morphine tolerance. The present study investigates the role of central ET receptors in morphine withdrawal in neonatal rats. The aim was to determine if activation of G-proteins coupled to opioid and ET receptors by morphine and various ET receptor modulators is affected during morphine withdrawal in neonatal rats.

Pregnant female rats were rendered tolerant to morphine by chronic exposure to morphine pellets over seven days. On day 8, pellets were removed and rats were allowed to undergo withdrawal for 24h. Rat pups were delivered by cesarean section. G-protein stimulation induced by morphine; ET-1; ETA receptor antagonist, BMS182874; and ETB receptor agonist, IRL1620, were determined in brains of neonatal rats undergoing morphine withdrawal by [35S]GTP$\gamma$S binding assay. Morphine produced higher ($P<0.05$) maximal stimulation of G-protein in morphine withdrawal group (83.60%) compared to control (66.81%). ET-1-induced G-protein stimulation was also altered, and EC50 during morphine withdrawal (170.60nM) was significantly higher than placebo (62.5nM, $P<0.05$). ETA receptor antagonist, BMS182874-induced maximal stimulation in morphine withdrawal group (86.07%, EC50=31.25nM) was significantly higher than placebo group (EC50>1000nM). ETB agonist, IRL1620-induced G-protein stimulation was similar in placebo (73.43%, EC50=13.26nM) and morphine withdrawal (75.08%, EC50=11.70nM), respectively. This the first report indicating involvement of central ETA receptors in neonatal morphine withdrawal.

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**Endothelin-2 is concentrated near the basement membrane in mouse intestine and upregulated in experimental colitis**

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Endothelin (ET)-2 / vasoactive intestinal contractor (VIC), an ET family peptide, is highly expressed in intestine. We elucidated the localization of ET-2/VIC in mouse gastrointestinal tract. Immunohistochemical analysis revealed that ET-2/VIC-like immunoreactivity mainly in epithelial cells of the mucosa throughout the normal gastrointestinal tract. Intracellularly, ET-2/VIC was concentrated close to the basement membrane of intestinal epithelial cells. A weak ET-2/VIC-like immunoreactivity was also localized to some neurofibers and the myenteric plexus of the muscle layer, coexpressing with vasoactive intestinal peptide. ET-2/VIC-like immunoreactivity was also detected at Brunner's glands of the duodenum, follicle-associated epithelium of Peyer's patch and some fundic gland cells of the stomach. In contrast, ET-1-like immunoreactivity was uniformly detected in epithelial cells. In dextran sulphate sodium-induced colitis, colonic ET-2/VIC was upregulated during the late stage of inflammation. These results suggest that in intestinal epithelial cells ET-2/VIC is secreted into the lamina propria of the villus and the dome region in Peyer's patch, and that it modulates immune cells in these sites for mucosal defense.

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**Endothelin (ET)-1 and nitric oxide (NO) were altered accompanied with alteration of inflammatory markers in hepatic tissue in sepsis in a time-dependent manner**

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Sepsis is a heterogeneous class of syndromes and septic shock, a severe form of sepsis, is associated with the development of progressive damage in multiple organs. The present study examined the time-dependent alterations of ET-1, NO and inflammatory cytokines, such as TNF-$\alpha$ in liver tissue in a septic rat model. Normal male Wistar rats at age 15 wks were administered with lipopolysaccharide (LPS: 15 mg/kg) and then sacrificed at different time points (1h, 3h, 6h and 10h). A number of rats without LPS administration was considered as control group. Both systolic and diastolic pressures were drastically fallen at 1 h after LPS administration and then gradually became normal though still at 10 h of LPS administration blood pressures were lower compared to control rats. Administration of LPS resulted in increases in the serum levels of TNF-$\alpha$ (maximum at 1 h after LPS, 1200-fold compared to control rats), and ET-1 (maximum at 3 h after LPS, 25-fold compared to control rats). Time-dependently, the features of acute liver injury, such as the infiltration of inflammatory cells, hepatocytic necrosis were seen in LPS administered rats. Plasma bilirubin, GOT and GPT levels were also significantly changed with the time of sepsis. A 28-fold increase in ET-1 level was observed in liver tissue at 10 h after LPS administration while a peak increase of 14-fold ET-1 mRNA level was seen at 1 h after LPS administration in liver tissue. Hepatic tissue TNF-$\alpha$ was peaked (4.5-fold) at 1 hour of sepsis by ELISA. Although a compensatory increase in NO and eNOS levels were seen in liver tissue as endotoxemia progressed, but a concomitant iNOS induction questioned the availability of functional NO. In sepsis at first the hepatic $ET_AR$ expression was downregulated but with time the expression level was increased, while $ET_BR$ showed a time-dependent decrease in expression in liver tissue. The present findings suggest that there might be a loss of balance among the ET-1, NO and inflammatory cytokine in septic liver in different time points, which could contribute to the pathogenesis of acute liver injury in endotoxemia.

