Am J Physiol Cell Physiol 303: C179–C191, 2012.
First published May 16, 2012; doi:10.1152/ajpcell.00008.2012.

Upregulation of thrombospondin-1 expression by leptin in vascular smooth muscle cells via JAK2- and MAPK-dependent pathways

Ronaldo J. Chavez,* Rebecca M. Haney,* Rene H. Cuadra, Rituparna Ganguly, Ravi K. Adapala, Charles K. Thodeti, and Priya Raman

Department of Integrative Medical Sciences, Northeast Ohio Medical University, Rootstown, Ohio

Submitted 4 January 2012; accepted in final form 9 May 2012

Chavez RJ, Haney RM, Cuadra RH, Ganguly R, Adapala RK, Thodeti CK, Raman P. Upregulation of thrombospondin-1 expression by leptin in vascular smooth muscle cells via JAK2- and MAPK-dependent pathways. *Am J Physiol Cell Physiol* 303: C179–C191, 2012. First published May 16, 2012; doi:10.1152/ajpcell.00008.2012.—Hyperleptinemia, characteristic of diabetes and a hallmark feature of human obesity, contributes to the increased risk of atherosclerotic complications. However, molecular mechanisms mediating leptin-induced atherogenesis and gene expression in vascular cells remain incompletely understood. Accumulating evidence documents a critical role of a potent antiangiogenic and proatherogenic matricellular protein, thrombospondin-1 (TSP-1), in atherosclerosis. Although previous studies reported elevated TSP-1 levels in both diabetic and obese patients and rodent models, there is no direct information on TSP-1 expression in vascular cells in response to leptin. In the present study, we show that leptin upregulates TSP-1 expression in cultured human aortic smooth muscle cells (HASMC) in vitro, and this increase occurs at the level of transcription, revealed by mRNA stability and TSP-1 promoter-reporter assays. Utilizing specific pharmacological inhibitors and siRNA approaches, we demonstrate that upregulation of TSP-1 expression by leptin is mediated by JAK2/ERK/JNK-dependent mechanisms. Furthermore, we report that while ERK and JNK are required for both the constitutive and leptin-induced expression of TSP-1, JAK-2 appears to be specifically involved in leptin-mediated TSP-1 upregulation. Finally, we found that increased HASMC migration and proliferation in response to leptin is significantly inhibited by a TSP-1 blocking antibody, thereby revealing the physiological significance of leptin-TSP-1 crosstalk. Taken together, these findings demonstrate, for the first time, that leptin has a direct regulatory effect on TSP-1 expression in HASMCs, underscoring a novel role of TSP-1 in hyperleptinemia-induced atherosclerotic complications.

leptin; thrombospondin-1; vascular smooth muscle cell; Janus kinase 2; mitogen-activated protein kinase

THROMBOSPONDIN-1 (TSP-1) belongs to a family of secreted extracellular matrix proteins, implicated in cell-cell and cell-matrix interactions. It has been established that these matricellular glycoproteins play an important role in numerous biological processes, such as cell adhesion, migration and proliferation, angiogenesis, inflammation, atherosclerosis, and thrombosis (1, 2, 7, 23). TSP-1 is a potent antiangiogenic and proatherogenic protein with differential cell type-specific effects, e.g., increased proliferation of vascular smooth muscle cells (VSMC) and apoptosis of endothelial cells (EC) (48). An increased expression of TSP-1 was reported in the injured vascular wall (42, 45) and early atherosclerotic lesions (43), and TSP-1 is among the immediate early genes and growth factor genes expressed by VSMCs (28). The TSP protein family has been genetically linked to the development of atherosclerotic vascular disease (54), and previous studies have suggested a role for TSP-1 in restenosis (9, 20). More recently, using the TSP-$1^{-/-}$/$ApoE^{-/-}$ double-knockout mice, it was shown that TSP-1 facilitates initiation of atherosclerotic lesions (30, 50). Multiple studies have also documented that TSP-1 is upregulated in diabetic patients and animal models (31, 34, 49) as well as in obese humans and obese rodent models (19, 40, 56), suggesting a role for TSP-1 in insulin resistance and obesity.

Hyperleptinemia, characteristic of type 2 diabetes and a hallmark feature of obesity, is well-recognized as a pathophysiological trigger attributing to the increased risk of cardiovascular complications. Emerging evidence suggests that several of the peripheral effects of leptin occur independently of its role in metabolic regulation. Previous studies have reported a direct association between plasma leptin concentrations and the intima-media thickness of the common carotid artery, an early marker of atherosclerosis (13). Numerous human (3, 13, 14) and animal studies (4, 12) have also established that increased plasma leptin levels contribute to the pathogenesis of atherosclerosis, underscoring the importance of hyperleptinemia in vascular complications associated with both diabetes and obesity. It has been suggested that leptin promotes atherogenesis by exerting a direct effect on the endothelium, VSMC, and macrophages (33, 36). Leptin exhibits several proatherogenic properties, including EC dysfunction (37, 51), platelet aggregation (22), vascular calcification (35, 58), increased migration and proliferation of VSMC (25, 33), and generation of reactive oxygen species (8, 25, 57). Leptin is also known to regulate the expression of a number of vascular genes, including growth factors, cytokines, and extracellular matrix proteins (17, 24, 25, 36, 37, 41, 57); many of these genes, in turn, have been associated with the development of atherosclerosis and abnormal angiogenesis. However, a precise mechanistic understanding of leptin-induced atherogenesis and mechanisms activated by leptin that modulate changes in the gene expression profile in vascular cells remain controversial and incomplete. While recent studies demonstrate an important role of TSP-1 in obesity-associated insulin resistance, the putative role of TSP-1 in hyperleptinemia-induced macrovascular complications has not been clearly defined. In the present study, we sought to investigate a potential cross talk between leptin and TSP-1 in human aortic smooth muscle cells (HASMC) and to elucidate the role of TSP-1 in leptin-induced vascular cell function. In particular, we have postulated that an upregulation of TSP-1 expression by leptin may lead to macrovascular complications associated with hyperleptinemia. Additionally, we have deter-

*R. J. Chavez and R. M. Haney contributed equally to this study.

Addressfor reprint requests and other correspondence: P. Raman, Dept. of Integrative Medical Sciences, Northeast Ohio Medical Univ. (NEOMED), 4209 State Route 44, Rootstown, OH 44272-0095 (email: praman@neomed.edu).

mined specific signaling mechanism(s) underlying such regulation.

MATERIALS AND METHODS

Cell culture. Primary HASMC isolates were cultured in DMEM/F12 medium containing 10% FBS at 37°C in 5% CO_2 atmosphere. Cells with passage numbers between 6 and 18 were used in all experiments. Cells were placed in low-glucose (5 mM) DMEM supplemented with 0.2% FBS, 24 h prior to treatments, and were then stimulated with recombinant human leptin (endotoxin level < 1.0 EU per 1 μg of protein, as determined by the LAL method; Peprotech) as described in the figure legends. For pharmacological inhibition of specific signaling pathways, cells were preincubated with different inhibitors for 2 h prior to stimulation with leptin. The specific signaling inhibitors used in this study were PD-98059(30 μM, ERK inhibitor), SP-600125 (30 μM, JNK inhibitor), SB-203580 (5 μM, p38 MAPK inhibitor), and AG-490 (30 μM, JAK2 inhibitor).

Northern blotting. Effect of leptin on TSP-1 mRNA levels was determined by Northern blotting as previously described (39). Briefly, total RNA was extracted using Trizol reagent (Invitrogen), and the isolated RNA was stored in diethyl pyrocarbonate-treated water at −80°C. Prior to use, the purity and concentration of the RNA sample was determined by measuring the optical density at 260 and 280 nm. The optical density ratio at 260/280 for all RNA samples used in this study ranged from 1.8 to 2.1. For Northern blot analyses, 10 μg of SMC RNA was electrophoresed in 16.7% agarose-formaldehyde gels, transferred to nylon membranes (PerkinElmer Life Sciences), and hybridized to ^{32}P-labeled TSP-1 cDNA probe, according to the method described previously (39). The membranes were exposed to HyBlot CL autoradiography films (Denville Scientific) at −80°C for varying periods of time. Signal intensity of Northern blots was quantified using ImageJ or Adobe Photoshop software. Ethidium bromide staining of ribosomal RNA was used to control for RNA loading in samples.

Western blotting. Whole cell lysates were prepared using 1X RIPA buffer supplemented with protease inhibitors. Cell pellets were resuspended in protein sample buffer, boiled for 10 min, and samples were resolved in 10% SDS-PAGE gel. Western blotting was performed using either human anti-TSP-1 (1:2,000 clone AB11, Thermofisher) or mouse anti-TSP-1 (1:1,000 clone AB4, Thermofisher), anti-ERK1/2 (1:10,000, Cell Signaling), anti-JNK (1:2,000, Cell Signaling), and anti-PCNA (1:300, Abcam) antibodies. The membranes were stripped and reprobed with anti-β-actin or anti-tubulin (Abcam) to control for protein loading in samples. The membranes were also stained with Ponceau S to determine equal protein loading. Quantification of Western blots was performed using ImageJ or Adobe Photoshop software.

siRNA transfection. HASMC were plated in 24-well clusters in DMEM/F12 medium containing 10% FBS; 80–90% confluent cells were transfected with either control scrambled siRNA or with ERK1/2- or JNK-specific siRNA for gene silencing of the corresponding MAPK signaling pathways, as indicated in the figure legends. The siRNA was delivered to the cells using the silentFect Lipid Reagent (Bio-Rad) following the manufacturer's protocol. Briefly, 15–60 min prior to transfection, cells were placed in fresh serum-containing growth medium in the absence of antibiotics. Cells were transfected with 20 nM different siRNAs or scrambled siRNA (Cell Signaling). Forty-eight hours posttransfection, the silentFect-siRNA complex containing medium was changed to low-glucose (5 mM) DMEM containing 0.2% FBS, and the cells were preincubated in this low-glucose, low-serum medium for 24 h. Following this, the cells were further incubated with or without 100 ng/ml leptin, and transfected cells were harvested after 3 h. To detect protein expression of total ERK, JNK, and TSP-1, whole cell lysates were prepared from the transfected cells and subjected to Western blotting as described above.