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**Tactile Allodynia Produced by Low Concentrations of ET-1 Involves TRPV1 Receptors and Differs from Pain Induction by Higher ET-1 Concentrations**

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When injected subdermally into the hind paw at 200μM (2 nmoles/paw), endothelin-1 (ET-1) is known to cause robust hindpaw flinching (HPF) and paw licking, and selective generation of impulses in primary nociceptors. Here we report that a much lower [ET-1] sensitizes the paw to mechanical stimulation (by von Frey hairs, VFH) and that this sensitization involves TRPV1 receptors in the paw. Injection of 10 μM ET-1 (0.1nmole/paw) causes only marginal HPF but rapidly (20 min post inj.) drops the paw withdrawal threshold (PWT) to VFH, to ~40% of pre-injection baseline. Such *tactile allodynia* persists for at least 5 h. In rats pre-injected with TRPV1-specific drugs, 1.33mM capsazepine (CPZ) or 0.1μM iodinated-resiniferatoxin (I-RTX) (in PBS + 10% DMSO/0.3% Tween-80, 15 min before ET-1), the fast initial drop in PWT occurs (to 40.1% or to 19.2% of baseline, respectively), but this earliest reduction then regresses monotonically back to the pre-injection value whereas that from ET-1 alone continues to fall for the next 90 min. The allodynia from ET-1+CPZ is significantly less than that from ET-1 alone, from 50 to 300 min after ET-1's delivery, and allodynia from ET-1+I-RTX fully reverses (the threshold returns to 91-98% of the baseline level) by 240-300 min post inj. Injection of PBS (vehicle of ET-1) alone also reduces PWT, but by only half as much as ET-1. Overt HPF, induced by 200 μM ET-1, is not attenuated by CPZ, and is increased super-additively in the presence of I-RTX (54±13 flinches/60 min), vs. 15±1 (ET-1 alone), and 9±1 (I-RTX alone). These findings show that: 1. low [ET-1] causes tactile allodynia, which 2. has a different time-course and pharmacology than induced nociception and 3. involves to a significant degree the participation of TRPV1 receptors in the paw. Supported by CA-080153.

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**ET-1 modulation in human microvascular endothelial cells after human herpes virus 8 infection**

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Endothelin-1 (ET-1) is an angiogenic factor that, among other, is secreted by endothelial cells during development of several neoplasias. In particular, Kaposi Sarcoma (KS) skin lesions show overexpression of the ET-1 system. Spindle cells, which characterize tumour lesions, are of endothelial origin and during disease are infected by Human Herpes virus 8 (HHV-8). The majority of these cells are latently infected, suggesting that latent genes are sufficient for maintenance of viral infection and development of KS. The establishment of a reliable infection system is required to better understand the role of viral and cellular angiogenetic factors involved in KS progression. For this purpose, we used Human Microvascular Endothelial Cells (HMEC) to establish an ET-1-producing model of infection with HHV-8. Viral particles purified from BCBL-1 cells were used to infect HMEC monolayer and infection was assessed by PCR, Western Blot and confocal microscopy. Mitochondrial activity and cell viability, measured at 24 and 48 hours post infection by MTT tetrazolium assay, was reduced in HHV-8 infected cells compared to control. The same decreasing trend was noticed after HMEC stimulation with IFN$\gamma$ and IL6. Endothelins production was measured in culture media collected at 24 and 48 hours post infection. Preliminary data show that HHV-8 might determine a slight decrease of endothelin precursor big-endothelin-1 and increasing levels of ET-1. Similar data were obtained when the cells are cultured in presence of IL6. Infected cells were maintained in culture for three weeks after infection and cell proliferation was measured by counting viable cells. HHV-8 infected cells were able to grow significantly faster than control. These results indicate that this model will be useful to further characterize the effects of HHV-8 in early and late phase of infection, and to determine its ability to interfere with endothelin system.

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**Endothelin-converting enzyme-1 (ECE-1), its isoforms and prostate cancer**

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Endothelin-1 (ET-1) can influence cancer invasion and metastasis. With respect to prostate cancer (PC), plasma concentrations of ET-1 are significantly elevated in men with metastatic disease. ET-1 also contributes to the transition of hormonally regulated androgen-dependent to independent PC. ET-1 is generated from big-ET-1, by endothelin-converting enzyme (ECE-1). There are 4 distinct isoforms, ECE-1a, ECE-1b, ECE-1c and ECE-1d. This study investigated a role for ET-1 regulation via expression of specific ECE-1 isoforms in prostate cancer stromal-epithelial components using a Matrigel co-culture invasion model. Androgen-sensitive LNCaP, androgen-independent PC-3, Du145, and non-malignant PNT1-a, PNT2-C2 and P4E6 prostate cell lines were used. Malignant and benign stromal cells derived from radical prostatectomies were also exploited. Previously we reported that inhibition of endogenous ECE-1 activity significantly reduced PC cell invasion and supplementation with ET-1 significantly increased PC invasion. In this study, examination of ECE-1 isoforms revealed mRNA for each isoform in all cell lines tested but only ECE-1c protein was detected and only in PC-3, Du145 and PNT2-C2 cells. Transient over-expression of ECE-1c in PC-3 cells increased invasion by 20%. Interestingly, over-expression of ECE-1a or ECE-1b decreased invasion by ~50%. However, transient expression of ECE-1c increased invasion of non-invasive PNT1-a cells by 150%. Co-transfection of either ECE-1a or ECE-1b with ECE-1c completely suppressed the effect of ECE-1c. We considered that ECE-1c might exert its observed influence on invasion by regulating ET-1 levels and subsequent levels of ET-induced phosphorylation of focal adhesion kinase (FAK). Addition of an ECE-1 specific inhibitor reduced FAK phosphorylation significantly. We therefore conclude that ECE-1c influences cell invasion via ET-1 mediated FAK phosphorylation. We are further investigating the mechanism by which ECE-1a and ECE-1b are able to suppress invasion.