Inhibition of transcriptional or translational pathways. Confluent HASMC were preincubated with either 5 μg/ml actinomycin D (Act D), a transcriptional inhibitor, or 5 μg/ml cycloheximide (CHX), a translational inhibitor, for 30 min. This was followed by incubation for 3 h with or without 100 ng/ml leptin, and cells were then harvested for isolation of RNA. Total RNA was extracted and used for Northern blotting as described above.

mRNA stability assay. Control cells or leptin-stimulated (3 h) cells were treated with 5 μg/ml Act D and then lysed at different time points from 1 to 4 h following initiation of Act D treatment. Total RNA was extracted and used for Northern blotting as described above.

Cell transfection and luciferase assay. HASMC were plated in 24-well clusters (Costar) in 10% FBS, DMEM/F12 media. At 70–80% confluency, cells were transiently transfected with either −1,270/+750 TSP-1 gene (THBS1) promoter linked to a luciferase reporter gene construct or with pGL3 vector control. The transfection procedure was carried out using Lipofectin Reagent (Invitrogen), according to manufacturer's instructions. At 6 h posttransfection, the plasmid-DNA containing medium was changed to low-glucose (5 mM) DMEM containing 10% FBS, and the transfected cells were further incubated with 100 ng/ml leptin. For inhibitor studies, cells were incubated with leptin in the presence or absence of inhibitors of different signaling pathways as indicated in the figure legends. After 42 h of incubation, cell extracts were assayed for luciferase activity using a luciferase assay kit (Promega). Protein concentrations in cell lysates were analyzed using the BCA protein assay reagent (Pierce), and the activity of luciferase was normalized to total protein concentrations in lysates.

Animals. All procedures were conducted in accordance with and were approved by the Institutional Animal Care and Use Committee of Northeast Ohio Medical University (NEOMED). Male C57BL/6J mice and TSP-1$^{-/-}$ mice, in C57BL/6J background (at 9 wk of age) from Jackson Laboratories (Bar Harbor, ME) were treated daily with 5 μg/g recombinant mouse leptin (endotoxin level < 1.0 EU per 1 μg of the protein as determined by the LAL method, R&D Systems) by intraperitoneal injections for 10 days. This dose regimen of leptin was chosen based on protocols used previously to reduce body weight and achieve fertility in leptin-deficient (*ob/ob*) mice (26). Age-matched control mice received saline (vehicle) only by daily intraperitoneal injections. Mice were housed in a temperature-controlled room with a 12:12-h light/dark cycle and had adequate access to food and water ad libitum.

TSP-1 immunostaining. Freshly frozen aortic tissue sections were stained with biotinylated anti-TSP-1 antibody (AB4-BO, Thermofisher) using Vectastain Elite ABC Kit- Mouse IgG (Vector Labs.). TSP-1 expression was visualized with diaminobenzidine (15) peroxidase substrate kit (Vector Labs). A negative control without the anti-TSP-1 primary antibody was processed simultaneously for each section. Staining of matching sets of tissues from saline- and leptin-treated mice was done simultaneously. Sections were counterstained with hematoxylin and eosin to allow identification of the intimal, medial, and adventitial layers of the vessel wall.

Preparation of tissue lysates. Aortic vessel, heart, and fat pad tissues collected from all mice were homogenized in SDS lysis buffer (per 100 ml: 0.76 g Tris-base, 0.074 g disodium EDTA, 2.3 g SDS, and 10% glycerol) containing protease inhibitors and incubated for 30 min at 4°C. After 30 min of centrifugation at 16,000 rpm, the supernatants were used for Western blotting as described earlier.

Migration of cultured smooth muscle cells. HASMC migration was determined using the scratch-wound assay (10). Briefly, HASMCs were plated onto six-well cell culture plates and cultured to a confluent monolayer. The monolayer was wounded using a sterile 200-μl pipette tip making a scratch in the middle of the well. The cells were then washed with serum-free DMEM, random areas on the scratch-wound were marked with pen, and images were taken prior to any treatments. Following this baseline measurement, cells were incubated with or without 100 ng/ml recombinant human leptin in the

presence or absence of 10 μg/ml anti-TSP-1 antibody (clone AB 11, Thermofisher) or purified IgG (negative control antibody), and images were taken 24 h later to determine the distance of each scratch-wound closure. All images of wounding were taken using an Olympus microscope equipped with a CCD camera and Q Capture Pro software, and ImageJ was used to measure the area of the wound.

Proliferation of cultured smooth muscle cells. HASMC were plated in 96-well clusters (2,000 cells/well) in 10% FBS, DMEM/F12 medium. Twenty-four hours later, the medium was changed to low glucose (5 mM) DMEM containing 0.2% FBS, and treatments were initiated as follows. Cells were preincubated for 4 h with 10 μg/ml of anti-TSP-1 antibody (clone AB11, Thermofisher) or purified IgG (negative control antibody) followed by further incubation with or without recombinant human leptin (100–1,000 ng/ml). After 4 days of treatment, cell proliferation was determined using the Cell Proliferation Reagent WST-1, a nonradioactive colorimetric assay for quantitation of cell proliferation, according to the manufacturer's instructions (Roche).

Statistical analysis. All results are presented as means ± SE from at least three independent experiments, with two to four replicates of control and leptin-treated cells within each individual experiment. Significant differences between means were determined by a Student's *t*-test; $P \leq 0.05$ considered statistically significant.

RESULTS

High leptin concentrations upregulate TSP-1 expression in HASMCs in vitro. We previously demonstrated that high glucose stimulates TSP-1 expression in HASMC (39). In the present study, we evaluated whether high leptin concentrations, mimicking hyperleptinemic conditions characteristic of diabetes and obesity, upregulate TSP-1 expression in HASMCs. Our results showed that treatment of HASMC with leptin (100 ng/ml) significantly increased TSP-1 mRNA expression, as early as within an hour of incubation (Fig. 1*A*). The maximal response was observed at 3 h of 100 ng/ml leptin treatment (3.5-fold). In addition, there was a dose-dependent (1–100 ng/ml) increase in TSP-1 mRNA levels, with statistically significant increases observed only with 10 and 100 ng/ml of leptin (Fig. 1*B*). TSP-1 protein expression was also upregulated by high leptin treatment at both 3 and 6 h of incubation with leptin compared with untreated cells. Consistent with its effects on the TSP-1 mRNA level, the maximal TSP-1 protein expression occurred at 3 h of 100 ng/ml leptin treatment (4-fold, Fig. 1, *C* and *D*). These findings clearly demonstrate that leptin has

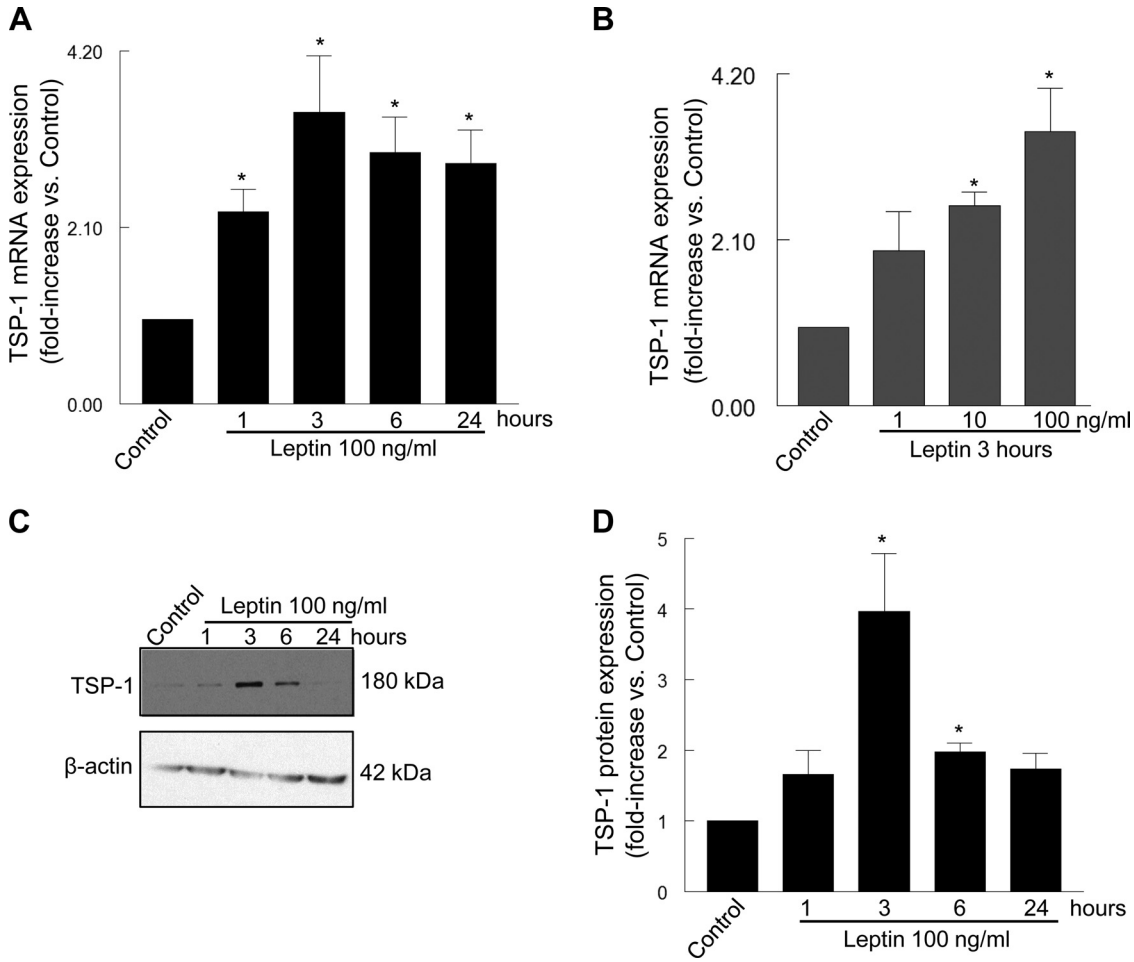


Fig. 1. Leptin in vitro stimulates thrombospondin-1 (TSP-1) expression in human aortic smooth muscle cells (HASMCs). HASMC were incubated with 100 ng/ml leptin for 1, 3, 6, and 24 h, as appropriate; total RNA and whole cell lysates were used in Northern and Western blotting, respectively. *A*: time-dependent effect of 100 ng/ml leptin on TSP-1 mRNA expression; shown is the densitometric quantification of Northern blots (n = 3–5). *B*: dose-dependent effect of leptin on TSP-1 mRNA expression at 3 h of incubation; shown is the densitometric quantification of Northern blots (n=3–5). *C*: time-dependent effect of leptin (100 ng/ml) on TSP-1 protein expression; shown is a representative Western blot for TSP-1 (top panel) and β-actin (loading control, bottom panel). *D*: densitometric quantification of Western blots (n = 4–5). All results are expressed as means ± SE. *$P \leq 0.05$ vs. control.

a direct stimulatory effect on TSP-1 expression in HASMCs. All subsequent experiments were performed after 3 h of leptin treatment, since the maximum effect on TSP-1 expression was achieved at this time point.

MAPK signaling mediates constitutive and leptin-induced TSP-1 expression in HASMCs. We next investigated the signaling mechanisms that mediate upregulation of TSP-1 expression by leptin in HASMC. Activation of mitogen-activated protein kinase (MAPK) is one of the major signal transduction cascades widely attributed to the actions of leptin (18). To delineate its underlying role in leptin-induced TSP-1 expression, we used specific inhibitors blocking each of the MAPK family members, namely, extracellular signal-regulated kinases (ERK), stress-activated protein kinase/c-Jun-NH_2-terminal kinases (SAPK/JNK), and p38-MAPK. Specifically, pretreatment of cells with PD-98059 (ERK inhibitor) and SP-600125 (JNK inhibitor) significantly inhibited leptin-induced increase in TSP-1 mRNA expression by 77% and 82%, respectively (Fig. 2*A*), as quantified by densitometric scans of the Northern blots. Consistent with their effects on the mRNA levels, both PD-98059 and SP-600125 remarkably attenuated TSP-1 protein expression stimulated by leptin (81% and 92%, respectively) (Fig. 2*B*). However, we found only a marginal inhibition in leptin-induced TSP-1 expression with SB-203580, a p38-specific inhibitor (data not shown). Additionally, we have observed that pretreatment with both ERK- and JNK-specific inhibitors resulted in a significant decrease in the basal expression of TSP-1 mRNA and protein levels (Fig. 2). To further confirm the role of ERK- and JNK-dependent mechanisms in leptin-induced TSP-1 upregulation, we next utilized siRNA gene silencing approaches to specifically knockdown ERK and JNK isoforms in HASMC. Transient transfection of HASMC with siRNAs targeted against ERK and JNK significantly decreased the levels of ERK and JNK protein expression, respectively, compared with cells transfected with a control scrambled siRNA, confirming the specific knockdown of ERK and JNK proteins by the used siRNAs. Importantly, knockdown of either ERK or JNK completely blocked leptin-induced increase in TSP-1 protein expression (Fig. 3), further confirming a role for ERK and JNK in upregulation of TSP-1 expression by leptin.

Transcriptional regulation of TSP-1 mRNA by high leptin concentration in HASMCs. We have observed that pretreatment of HASMC with actinomycin D (Act D), a transcriptional inhibitor, but not with cycloheximide (CHX), a translational inhibitor, significantly inhibits leptin-induced increase in TSP-1 mRNA expression (Fig. 4*A*). To determine whether leptin induces TSP-1 mRNA expression by increasing the transcription or stability of mRNA, we performed an Act D chase experiment. While TSP-1 mRNA levels were increased after stimulation with leptin (Fig. 4*B*, top panel), incubation with leptin did not alter the stability of TSP-1 mRNA (Fig. 4*B*, bottom panel), suggesting that leptin-induced increase in TSP-1 mRNA is not due to an increase in the mRNA stability. Furthermore, in cells transiently transfected with −1,270/+750 pTHBS1 promoter-luciferase reporter gene construct, incubation with leptin (100 ng/ml) resulted in a robust increase in the luciferase activity of the THBS1 promoter (3.4-fold) compared with untreated cells; in contrast, leptin did not have any effect on cells transfected with pGL3 vector control (Fig.

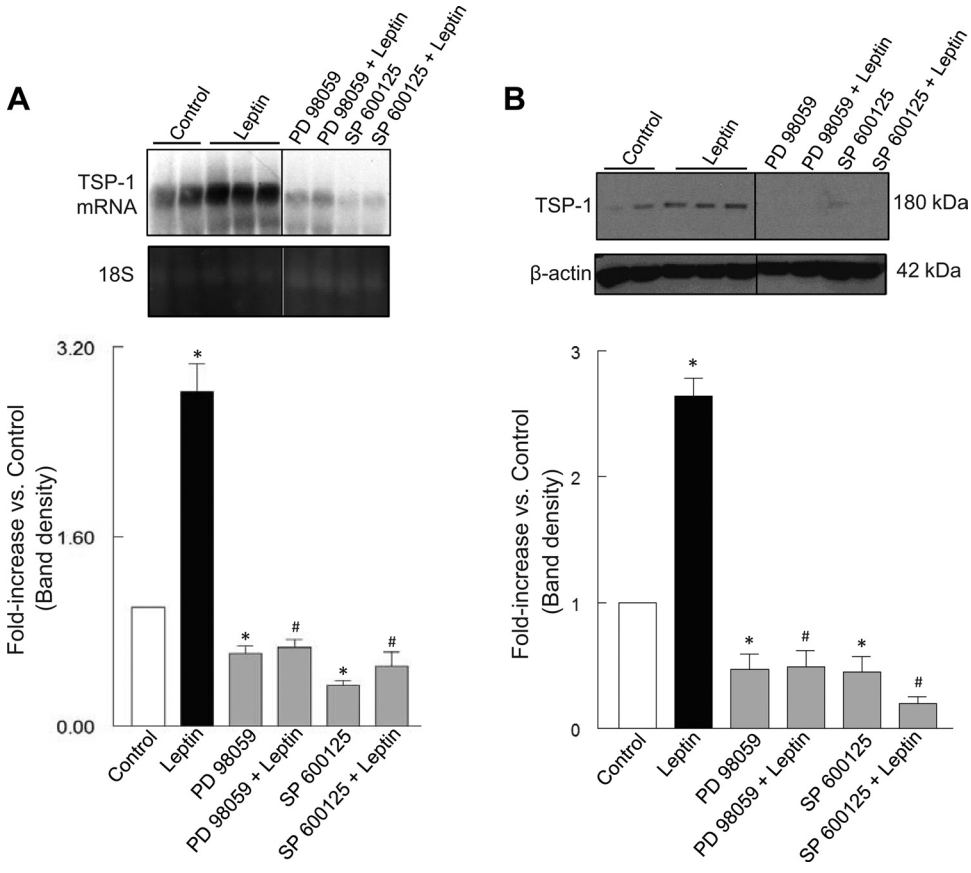


Fig. 2. MAPK inhibitors attenuate leptin-induced TSP-1 mRNA and protein expression in HASMC. Following a 2-h preincubation with 30 μM PD-98059 (ERK inhibitor) or SP-600125 (JNK inhibitor), HASMCs were treated with 100 ng/ml leptin for 3 h; isolated RNA and protein lysates were used for Northern (*A*) and Western blotting (*B*), respectively. Top panels: representative Northern and Western blots. Note that the lane images show mRNA or proteins detected on a representative single blot; however, they were rearranged for clarity of presentation. Bottom panels: quantification of blots from 4–5 independent experiments. Values are means ± SE. *$P < 0.05$ vs. control; #$P < 0.05$ vs. leptin.