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**The green tea polyphenol epigallocatechin-3-gallate inhibits the endothelin axis in ovarian carcinoma**

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The green tea polyphenol epigallocatechin-3-gallate (EGCG) has been shown to prevent cancer. However, a precise mechanism for tumor growth inhibition has not yet been clearly described. The endothelin A receptor ($ET_AR$)/endothelin-1 (ET-1) autocrine pathway is overexpressed in ovarian carcinoma and triggers tumor growth, survival, neoangiogenesis, and invasion indicating that $ET_AR$-inhibitory agents may be of therapeutic value. In the present study, we investigated the effects of EGCG on ET-1/$ET_AR$ expression and signaling pathway in HEY and OVCA 433 ovarian carcinoma cell lines. Treatment with EGCG inhibited $ET_AR$ and ET-1 expression, at mRNA and protein levels, reduced the basal and ET-1-induced cell proliferation and invasion. Remarkably, EGCG treatment resulted in a reduction of basal and ET-1-induced mediators of angiogenesis, such as cyclooxygenase (COX)-1 and COX-2, prostaglandin E2, vascular endothelial growth factor (VEGF) and matrix-metalloproteinase activity. The EGCG-induced inhibitory effects were associated with a reduction of $ET_AR$-dependent activation of the p42/44 and p38 mitogen-activated protein kinases and phosphatidylinositol-3-kinase pathway. Finally, in ovarian carcinoma xenografts, tumor growth was significantly inhibited by oral administration of green tea. This effect was associated with a significant reduction in the ET-1, $ET_AR$, and VEGF mRNA and protein expression, as well as with a decreased in the microvessel density and proliferation index. These results provide a novel insight into the mechanism by which EGCG, affecting multiple $ET_AR$-dependent pathways may inhibit ovarian carcinoma growth suggesting that EGCG may be useful in preventing and treating ovarian carcinoma in which activation of $ET_AR$ by ET-1 plays a critical role in tumor growth and progression. Supported by AIRC, CNR-MIR, Ministero della Salute

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**ZD4054, a specific endothelin A receptor antagonist, inhibits tumor growth and enhances cytotoxicity of paclitaxel in ovarian carcinoma in vitro and in vivo**

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Endothelin-1 (ET-1) is a multifunctional peptide that plays a critical role in the growth and progression of several malignancies. Ovarian carcinomas secrete ET-1 and overexpress endothelin A receptors ($ET_AR$) indicating the presence of an autocrine loop, which triggers tumor growth, survival, angiogenesis and increased invasiveness. Targeting $ET_AR$ may therefore offer improvements in the treatment of ovarian and other tumors. We examined in vitro and in vivo the effect of ZD4054, a potent antagonist of $ET_AR$ in early clinical development for the treatment of cancer, in mono- and combination therapy with a taxane. ZD4054 (1 μM) effectively inhibits cell proliferation, reduces vascular endothelial growth factor (VEGF) secretion by 35% compared to baseline and enhances paclitaxel-induced apoptosis in HEY and OVCA 433 ovarian carcinoma cell lines. We therefore explored the therapeutic efficacy of ZD4054 on HEY ovarian carcinoma xenografts in athymic mice. ZD4054 as monotherapy was capable of inhibiting the tumor growth at doses ranging from 10 to 50 mg/kg/day i.p. administered for three weeks. ZD4054 (25 mg/kg/day i.p. for 21 days) induced similar inhibition of tumor growth as paclitaxel (20 mg/kg i.v Q 4X3 i.e every 4 days for 3 doses i.e. on days 1, 5 and 9) with a reduction of 65% compared with control. The co-administration of ZD4054 (10 mg/kg/day) with paclitaxel (20 mg/kg i.v Q 4X3) enhanced the efficacy of paclitaxel and was effective in potentiating the antitumor effects of the chemotherapy leading to partial or complete tumor regression. These findings demonstrate that interruption of $ET_AR$ signaling represents a promising therapeutic strategy in ovarian carcinoma. In this context, the specific $ET_A$ antagonist ZD4054 is a candidate for hypothesis testing in the clinic as an antitumor agent in ovarian cancer patients, either as monotherapy or in combination with taxane therapy. Supported by AIRC, Ministero della Salute, CNR-MIUR and AstraZeneca.

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**Endothelin-1 modulates cell survival of squamous cell carcinoma**

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Endothelin-1 (ET-1) aids tumour growth and the progression of various tumours by protecting cells from apoptosis. Furthermore, ET-1 may modulate the balance between cell proliferation and apoptosis to maintain tissue homeostasis since there were some previous reports to suggest that ET-1 might induce apoptosis of several cancer cell lines. Apoptosis is the predominant mechanism of cytotoxicity induced by chemotherapeutic agents. Therefore, it would be valuable to understand the function of ET-1 in cancer cells in order to develop a more effective therapy. Recently, we provided the evidence that ET-1 is expressed in oral squamous cell carcinoma (SCC) cells. The aim of this study was to examine whether ET-1 plays a role to modulate apoptosis in these cells (SCC25). The effects of ET-1 on proliferation and apoptosis of SCC25 that was grown in medium with serum or serum-free medium with/without an inducer (paclitaxel) for apoptosis were evaluated by Cell Proliferation assay and Annexin-V Apoptosis assay. Our results demonstrated that the addition of ET-1 promoted proliferation of serum-starved cells in a concentration-dependent manner, and markedly inhibited paclitaxel-induced apoptosis. On the other hand, when SCC25 was grown in medium with serum, it was demonstrated that the addition of ET-1 inhibited the proliferation of the cells and induced apoptosis of the cells as well as the effect of paclitaxel. These effects of ET-1 were decreased by pre-treatment of cells with an endothelin A receptor antagonist BQ123 or an B receptor antagonist BQ788. These results established a novel role of ET-1 that modulates survival of oral SCC cells according to cell viability. Moreover, it was suggested that a chemotherapeutic for blockage of the action of ET-1, such as specific endothelin receptor antagonists might provide a more effective approach to the treatment of oral SCC cells that is aimed to induce apoptosis in combination therapy.

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**ZD4054 reduces ET-1-induced forearm vasoconstriction in healthy men**

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In prostate cancer, activation of ETA by ET-1 is thought to drive cancer cell growth, while activation of ETB is thought to drive cancer cell death. ZD4054 is an orally active ET-1 antagonist under early clinical evaluation for hormone refractory prostate cancer. In preclinical studies, ZD4054 is a potent, specific ETA antagonist. The aim of this study was to show whether ZD4054 effectively blocks ET-1 activity through ETA in vivo by assessing the effect of a single, oral dose on forearm vasoconstriction in response to brachial artery infusion of ET-1. With this model, which is generally considered the gold standard for investigation of ETA blockade in man, ET-1 is known to cause vasoconstriction, predominantly by activation of ETA on the vascular smooth muscle. Inhibition of this effect would provide clinical evidence of ETA blockade. We conducted a single dose, placebo-controlled, double-blind, randomized study in 8 healthy men who previously demonstrated a mean 25-70% reduction in forearm blood flow (FBF) in response to 120 min brachial artery infusion of ET-1. Over non-consecutive days, each volunteer received 2 oral doses of ZD4054 (10mg and 30mg) and placebo. A 120 min brachial artery infusion of ET-1 (2.5 pmol/min) was commenced 2 h post-dosing with ZD4054/placebo, and the degree of forearm vasoconstriction (from 90-120 min of infusion) was compared between groups. In subjects given placebo, FBF was reduced by 40% in response to ET-1. Administration of ZD4054 (30 mg) to 6 subjects produced an absolute reduction in vasoconstriction of 24% (90% CI 10, 38; p=0.01) representing a 63% decrease in vasoconstriction relative to placebo. ZD4054 (10 mg) resulted in a relative decrease in vasoconstriction of 14% (p=0.10). The ability of ZD4054 to reduce ET-1 induced vasoconstriction, compared with placebo, provides clinical evidence that ZD4054 antagonizes ETA in man. This, coupled with previous data demonstrating no clinical/preclinical evidence of ETB blockade, suggests that ZD4054 is a specific ETA antagonist in man. Since ET-1, acting through ETA may be an important driver of oncogenesis, these results provide a rationale for further evaluation of ZD4054 as a cancer therapy.

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**ETB receptor agonist, IRL 1620, does not alter paclitaxel plasma pharmacokinetics and toxicology in tumor bearing rats**

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The present study was conducted to determine whether ETB receptor agonist, IRL 1620 alters pharmacokinetics of paclitaxel. We have found that intravenous administration of IRL 1620 to tumor bearing rats increased blood perfusion and enhances delivery of chemotherapeutic agents to the tumor tissue. The present study was conducted to determine whether IRL 1620 alters pharmacokinetics and toxicity of paclitaxel. Breast tumors were induced in female Sprague Dawley rats by MNU (50 mg/kg, i.p). Saline (0.3 ml/kg, i.v.) or IRL 1620 (3 nmol/kg, i.v.), was administered to the tumor bearing rats. [3H]-Paclitaxel (40 µCi/rat, i.v.) was administered 15 minutes after saline or IRL 1620 and serial plasma samples were collected till 24 hours. [3H]-Paclitaxel radioactivity in the plasma samples was measured by liquid scintillation counting. Data was fit to a 3-compartment model and pharmacokinetic parameters were generated using WinNonlin software. In a separate study, rats were treated with saline (0.3 ml/kg, i.v.) or IRL 1620 (3 nmol/kg, i.v.) and paclitaxel (1 and 5 mg/kg, i.v.) for a total of five doses. After 30 days, the rats were sacrificed and a complete blood chemistry test was performed to measure blood cell counts, electrolyte levels, liver, and kidney functions. IRL 1620 did not produce any change in the plasma pharmacokinetic profile of tumor bearing rats. The AUC0-8 (9.43 ± 3.18 µg-h/mL), clearance (0.69 ± 0.17 L/h/kg), volume of distribution (10.31 ± 4.54 L/kg), and half life (1.0 ± 0.32 hr) of the tumor rats treated with [3H]-Paclitaxel were not significantly different in the rats treated with IRL 1620 and [3H]-Paclitaxel. Hematological, liver, and kidney function parameters were found to be similar in groups treated with vehicle and IRL 1620. ETB receptor agonist does not alter paclitaxel plasma pharmacokinetics or its toxicological profile and can therefore be safely used to deliver paclitaxel to the tumor tissue.