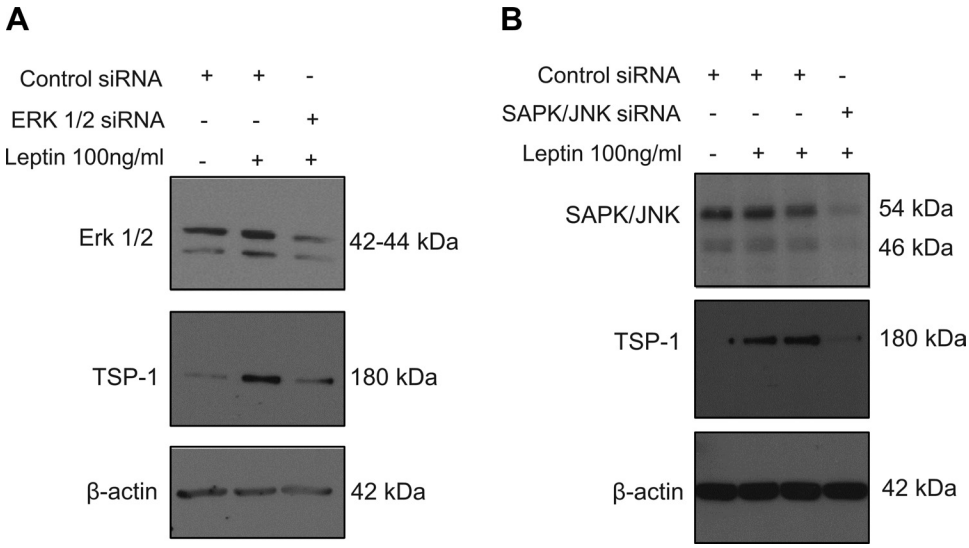


Fig. 3. siRNA-mediated gene silencing of ERK (*A*) or JNK (*B*) blocks leptin-induced TSP-1 expression in HASMC. siRNA for ERK, JNK, or control scrambled siRNA were delivered to HASMC using silentFect Lipid Reagent (Bio-Rad); transfected cells were incubated with or without 100 ng/ml leptin for 3 h. Shown are representative Western blots from 3 independent experiments. β-Actin was used as the loading control.

4*C*). Taken together, these results clearly demonstrate that leptin activates TSP-1 gene at the level of transcription in HASMCs. Additionally, we have found that specific MAPK inhibitors blocked leptin-induced THBS1 promoter activity, and this was consistent with their effects on the endogenous TSP-1 mRNA levels induced by leptin. Specifically, PD-98059 and SP-600125 significantly attenuated THBS1 promoter activity (>50%) compared with leptin-treated cells, thereby suggesting a role for MAPK-dependent mechanisms in transcriptional upregulation of TSP-1 by leptin (Fig. 4*D*).

JAK2-dependent signaling is more specific for leptin-induced upregulation of TSP-1 expression in HASMCs. Our data demonstrate that ERK and JNK regulate both the constitutive and leptin-induced expression of TSP-1 in HASMC. To further elucidate a signaling mechanism that is more specific to leptin-mediated upregulation of TSP-1, we focused on the Janus kinase 2 (JAK2)-dependent pathway, previously linked to leptin-mediated signaling. AG-490, a JAK2-specific inhibitor, significantly inhibited TSP-1 mRNA (67%, Fig. 5*A*), protein expression (51%, Fig. 5*B*), and THBS1 promoter activity (53%, Fig. 5*C*) induced by leptin in HASMC, compared with untreated cells. However, unlike ERK- and JNK-specific inhibitors, AG-490 did not have any effect on the basal level of expression of the endogenous TSP-1.

Leptin stimulates TSP-1 expression in vivo. To assess a direct effect of leptin treatment in vivo (at concentrations previously shown to promote atherosclerosis in ApoE$^{-/-}$ mice, a mouse model of hyperlipidemia, and effective in achieving weight loss and fertility in *ob/ob* mice) on TSP-1 expression in the walls of the large blood vessels, C57BL/6J mice were treated with daily intraperitoneal injections of recombinant mouse leptin (5 μg/g body wt) or saline (vehicle control) for a period of 10 days. Uninjured aorta was collected from all animals, and the expression and distribution of TSP-1 along the blood vessel wall was compared between leptin-treated and control mice. The TSP-1 expression was remarkably elevated in the vascular walls of the leptin-treated mice (Fig. 6), and the increase was localized to both the medial and adventitial layers of the vessel wall. Immunohistochemistry studies revealed a positive staining for TSP-1 in luminal EC as well as cells of the media and adventitia (Fig. 6, *A* and *B*). A significant difference in TSP-1 expression within the vascular walls of leptin-treated vs. control mice was also confirmed by Western blotting of the protein lysates from the aortic vessel tissue. While TSP-1 was abundant in the aorta of mice treated with leptin, protein expression was barely detectable in the vessel tissues of saline-treated animals (Fig. 6*D*). As reported earlier (49), an additional lower band representing proteolytically processed TSP-1 was also detected by the antibody (AB4, Thermofisher). Densitometric quantification of Western blots further confirmed the significant increase (3.7-fold) in TSP-1 levels in the large vessels of mice treated with leptin (Fig. 6*E*). To further determine whether the effects of leptin in vivo on TSP-1 expression are vascular specific, we measured TSP-1 protein expression in heart and fat pad tissue lysates obtained from saline- and leptin-treated mice. Western blotting revealed that unlike its effect in the aortic vessel wall, leptin in vivo did not have any effect on TSP-1 expression in the heart (Fig. 6*F*). Additionally, TSP-1 expression was undetectable in fat pad tissue lysates from both control and leptin-treated mice (data not shown). Taken together, these findings clearly demonstrate that exogenous leptin stimulates TSP-1 production in the intact large blood vessels of normal C57BL/6J mice.

SMC migration and proliferation induced by high leptin concentrations in HASMCs is mediated via TSP-1. Leptin has been previously reported to induce migration and proliferation of VSMC (25, 33). Consistently, increasing evidence has also demonstrated an important role for TSP-1 in activation, migration, and proliferation of aortic SMCs, in turn, triggering neointimal formation upon vascular injury (29). Therefore, we investigated a potential role of TSP-1 in modulation of VSMC function induced by leptin. Leptin (100 ng/ml) significantly increased HASMC migration (Fig. 7*A*). Concomitant with our hypothesis that leptin-induced migration is modulated by increased levels of TSP-1, incubation with an anti-TSP-1 blocking antibody (10 μg/ml) significantly inhibited leptin-induced SMC migration by 63% compared with cells treated with leptin alone. In contrast, treatment with IgG control antibody (10 μg/ml) did not have any effect on leptin-induced SMC migration (Fig. 7*A*). Quantification of scratch-

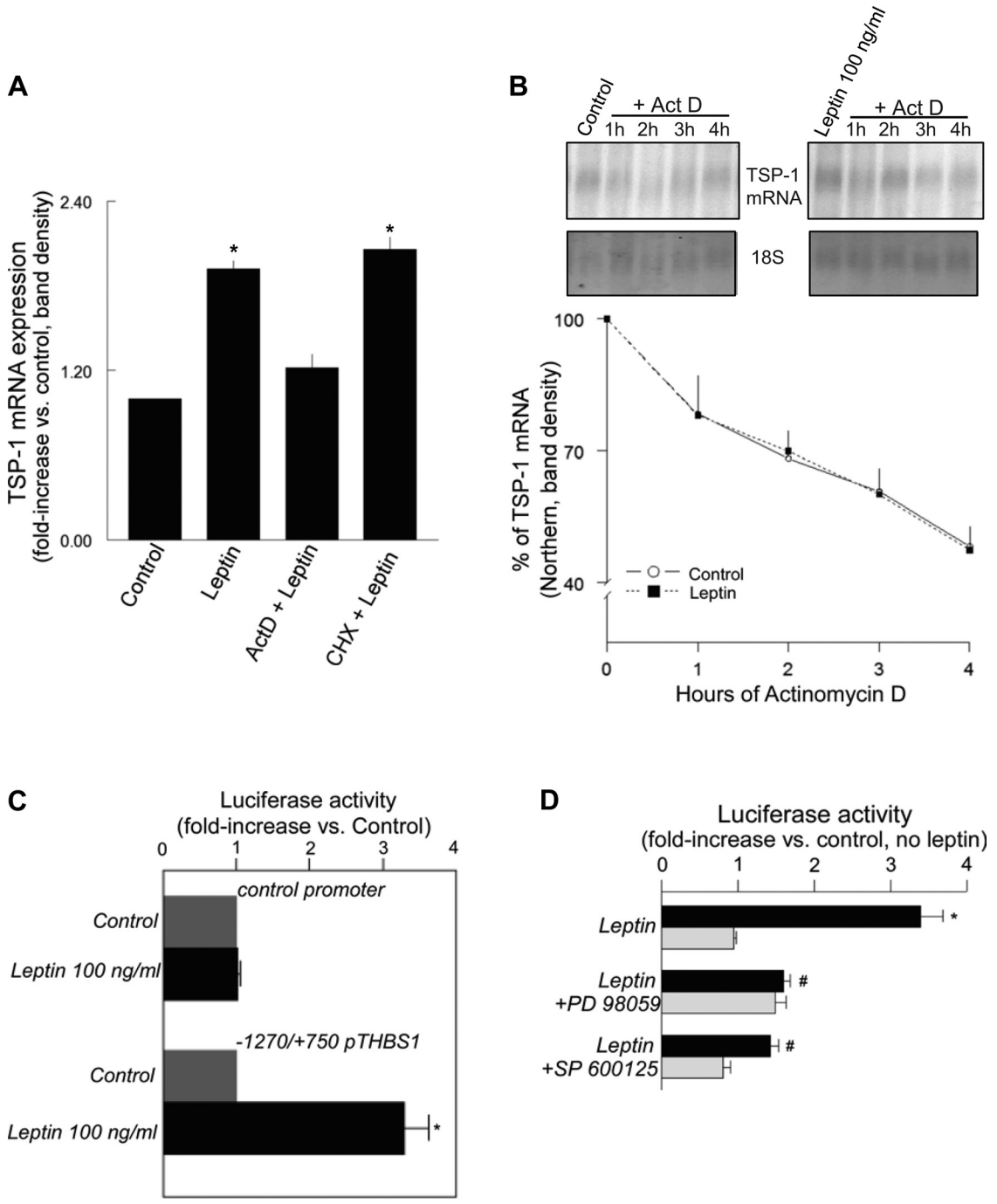


Fig. 4. Leptin activates TSP-1 gene transcription in HASMC. *A*: actinomycin D (Act D, transcriptional inhibitor), but not cycloheximide (CHX, translational inhibitor), inhibits leptin-induced increase in TSP-1 mRNA expression. Cells were preincubated for 30 min with either 5 μg/ml Act D or CHX, followed by incubation with leptin (100 ng/ml) for 3 h; total RNA was used in Northern blotting. Shown is the densitometric quantification of Northern blots. Results are means ± SE from 3 independent experiments. *$P \leq 0.05$ vs. control. *B*: leptin treatment did not increase TSP-1 mRNA stability. HASMC were incubated with or without 100 ng/ml leptin for 3 h, followed by treatment with 5 μg/ml Act D; cells were lysed at 1–4 h after initiation of Act D treatment. Top panel: representative Northern blot; bottom panel: quantification of Northern blots ($n = 4$). *C*: leptin stimulation increases the activity of −1,270/+750 pTHBS1 promoter. HASMC were transiently transfected with −1,270/+750 pTHBS1 promoter-luciferase reporter construct or pGL3 vector control. After a 6-h recovery, media were aspirated and cells were treated with or without 100 ng/ml leptin. Luciferase activity was measured 42 h later. Results are expressed as means ± SE ($n = 6$). *$P < 0.01$ vs. control (−1,270/+750 pTHBS1). *D*: MAPK inhibitors decrease leptin-induced THBS1 promoter activity. HASMC transfected with −1,270/+750 pTHBS1 promoter (dark bars) or pGL3 vector control (light bars) were incubated with or without leptin (100 ng/ml) in presence or absence of 30 μM PD-98059 or SP-600125; luciferase activity was measured 42 h later. Results are expressed as means ± SE ($n = 6$). *$P \leq 0.05$ vs. control (no leptin, THBS1 promoter); #$P \leq 0.005$ vs. leptin (THBS1 promoter).

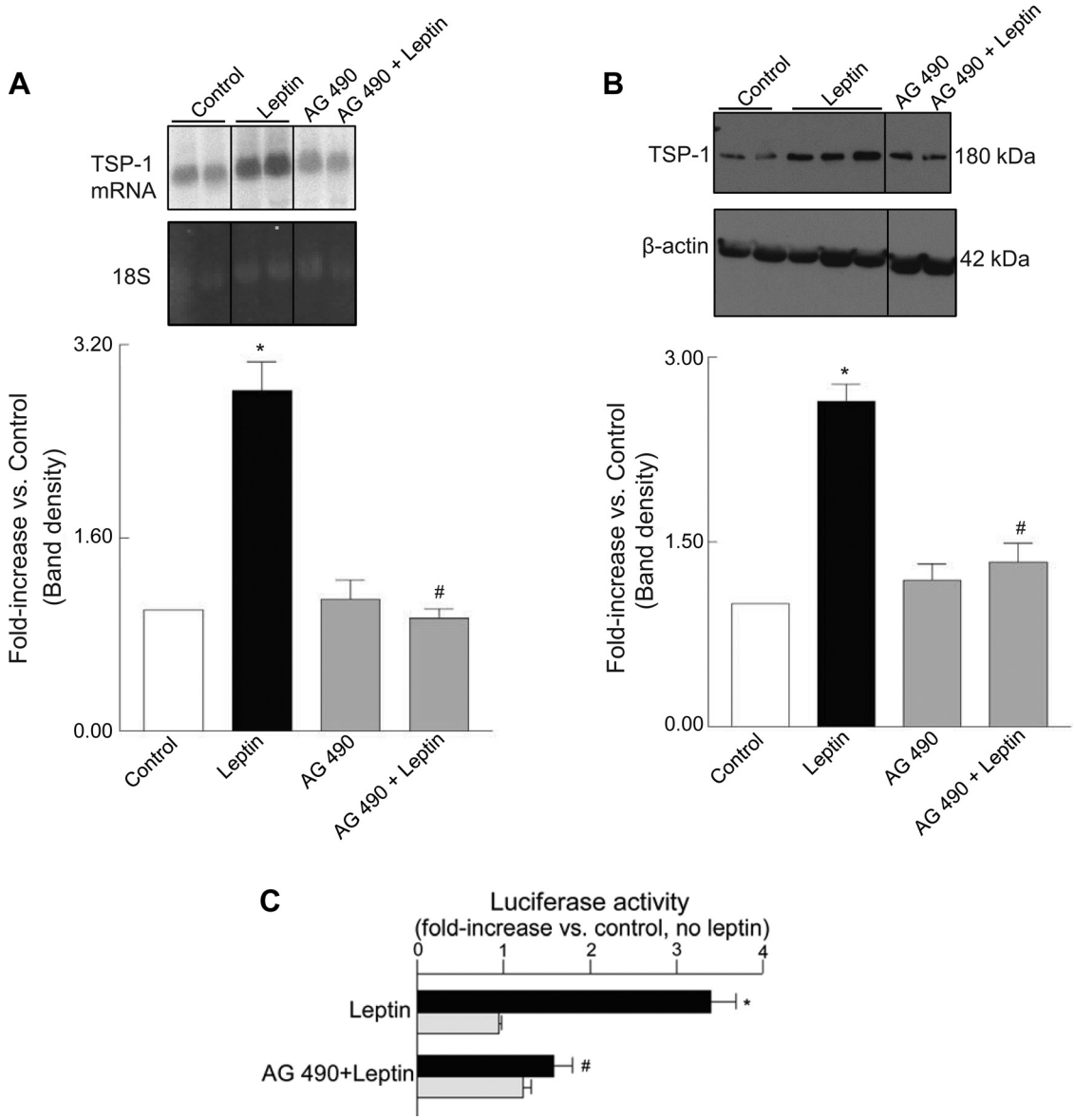


Fig. 5. JAK2-specific inhibitor attenuates leptin-induced TSP-1 expression in HASMC. HASMC were preincubated with 30 μM AG-490 (JAK2 inhibitor) for 2 h followed by treatment with or without leptin (100 ng/ml) for 3 h. *A*, top panel: representative Northern blot showing TSP-1 mRNA expression, Note that the lane images show mRNA detected on a representative single blot; however, they were rearranged for clarity of presentation. *B*, top panel: representative Western blot showing TSP-1 protein expression. Note that the lane images show proteins detected on a representative single blot; however, they were rearranged for clarity of presentation. *A* and *B*, bottom panels: quantification of densitometric scans ($n = 4–6$). Values are means ± SE. *$P \leq 0.05$ vs. control, #$P \leq 0.05$ vs. leptin. *C*: AG-490 inhibits leptin-induced increase in THBS1 promoter activity. HASMC transiently transfected with −1,270/+750 pTHBS1 (dark bars) or pGL3 vector (light bars) were incubated with or without leptin (100 ng/ml) in presence or absence of 30 μM AG 490, and luciferase activity was measured in cell lysates 42 h later. Results are expressed as means ± SE ($n = 6$). *$P \leq 0.05$ vs. control (no leptin, THBS1 promoter); #$P \leq 0.005$ vs. leptin (THBS1 promoter).

wound closures confirmed the role of TSP-1 in SMC migration induced by leptin (Fig. 7*B*). In parallel experiments using serum-starved cells, leptin (100–1,000 ng/ml) produced a dose-dependent increase in HASMC proliferation (6.6- to 9-fold). Although leptin-induced SMC proliferation was significantly attenuated by an anti-TSP-1 blocking antibody, there was a complete lack of effect on SMC proliferation stimulated by leptin in response to IgG control antibody (Fig. 7*C*). Taken together, these findings clearly demonstrate that leptin-induced migration and proliferation of HASMC is mediated, at least in part, by TSP-1. To further confirm these in vitro data, we compared the effect of leptin treatment in vivo on the expression level of a proliferation marker, proliferating cell nuclear antigen (PCNA), in the vessel walls of wild-type and TSP-$1^{-/-}$ mice. As shown by Western blotting, we found that leptin treatment in vivo significantly increased PCNA expression in the aortic vessel walls of wild-type mice (2.9-fold vs. untreated mice). Importantly, in contrast to the wild-type mice, leptin in vivo failed to produce a significant increase in PCNA expression in the vascular walls of TSP-$1^{-/-}$ mice (Fig. 7, *D* and *E*). These results support our in vitro findings