P-137

**Endothelin-1 Stimulates Osteoblast Production of Interleukin-6, Connective Tissue Growth Factor (CTGF) and Cyr61**

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Tumor-produced ET-1 is a causal agent in the pathogenesis and progression of osteoblastic metastases by stimulating osteoblast activity via the ETAR. ET-1 stimulates osteoblast proliferation and new bone formation in neonatal mouse calvariae, an effect that is blocked by ETAR antagonists. Breast cancer cell lines that secrete ET-1 produce osteoblastic metastases in nude mice and such metastases are blocked by the ETAR antagonist ABT-627. Targets of ET-1 action in osteoblasts with the potential to enhance bone metastases were identified by gene microarray analysis and confirmed by quantitative RT PCR. Transcripts for IL-6 and the CCN proteins Cyr61 and CTGF demonstrated a rapid increase with ET-1 treatment. The increase in IL-6 and Cyr61 mRNA was blocked by ABT-627, but not by an antagonist selective for the endothelin B receptor. IL-6 is a potent osteoclast activating factor but its role in osteoblast function is not clear. ET-1 treatment of murine calvarial organ cultures results in a two-fold increase in IL-6 protein secretion. However, an IL-6 neutralizing antibody did not block the effects of ET-1 to stimulate new bone formation in neonatal mouse calvariae. These data suggest that IL-6 is not a direct mediator of new bone formation induced by ET-1. CTGF is an established stimulator of osteoblast proliferation and bone formation, but an action of Cyr61 on osteoblasts has not been reported. Human Cyr61 expressed under control of the CMV promoter was delivered to calvarial organ cultures by transduction with a lentiviral vector for 48 hours. Cyr61 lentivirus resulted in significant new bone formation and increased osteoblast numbers. Our results demonstrate that ET-1 induces osteoblast expression of Il-6, Cyr61 and CTGF. These effects appear to be mediated by ETAR. Cyr61 and CTGF, but not IL-6, may directly mediate the effects of ET-1 osteoblast activity.

# LATE-BREAKING ABSTRACTS

LB-001

**Endogenous endothelin-1 is required for cardiomyocyte survival in vivo**

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Endothelin-1 (ET-1) has potent vasoconstrictor and hypertrophic actions. Pharmacologic antagonists of endothelin receptors attenuate cardiac hypertrophy, have been approved for treatment of pulmonary hypertension, and are under investigation for treatment of heart failure. We have previously shown that cardiomyocyte-specific deletion of ET-1 reduces the hypertrophic response to thyroid hormone. These ET-1 deficient mice are phenotypically normal when young. Remarkably, as the mice age, or when young animals are subjected to aortic banding, they develop an unexpected phenotype of progressive systolic dysfunction and cardiac dilation. Echocardiography, necropsy, histology and molecular phenotype confirm a dilated cardiomyopathy. TUNEL analysis reveals greater abundance of apoptotic nuclei in the ET-1 deficient hearts. Transcriptional and Western analysis suggests enhanced TNF mediated apoptosis with increases in caspase-8 and caspase-3 activity. These ET-1 deficient hearts also have diminished ERK1/2 phosphorylation and NFkB activity, resulting in diminution of downstream inhibitors of TNF signaling. We conclude that local endothelin-1 gene expression is necessary to maintain normal cardiac function and cardiomyocyte survival in mice with both age and hemodynamic stress. This cardiac protective effect is mediated by paracrine ET-1 modulation of TNF-related apoptosis, and NFkB connects these signaling pathways.

LB-002

**Modification of the endothelin system in Alzheimer's disease**

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Endothelin (ET), a 21-amino acid peptide, belongs to a family including ET-1, ET-2 and ET-3. These peptide isoforms and their G-coupled-protein receptors ET-A and ET-B are widely distributed in the CNS. ET is a potent vasoconstrictor even in the brain microvasculature. Cerebral vessels display potent vasoconstriction upon ET-A activation and respond to ET-B stimulation with NO-mediated vasodilatation. In the brain, the presence of ET receptors is not restricted to the cerebral vasculature, they are also localized on neurons and glial cells. The precise role ET in the CNS remains unclear. Some findings indicate that ET could directly or indirectly contribute to neuronal injury through ET-A. On the contrary, ET-B seems to be involved in neuronal survival. The endothelin converting enzyme (ECE) was shown to degrade the amyloid peptide. In addition to its potent vasoconstrictor activity, ET could have a crucial role in neuroprotection/ neurodegeneration. We will show that the levels of ET-A, ET-B and ECE in N2A neurons are increased in the presence of the amyloid beta peptide (25-35) (Aß25-35). Interestingly, the expression of ET-A, ET-B receptors and ECE is higher in the hippocampus and frontal cortex from Alzheimer's patients than in age-matched controls where the Aß is elevated. Thus an alteration of these receptor expressions by the Aß could be involved in the pathophysiology of Alzheimer's disease. A better understanding of the role of the ET system in the CNS, under some pathological conditions may open new ET-targeted therapeutic avenues. Brain tissue were provided by the Douglas Hospital research Center Brain Bank, Verdun, Quebec, Canada.