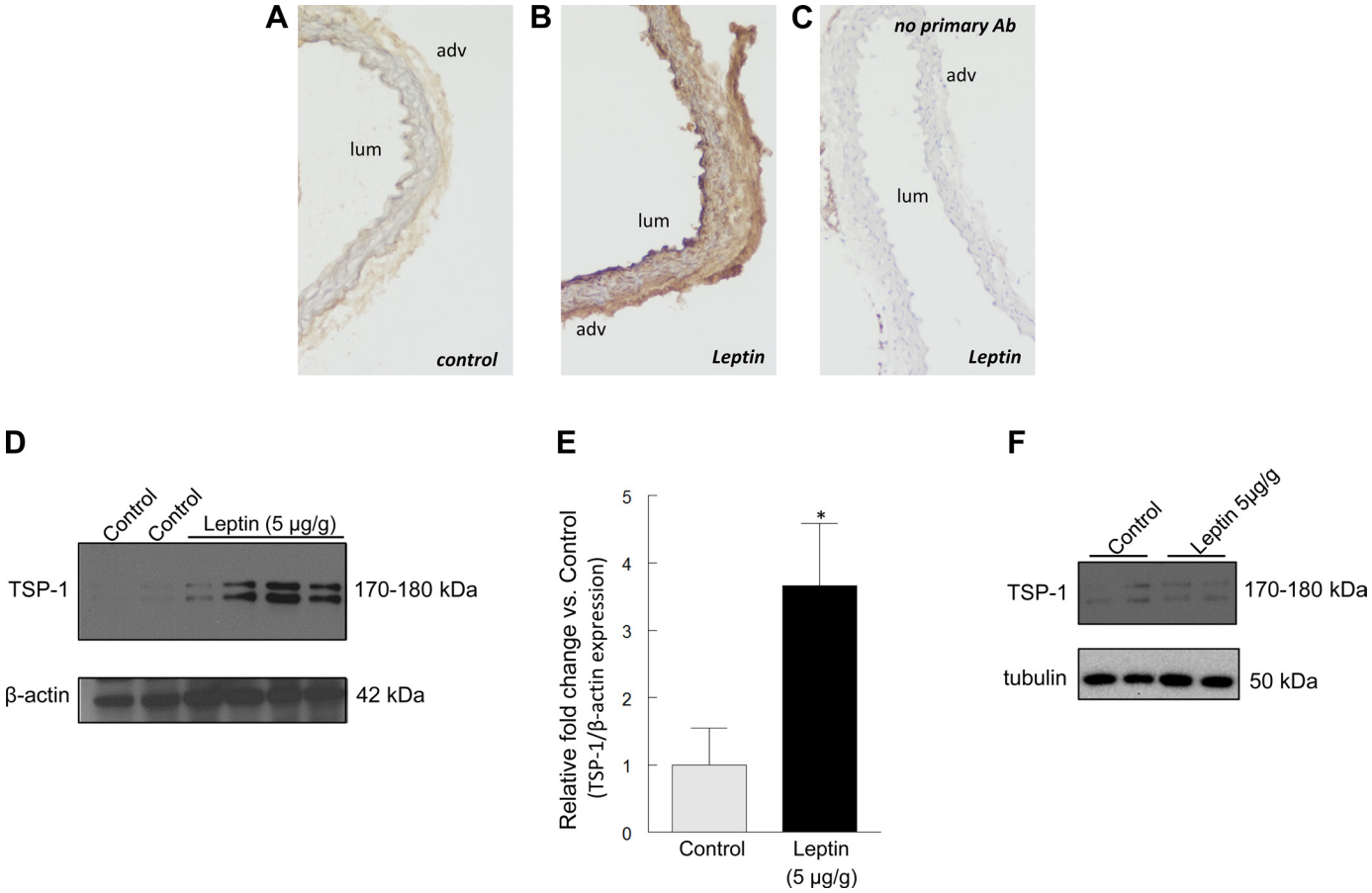


Fig. 6. Leptin in vivo increases TSP-1 expression in the aortic vessel wall. Male C57BL/6J mice were injected ip with recombinant mouse leptin (5 μg/g body wt) or saline for 10 days. The aortic vessel tissue was harvested at end point and used for immunostaining and Western blotting as described in MATERIALS AND METHODS. Shown are representative images for immunostaining of aortas derived from saline-treated (*A*) and leptin-treated (*B*) mice. *C*: representative negative control processed without the primary antibody (anti-TSP-1); magnification, ×10. Luminal (lum) and adventitial (adv) sides of vessels are marked, and hematoxylin and eosin was used for counterstaining. *D*: representative Western blots for TSP-1 (top panel) and β-actin (loading control, bottom panel); each lane represents an individual mouse. *E*: relative fold changes in TSP-1 expression normalized to β-actin. Values are means ± SE. $*P \leq 0.05$ vs. control (n = 3–5 mice). *F*: leptin in vivo did not stimulate TSP-1 expression in heart tissue. Mice were treated as described above; heart tissue was harvested at end point and used for Western blotting as described in MATERIALS AND METHODS. Shown are representative Western blots for TSP-1 (top panel) and tubulin (loading control, bottom panel); each lane represents an individual mouse (n = 3 mice).

and clearly demonstrate that leptin-induced proliferation in the aortic vessel wall is mediated, at least in part, via TSP-1.

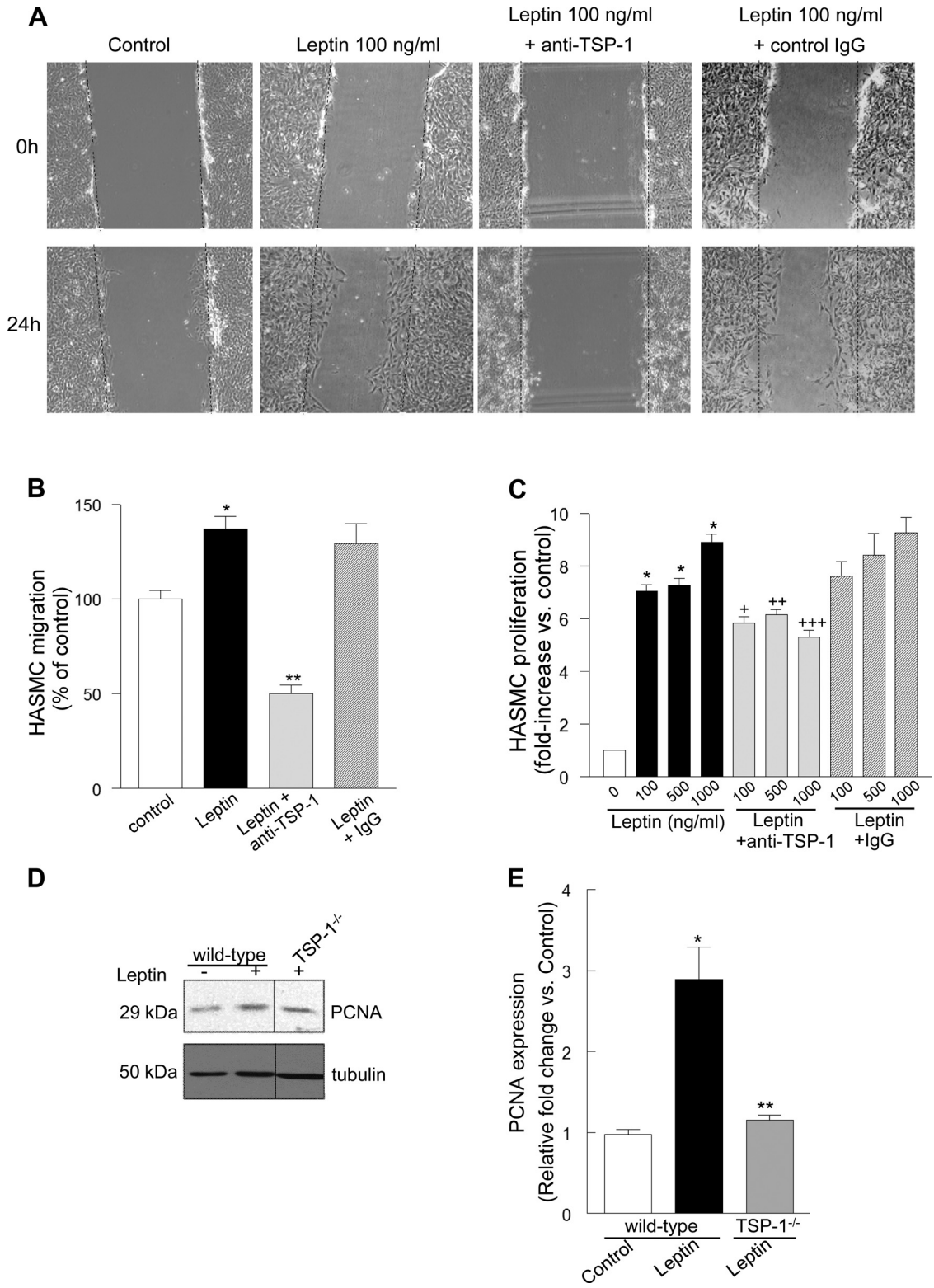
DISCUSSION

In the present study, we show for the first time that leptin, at clinically relevant concentrations measureable in obese and diabetic individuals, has a direct stimulatory effect on the matricellular protein, TSP-1, expression in HASMCs in culture. We further show that this upregulation of TSP-1 by leptin is at the transcriptional level. Overall, our results suggest TSP-1 as an important mediator of leptin-induced vascular dysfunction.

There are several lines of published evidence suggesting a causal link between hyperleptinemia and macrovascular atherosclerotic disease. An increased expression of leptin receptor has been documented in the injured neointima, media, and

Fig. 7. TSP-1 blocking antibody inhibits leptin-induced migration and proliferation in HASMC. *A*: HASMC were incubated with or without 100 ng/ml leptin in presence of 10 μg/ml anti-TSP-1 antibody or purified IgG (negative control antibody). Cell migration was determined using scratch-wound assay as described in MATERIALS AND METHODS; shown is a representative image. *B*: the distance of the scratch wound closure was measured using ImageJ software. Shown is the quantification of migration. Values are means ± SE (n = 3); $*P \leq 0.01$ vs. control; $**P \leq 0.0001$ vs. leptin. *C*: HASMC were preincubated for 4 h in serum-free low glucose (5 mM) DMEM with anti-TSP-1 or purified IgG, as in *A*. Cells were then stimulated with leptin (100–1,000 ng/ml), and cell proliferation was measured 4 days poststimulation as described in MATERIALS AND METHODS. Results are means ± SE (n = 4); $*P \leq 0.05$ vs. control; $+P < 0.02$ vs. leptin 100 ng/ml; $++P < 0.04$ vs. leptin 500 ng/ml; $+++P \leq 0.05$ vs. leptin 1,000 ng/ml. *D*: leptin in vivo failed to increase PCNA expression in aortic vessel walls of TSP-$1^{-/-}$ mice. Wild-type and TSP-$1^{-/-}$ mice were treated with recombinant mouse leptin as described in Fig. 6; the aortic vessel tissue was harvested at end point and used for Western blotting as described in MATERIALS AND METHODS. Shown are representative Western blots for PCNA (top panel) and tubulin (loading control, bottom panel); each lane represents an individual mouse. Note that the lane images show proteins detected on a representative single blot; however, they were rearranged for clarity of presentation. *E*: relative fold change in PCNA expression vs. control (untreated) mice. Values are means ± SE. $*P < 0.01$ vs. control; $**P < 0.02$ vs. wild-type + leptin (n = 3–5 mice).