LB-003

**The three endothelin receptors in the killifish, *Fundulus heteroclitus*: Physiological and phylogenetic relationships**

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The endothelin (ET) signaling cascade is traditionally viewed as three paracrines, ET-1, ET-2 and ET-3 that bind to two membrane bound receptors, ETA and ETB. Generally, when ETs bind to receptors on vascular smooth muscle (VSM) cells constriction is observed; however if ET binds to endothelial ETB, nitric oxide (NO) and prostaglandins (PGs) are produced leading to relaxation of the underlying VSM. ET stimulation of NO and PGs can also result in inhibition of ion transport in the mammalian kidney and recently this cascade was hypothesized to function similarly in the epithelial lining of the operculum from the fish *Fundulus heteroclitus* (killifish). These ET signaling cascades are well conserved throughout the vertebrates. Interestingly, in non-mammalian vertebrates there are three ET receptors (ETRs), ETA, ETB1, and ETB2 (ETC in amphibians). Our phylogenetic analysis supports previous findings that these ETRs are produced by separate genes and are not splice variants. In mammals there is physiological evidence for two ETB receptors, but only one ETB gene has been cloned. Studies using ETR agonists and the antagonists BQ-123 (ETA-selective) and PD142893 (non-selective) suggest that the ETB that leads to VSM dilation is pharmacologically different from the ETB that leads to VSM constriction. Examination of GenBank and the vertebrate Genome projects has found ETA and ETB genes in the mammals, but ETA, ETB and ETB2 in the chicken, frog and fishes, suggesting that there is only one ETB gene in mammals, but two ETB genes in all other vertebrates. Using standard cloning and sequencing, we have partially sequenced the ETRs from killifish gill cDNA. To further characterize these three receptors, tissue distribution and quantitative PCR mRNA analyses were performed. Given that animals from fishes to birds have three ETRs while mammals have only two, we hypothesize that mammals have lost the ETB2 gene. Future experiments to further characterize the ETB2 are needed and may help elucidate why mammals have apparently lost this receptor.

LB-004

**Expression And Localization of The Endothelin System in Cisplatin-Induced Acute Renal Failure in Mice**

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In animal models such as ischemic acute renal failure (ARF), renal fibrosis, and renal hypertension endothelin-1 (ET-1) was unequivocally elevated in the kidney, whereas ET receptors (ETRA and ETRB) was variably expressed. We characterized changes in the expression and localization of the ET system in nephrotoxic ARF induced by cisplatin. Mice were injected with 16 mg cisplatin/kg at a single dose. Quantification of mRNA and protein expression was performed by real-time RT-PCR and Western blot, respectively. Conventional staining showed that renal injuries by cisplatin were mostly confined to cortical proximal tubules. ET-1 transcripts were about eight times higher in cisplatin-treated mice than in controls. Tissue ET-1 contents measured using ELISA increased 1.5-fold. Western blot also demonstrated 2-fold upregulation of ET-1. Most of the increased immunoreactive ET-1 was localized in damaged tubules. The expression of ETRA mRNA between the cisplatin-treated group and the control was not different. Unexpectively, ETRA protein was twice more abundant in the cisplatin-treated group than in the control. In immunohistochemistry such an upregulation of ETRA was mainly found around the necrotic tubules of cisplatin-treated mice. Neither the expression of ETRB mRNA nor the abundance and immunoreactive level of ETRB were changed by cisplatin treatment. The findings suggest that the individual components of the ET system in the kidney are independently regulated in cisplatin-induced ARF.

LB-005

**Proteomics of ET-1 receptor signaling complexes in rat cardiac myocytes**

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Endothelin-1 (ET-1) regulates contractility and growth of the heart, and plays a central role in ischemic preconditioning and the onset of cardiac hypertrophy. The effects of ET-1 are mediated by binding one of two G-protein-coupled receptors (GPCRs): type A (ETAR) and type B (ETBR), both of which are present in mammalian ventricular muscle. GPCRs are known to interact functionally with many down stream effectors to form signaling cascades, but the extent to which such proteins interact physically is unkown. Here, we investigated ET-1 receptor associated proteins in rat cardiac myocytes by immunoprecipitating ET-1 receptors, isolating associated proteins by SDS-PAGE, and identifying them by peptide mass fingerprinting using MALDI-TOF mass spectrometry. Specificity of immunoprecipitation was addressed with IgG control beads, and mass spectrometry results were confirmed by Western blotting of key proteins. Effectors thought to function downstream of ET-1 receptors such as Gαq, Gαi, PLC-ß, PKC-ε, Na/H exchanger, and L-type Ca channels were readily detected. Proteins associated with other regulatory pathways were also prominent including i) AKAP--adenylate cyclase--PKA--cAMP phosphodiesterase, ii) PI3Kinase--Akt--Erk1/2--GSK3ß, iii) JAK--STAT, and iv) clathrin heavy chain--adaptor AP50--ßARKs--ßarrestin--dynamin--Rab GTPases--synaptotagmin. Other strong hits included numerous growth factor receptors, GTPase regulatory proteins, serine/threonine and tyrosine kinases/phosphatases, cytoskeletal/scaffolding proteins, and ion channels. Confidence in the selectivity of this method was enhanced by the observation that treatment of myocytes with ET-1 altered the composition of signaling complexes. This study provides the basis for future hypothesis-driven functional studies of ET-1 signaling in heart muscle.