atherosclerotic plaques, with enhanced expression in VSMC (5). An interaction with these receptors is typically considered as an initial step in leptin-induced atheroma formation. Plasma leptin levels are significantly elevated in both diabetic and obese individuals, and high circulating levels of leptin is recognized as an important risk factor for vascular complications, independent of metabolic and inflammatory dysregulation. In recent years, increasing evidence has assigned a critical role to the matricellular glycoprotein, TSP-1, in atherosclerosis, with an increased TSP-1 expression being reported in both diabetic patients and animal models (49). Additionally, we and others have shown that TSP-1 expression is upregulated by



high glucose in different vascular cells, including EC, VSMC, and fibroblasts (39, 49). More recently, both human and animal studies demonstrate elevated TSP-1 levels in the obese population as well (19, 40, 56). Surprisingly, there is no direct information on TSP-1 expression in vascular cells in response to leptin, and the goal of the present study was to provide some of this missing information. The present work demonstrates a time-dependent differential regulation of TSP-1 in response to high leptin concentrations, relevant to hyperleptinemia. Our findings that acute leptin exposure (3 h) increases both TSP-1 mRNA and protein expression suggest a transcriptional or posttranscriptional regulation of TSP-1 by leptin in HASMCs. On the other hand, upregulation of TSP-1 mRNA while decreasing the protein levels following long-term (24 h) leptin treatment could be attributed to protein degradation or silencing of the protein translational machinery. TSP-1, as a protein with significant proatherogenic and antiangiogenic action, is known to play a key role in early SMC activation following vascular injury. Previous studies have shown that vascular TSP-1 modulates distinct morphological features of the medial SMC, triggering migration and proliferation of SMCs with consequent neointimal and medial thickening (29). Our present work that TSP-1 expression is upregulated by leptin in HASMCs suggests that this effect may account for the atherogenic function of leptin contributing to the pathogenesis of atherosclerosis.

Leptin activates multiple signal transduction pathways, including MAPK and JAK/STAT (16, 18), but the molecular mechanisms responsible for the effects of leptin in vascular cells have been incompletely understood. It is well-established that both MAPK and JAK/STAT-mediated signaling play key roles in growth and proliferation of diverse cell systems (21). The MAPK pathway comprises three distinct highly interactive families of protein kinases, including ERK1/2, JNK, and p38. Accumulating evidence indicates that while ERK activation is a central player in VSMC proliferation, both JNK and p38 are also involved in various stress-induced cellular responses, including cell survival and apopotosis (21, 59). Zhan et al. (59) showed that all the three MAP kinases are important in regulation of VSMC migration. Previous studies have demonstrated that leptin can induce each of these MAP kinases in vascular cells, and several of the leptin-mediated responses are directly attributable to ERK, JNK, or p38. Both JNK and p38 activation have also been implicated in TSP-1 expression induced by angiotensin II and TGF-β in mesangial cells, VSMC, and pancreatic tumor cells (27, 32, 53). Additionally, McGillicuddy et al. (27) demonstrated that in VSMC, ERK1/2 signaling contributes to TSP-1 expression. We previously reported that activation of the hexosamine pathway of intracellular glucose metabolism and subsequent protein glycosylation mediate upregulation of TSP-1 expression by high glucose in VSMC (39). In the present study, we have shown that ERK or JNK inhibition significantly abrogated leptin-induced TSP-1 expression, both at the mRNA and protein levels. These observations suggest that in VSMC, both ERK and JNK signaling play dominant roles in upregulation of TSP-1 expression induced by leptin at the transcriptional or posttranscriptional levels. Our study thus demonstrates a role for MAPK in modulation of TSP-1 expression by hyperleptinemia that is distinct from the molecular mechanisms that control high glucose-induced TSP-1 expression in VSMCs.

Notably, we found that increased synthesis of TSP-1 upon acute leptin stimulation is direct and represents a transcriptional mechanism of upregulation of TSP-1 expression by leptin. The lack of an effect of a translational inhibitor, CHX, on leptin-induced TSP-1 mRNA levels suggests that de novo synthesis of proteins is not required for the leptin-mediated increase in endogenous TSP-1 mRNA levels. Further, the fact that leptin enhanced activation of THBS1 promoter coupled with its lack of effect on TSP-1 mRNA stability clearly demonstrate that upregulation of TSP-1 expression by leptin results from a transcriptional activation of the TSP-1 gene. Additionally, conditioned media from leptin (3 h)-stimulated HASMCs failed to increase TSP-1 expression, suggesting a direct effect of leptin on TSP-1 expression, but not mediated by any secreted factors (data not shown).

Our present work also reveals a critical role for JAK2 specific to leptin-mediated TSP-1 upregulation. JAK2, a component of the JAK/STAT pathway, was previously shown to be an important regulator of leptin-mediated responses in vascular cells (16, 18). In the present study, we demonstrate that while ERK- and JNK-specific inhibitors significantly decreased the constitutive expression of TSP-1, a JAK2-specific inhibitor decreased only leptin-induced TSP-1 expression with no apparent effect on the basal level of TSP-1 expression. These findings clearly indicate that in VSMC, while JAK2 is required for leptin-mediated TSP-1 upregulation, it is not sufficient for the constitutive expression of TSP-1. As a multifunctional extracellular matrix protein, regulation of TSP-1 may involve an interaction between multiple signaling cascades. Stimulation of MAPK and JAK2 pathways induces phosphorylation and activation of several transcription factors, including NF-κB, cJUN, AP-1, CREB, and STATs. Previous studies suggest that JAK2/STAT and MAPK may act as opportunistic partners, and nuclear translocation of activated STATs initiates target gene transcription upon recruitment of transcriptional factors, such as CREB, NF-κB, and AP-1, and binding of the resulting complex to specific DNA elements in the promoter of the target gene (16). We have previously reported that high glucose-induced activation of TSP-1 transcription in VSMCs is regulated cooperatively by a direct interaction between different transcription factors (38). Based on our findings in the present study, we speculate that JAK2 works upstream of the MAPK pathway, and an association between specific nuclear proteins, downstream of ERK, JNK, and JAK2 signaling, mediates synthesis of TSP-1 in response to hyperleptinemia in HASMCs.

Interestingly, in line with our in vitro findings, the present work also demonstrates that exogenous leptin, at concentrations sufficient to achieve weight loss in obese mice and adequate to promote atherosclerosis in hyperlipidemic mice, increases TSP-1 levels in the aortic vessel walls in mice, with marked accumulation of the protein in the medial layers. These observations are not only comparable to our in vitro findings but also distinctly support VSMC as an important local source of TSP-1 in response to hyperleptinemia. It is well known that multiple cell types can produce TSP-1 in response to various stimuli. Previous studies have indicated that the multidomain structure of TSP-1, capable of independent functionality, is largely responsible for its differential cell and tissue-specific effects (50). Such cell type-specific mechanisms of regulation of TSP-1 coupled with the involvement of distinct cell-surface

receptors and binding partners determine the overall fates of angiogenesis and SMC activation in a tissue. In the present work, detection of TSP-1 in the vessel wall by immunohistochemistry showing an increased expression in the luminal EC and extracellular matrix of the adventitia suggests the capacity of additional vascular cell types (EC, fibroblasts) to respond to leptin by increasing TSP-1 production. Future studies delineating the specific contribution of different vascular cell-types to increased TSP-1 synthesis induced by leptin as well as cell type-specific mechanisms that modulate TSP-1 regulation by leptin warrant investigation.

VSMC migration and proliferation are key events in the initiation and development of atherosclerosis, contributing to the increased arterial neointimal thickening. Numerous in vivo and in vitro studies have documented that leptin promotes neointimal hyperplasia and vascular remodeling by inducing VSMC migration, proliferation, hypertrophy, and calcification, thereby supporting a role for leptin in pathogenesis of vascular lesions (21, 46). TSP-1, abundantly expressed upon vascular injury, facilitates VSMC migration and proliferation, resulting in the formation of a neointima (11, 29). Our findings, that leptin upregulates TSP-1 expression in HASMC, support the notion that this effect, in turn, may trigger leptin-induced VSMC activation. Indeed, using an anti-TSP-1 antibody, previously shown to block SMC proliferation and reduce neointimal formation (11, 44), we have directly confirmed in this work that hyperleptinemia-induced migration and proliferation of SMC may be regulated, in part, by TSP-1. Furthermore, our in vivo experiments demonstrating the inability of leptin treatment to increase the expression of a proliferation marker, PCNA, in the aortic vessel walls of TSP-$1^{-/-}$ mice compared with wild-type mice lend additional support to a potential role of TSP-1, at least in part, in leptin-induced modulation of vascular function within the walls of the large blood vessel. While our results that leptin stimulates VSMC migration and proliferation are in total agreement with earlier reports (25, 33), these data are different from those of Bohlen et al. (6) who showed an inhibition of HASMC proliferation by leptin. However, while the aortic SMC used in our study were derived from healthy individuals, devoid of any arterial disease, most of the aortic SMC used by Bohlen et al. were obtained from an older, arterial diseased population. Previous studies have reported a differential effect of aging on VSMC response to injury (55), and this could be a possible explanation for the discrepancies between their work and our present findings.