LB-006

**Endothelin-1 mobilizes profilin-1 bound PIP2 in cardiac muscle**

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Phosphatidylinositol 4,5-bisphosphate (PIP2) is a key downstream substrate of the endothelin signaling pathway. PIP2 also plays an intricate role in regulating protein function at the membrane-cytoskeletal interface. However, the dynamic properties of distinct pools of PIP2 are poorly understood, especially PIP2 that is bound to cytoskeletal proteins. We investigated the effects of endothelin-1 (ET-1) stimulation on protein bound PIP2 in cardiac muscle. Isolated rat myocytes and homogenized mouse ventricles were exposed to 10 nM ET-1 for varying time periods and PIP2 bound proteins were analyzed using an anti-PIP2 antibody and Western Blotting. Several cytoskeletal proteins were found to contain tightly bound PIP2 including profilin-1 (~16 kD), CapZ (~30 kD) and -actinin (~106 kD). Interestingly, ET-1 reduced the amount of PIP2 bound to profilin-1 by 46% after 15 min, followed by a recovery to near basal levels after 60 min. ET-1 had no effect on CapZ or α-actinin bound PIP2 levels. To further explore the dynamics of PIP2 binding, brefeldin-A (BFA) was used to disrupt PIP2 binding to ADP-ribosylation factors (ARFs) and to impair receptor internalization. One μM BFA increased the PIP2 signal on profilin-1 by 54% after 15 min, followed by a decline to sub-basal levels after 60 min. Like ET-1, BFA had no effect on levels of PIP2 bound to CapZ or to α-actinin. Combined treatment with ET-1 and BFA resulted in a sustained drop in the PIP2 bound to profilin-1, but again not to CapZ or α-actinin. Taken together, the data indicate that profilin-1 binds PIP2 dynamically and may serve as a key regulator of the balance between cytoskeletal integrity and PIP2 availability for Ca2+/PKC signaling in the heart.

LB-007

**Hypoxia induces pulmonary arterial hypertension in endothelial cell-specific ETB receptor knockout mice**

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Pulmonary ETB receptors elicit both protective (vasodilator, and clearance) and deleterious (vasoconstrictor and pro-mitogenic) effects that may contribute to the pathogenesis of pulmonary arterial hypertension (PAH). We hypothesised that by decreasing vascular tone and limiting ETA receptor activation through clearance of ET-1, endothelial cell (EC) ETB receptors protect against the development of hypoxia-induced PAH. Mice with loxP sites flanking ETB receptor coding regions (FF/--) were crossed with Tie2-Cre mice to produce EC-specific ETB receptor knockout (KO) mice (FF/Tie2-Cre). Male mice were subjected to 2 weeks of either hypoxic (10% FiO2) or normoxic conditions. Right ventricular pressure (RVP) was measured by direct cardiac puncture. Right ventricular (RV) / left ventricle+septum (LV+S) ratio was measured as an index of RV hypertrophy. Pulmonary arteries (PAs) (~300µm ID) were removed and concentration responses to ET-1 (1fM-0.1µM) constructed using a wire myograph. Under normoxic conditions, there was no difference between systolic RVP in FF/Tie2-Cre mice (23.7±1.7 mmHg, n=9) and FF/-- controls (20.2±1.5 mmHg, n=10). Hypoxia induced an exaggerated increase in systolic RVP in FF/Tie2-Cre mice (34.4±1.2 mmHg, n=10) compared with FF/-- mice (24.6±1.4mmHg, n=10; $p<0.05$). Hypoxia resulted in increased RV/LV+S ratio in FF/Tie2-Cre mice (normoxia: 0.224±0.009; hypoxia: 0.285±0.017; $p<0.01$). In FF/-- mice the increase did not reach statistical significance (normoxia: 0.247±0.009; hypoxia: 0.282±0.012; $p>0.05$). Whilst ET-1 induced a dose dependent constriction of PAs from both FF/Tie2-Cre and FF/-- mice, under both normoxia and hypoxia, maximum responses were similar. EC ETB KO mice develop hypoxia induced PAH and RV hypertrophy that is not due to increased pulmonary vascular responsiveness to ET-1. Whilst we have shown that EC ETB receptors exert a protective effect during hypoxia that prevents the development of PAH, the mechanism by which this is achieved requires further investigation.

LB-008

**Endothelin-1 stimulates colon cancer adjacent submucosal fibroblasts**

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Background: Endothelin-1 is produced by and stimulates proliferation in colorectal cancer cells and is associated with early cancers and advanced disease. Fibroblasts are the key stromal cell in cancer and are involved in cancer initiation and progression. Endothelin-1 is known to activate fibroblasts from a number of tissues, but its effects on tumour adjacent colonic fibroblasts are poorly understood. Aims: To investigate the effect of endothelin-1 on submucosal colonic fibroblast proliferation, migration, contraction and protein expression and determine the receptor responsible for these effects. Methods: Proliferation, migration, contraction, and expression of MMP-2, MMP-3 and TIMP-1 were measured in the presence and absence of endothelin-1 and its specific receptor antagonists. Submucosal colonic fibroblast cell strains were extracted from areas adjacent to tumours from surgical excision specimens of colon. Results: Endogenous and exogenous endothelin-1 stimulates proliferation of fibroblasts via ETA receptors, exogenous endothelin-1 does not significantly increase proliferation compared to endogenous endothelin-1. Fibroblast migration is stimulated by endothelin-1 via ETA and ETB receptors. Contraction of fibroblasts is stimulated by endothelin-1 and mediated by ETA and ETB receptors. MMP-2/3 and TIMP-1 expression are stimulated by endothelin-1 predominantly via ETA receptors. Conclusion: Endothelin-1 stimulates proliferation, contraction, migration and matrix degrading protein expression. This suggests endothelin-1 is an important paracrine growth factor in colonic cancers and a potential anti-cancer stroma therapeutic target.

LB-009

**Endogenous endothelin controls activation of genes implicated in vascular bone formation in early autoimmune type 1 diabetes mellitus**

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Bone morphogenic proteins (BMP) are known to have anti-inflammatory and anti-proliferative properties within the vasculature. It is unknown to what extent transcriptional regulation of BMPs is affected during early stages of autoimmune type 1 diabetes mellitus and whether endothelin (ET)A-receptors are involved in this regulation. Female euglycemic non-obese-diabetic (NOD) and age-matched control mice were either killed at 16 weeks of age or treated with the selective ETA receptor antagonist BSF461314 for six weeks. Plasma glucose levels were measured before and after treatment and vascular gene expression of BMP-2, BMP-7, and BMP-type II receptor were determined in the aorta by quantitative real-time PCR. At 16 weeks of age plasma glucose levels of all animals were within the normal range, and aortic expression of all genes was comparable between NOD and control mice (n.s.). At age 22 weeks, NOD mice became diabetic and gene expression of vascular BMP-2, BMP-7 and BMP-type II receptor was almost doubled in diabetic NOD mice compared to non-diabetic controls ($p<0.05$). Concomitant treatment with BSF461314 significantly reduced expression of all three BMPs and lowered plasma glucose levels in NOD mice close to those of control mice (all $p<0.05$ vs. untreated). In conclusion vascular expression of BMP-2, BMP-7, and BMP type II receptor increases in early stages of autoimmune type 1 diabetes mellitus. Treatment of diabetic NOD mice with an orally active ETA receptor antagonist prevented hyperglycemia-associated activation of vascular BMPs and delayed the onset of autoimmune diabetes, suggesting a novel role of ET in glucose homeostasis and regulation of diabetes-associated induction of genes associated with vascular bone formation.

LB-010

**Bosentan, sildenafil and their combination in the monocrotaline model of pulmonary hypertension in rats**

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The dual endothelin receptor antagonist and the phosphodiesterase inhibitor sildenafil have both been shown to decrease pulmonary artery pressure in experimental and clinical pulmonary hypertension (PHT). The effects of bosentan, sildenafil, and their combination were evaluated in rats with monocrotaline (MCT)-induced PHT. Male Wistar rats (210-240 g) were randomly assigned to the following treatments: (1) s.c. injection of saline and no treatment (control, n=15); (2) MCT (60 mg/kg, s.c.) and no treatment (n=19); (3) MCT plus bosentan (300 mg/kg/day as food admix) (n=19); (4), MCT plus sildenafil (100 mg/kg/day in drinking water) (n=19); (5), MCT plus bosentan (300 mg/kg/day) and sildenafil (100 mg/kg/day) (n=19). The doses of bosentan and sildenafil were based on a prior dose-range finding study and chosen for having the same effect on mean pulmonary arterial pressure (MPAP). Over the 4-week treatment period and during terminal anesthesia, mortality was 0%, 53%, 11%, 11% and 0%, respectively, in the five different groups. Oral administration of bosentan, sildenafil, or their combination significantly attenuated the MCT-induced increase in MPAP by 21% ($p < 0.01$), 22% ($p < 0.01$), 42% ($p < 0.001$), respectively, compared to untreated PHT rats. Chronic oral administration of bosentan, sildenafil, or their combination for 4 weeks also significantly reduced right ventricular hypertrophy by 30%, 32%, and 37% ($p < 0.001$ compared to untreated rats). The three treatment options also led to a decrease in catecholamines and an increase in endothelium-dependent relaxation. Conclusion**:** Both bosentan and sildenafil are efficacious for decreasing pulmonary arterial pressure and right ventricular hypertrophy in rats with chronic PHT and the combination shows an additional effect.