Recent studies have suggested numerous counterbalancing effects of TSP-1 in vivo during early and later stages of atherosclerotic lesions. Thus, while vascular degenerative effects of TSP-1 in initial stages of plaque development may be mediated by limited EC survival, EC dysfunction, and enhanced SMC migration/proliferation, this may be counteracted at more advanced stages by the ability of TSP-1 to reduce collagen deposition and increase macrophage phagocytosis, resulting in suppression of plaque maturation and rupture (30, 50). In light of these earlier reports together with our present findings, it is reasonable to suggest that dramatically elevated TSP-1 levels in the vessel wall in response to hyperleptinemia may act as a pathophysiological trigger for initiation of lesions, contributing to accelerated atherosclerosis and restenosis in hyperleptinemia. Future studies are needed to investigate this TSP-1-dependent phenomenon during development and progression of atherosclerotic lesions in response to hyperleptinemia in an in vivo setting.

In conclusion, the present study is the first demonstration that leptin, at concentrations relevant to diabetes and obesity, has a direct regulatory effect on the matricellular protein, TSP-1, expression in HASMC, and this increase in TSP-1 production is mediated by JAK2/ERK/JNK-dependent mechanisms. Our results that leptin increases SMC migration/proliferation through stimulation of TSP-1 stress a crucial and potential role of TSP-1 in the context of important vascular functions of leptin. Overall, the present study suggests TSP-1 as a novel molecular link through which hyperleptinemia may modulate VSMC function in the large vessels, thus implicating TSP-1 as a potential therapeutic target for hyperleptinemia-induced macrovascular complications.

ACKNOWLEDGMENTS

We thank Dr. Olga I. Stenina (Cleveland Clinic) for providing the −1,270/+750 pTHBS1 promoter construct and the primary cultures of human aortic smooth muscle cells.

GRANTS

This work was supported by the American Heart Association Scientist Development Grant 0835190N (to P. Raman).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: R.J.C., R.M.H., R.H.C., R.G., R.K.A., and P.R. performed experiments; R.J.C., R.M.H., R.G., R.K.A., and P.R. analyzed data; R.J.C., R.M.H., and P.R. prepared figures; C.K.T. and P.R. interpreted results of experiments; C.K.T. and P.R. edited and revised manuscript; C.K.T. and P.R. approved final version of manuscript; P.R. conception and design of research; P.R. drafted manuscript.

REFERENCES

1. **Adams JC.** Thrombospondins: multifunctional regulators of cell interactions. *Annu Rev Cell Dev Biol* 17: 25–51, 2001.
2. **Adams JC, Lawler J.** The thrombospondins. *Int J Biochem Cell Biol* 36: 961–968, 2004.
3. **Beltowski J.** Leptin and atherosclerosis. *Atherosclerosis* 189: 47–60, 2006.
4. **Bodary PF, Gu S, Shen Y, Hasty AH, Buckler JM, Eitzman DT.** Recombinant leptin promotes atherosclerosis and thrombosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 25: e119–e122, 2005.
5. **Bodary PF, Shen Y, Ohman M, Bahrou KL, Vargas FB, Cudney SS, Wickenheiser KJ, Myers MG Jr, Eitzman DT.** Leptin regulates neointima formation after arterial injury through mechanisms independent of blood pressure and the leptin receptor/STAT3 signaling pathways involved in energy balance. *Arterioscler Thromb Vasc Biol* 27: 70–76, 2007.
6. **Bohlen F, Kratzsch J, Mueller M, Seidel B, Friedman-Einat M, Witzigmann H, Teupser D, Koerner A, Storck M, Thiery J.** Leptin inhibits cell growth of human vascular smooth muscle cells. *Vascul Pharmacol* 46: 67–71, 2007.
7. **Bornstein P.** Thrombospondins as matricellular modulators of cell function. *J Clin Invest* 107: 929–934, 2001.
8. **Bouloumie A, Marumo T, Lafontan M, Busse R.** Leptin induces oxidative stress in human endothelial cells. *FASEB J* 13: 1231–1238, 1999.
9. **Chamberlain J, Gunn J, Francis SE, Holt CM, Arnold ND, Cumberland DC, Ferguson MW, Crossman DC.** TGFbeta is active, and correlates with activators of TGFbeta, following porcine coronary angioplasty. *Cardiovasc Res* 50: 125–136, 2001.
10. **Chancellor TJ, Lee J, Thodeti CK, Lele T.** Actomyosin tension exerted on the nucleus through nesprin-1 connections influences endothelial cell

adhesion, migration, and cyclic strain-induced reorientation. *Biophys J* 99: 115–123.
11. **Chen D, Asahara T, Krasinski K, Witzenbichler B, Yang J, Magner M, Kearney M, Frazier WA, Isner JM, Andres V.** Antibody blockade of thrombospondin accelerates reendothelialization and reduces neointima formation in balloon-injured rat carotid artery. *Circulation* 100: 849–854, 1999.
12. **Chiba T, Shinozaki S, Nakazawa T, Kawakami A, Ai M, Kaneko E, Kitagawa M, Kondo K, Chait A, Shimokado K.** Leptin deficiency suppresses progression of atherosclerosis in apoE-deficient mice. *Atherosclerosis* 196: 68–75, 2008.
13. **Ciccone M, Vettor R, Pannacciulli N, Minenna A, Bellacicco M, Rizzon P, Giorgino R, De Pergola G.** Plasma leptin is independently associated with the intima-media thickness of the common carotid artery. *Int J Obes Relat Metab Disord* 25: 805–810, 2001.
14. **Correia ML, Haynes WG.** Leptin, obesity and cardiovascular disease. *Curr Opin Nephrol Hypertens* 13: 215–223, 2004.
15. **Dabir P, Marinic TE, Krukovets I, Stenina OI.** Aryl hydrocarbon receptor is activated by glucose and regulates the thrombospondin-1 gene promoter in endothelial cells. *Circ Res* 102: 1558–1565, 2008.
16. **Fruhbeck G.** Intracellular signalling pathways activated by leptin. *Biochem J* 393: 7–20, 2006.
17. **Gonzalez RR, Cherfils S, Escobar M, Yoo JH, Carino C, Styer AK, Sullivan BT, Sakamoto H, Olawaiye A, Serikawa T, Lynch MP, Rueda BR.** Leptin signaling promotes the growth of mammary tumors and increases the expression of vascular endothelial growth factor (VEGF) and its receptor type two (VEGF-R2). *J Biol Chem* 281: 26320–26328, 2006.
18. **Hegyi K, Fulop K, Kovacs K, Toth S, Falus A.** Leptin-induced signal transduction pathways. *Cell Biol Int* 28: 159–169, 2004.
19. **Hida K, Wada J, Zhang H, Hiragushi K, Tsuchiyama Y, Shikata K, Makino H.** Identification of genes specifically expressed in the accumulated visceral adipose tissue of OLETF rats. *J Lipid Res* 41: 1615–1622, 2000.
20. **Hoffmann J, Simon P, Zimmermann AK, Lemancyk M, Walter T, Beyer M, Hoffmeister HM, Ziemer G, Wendel HP.** Thrombospondin 1 as possible key factor in the hemocompatibility of endocoronary prostheses. *Biomaterials* 26: 5240–5250, 2005.
21. **Huang F, Xiong X, Wang H, You S, Zeng H.** Leptin-induced vascular smooth muscle cell proliferation via regulating cell cycle, activating ERK1/2 and NF-kappaB. *Acta Biochim Biophys Sin (Shanghai)* 42: 325–331.
22. **Konstantinides S, Schafer K, Koschnick S, Loskutoff DJ.** Leptin-dependent platelet aggregation and arterial thrombosis suggests a mechanism for atherothrombotic disease in obesity. *J Clin Invest* 108: 1533–1540, 2001.
23. **Lahav J.** The functions of thrombospondin and its involvement in physiology and pathophysiology. *Biochim Biophys Acta* 1182: 1–14, 1993.
24. **Leung JC, Chan LY, Tang SC, Chu KM, Lai KN.** Leptin induces TGF-beta synthesis through functional leptin receptor expressed by human peritoneal mesothelial cell. *Kidney Int* 69: 2078–2086, 2006.
25. **Li L, Mamputu JC, Wiernsperger N, Renier G.** Signaling pathways involved in human vascular smooth muscle cell proliferation and matrix metalloproteinase-2 expression induced by leptin: inhibitory effect of metformin. *Diabetes* 54: 2227–2234, 2005.
26. **Malik NM, Carter ND, Murray JF, Scaramuzzi RJ, Wilson CA, Stock MJ.** Leptin requirement for conception, implantation, and gestation in the mouse. *Endocrinology* 142: 5198–5202, 2001.
27. **McGillicuddy FC, O'Toole D, Hickey JA, Gallagher WM, Dawson KA, Keenan AK.** TGF-beta1-induced thrombospondin-1 expression through the p38 MAPK pathway is abolished by fluvastatin in human coronary artery smooth muscle cells. *Vascul Pharmacol* 44: 469–475, 2006.
28. **Miano JM, Vlasic N, Tota RR, Stemerman MB.** Smooth muscle cell immediate-early gene and growth factor activation follows vascular injury. A putative in vivo mechanism for autocrine growth. *Arterioscler Thromb* 13: 211–219, 1993.
29. **Moura R, Tjwa M, Vandervoort P, Cludts K, Hoylaerts MF.** Thrombospondin-1 activates medial smooth muscle cells and triggers neointima formation upon mouse carotid artery ligation. *Arterioscler Thromb Vasc Biol* 27: 2163–2169, 2007.
30. **Moura R, Tjwa M, Vandervoort P, Van Kerckhoven S, Holvoet P, Hoylaerts MF.** Thrombospondin-1 deficiency accelerates atherosclerotic plaque maturation in ApoE$^{-/-}$ mice. *Circ Res* 103: 1181–1189, 2008.
31. **Murphy M, Godson C, Cannon S, Kato S, Mackenzie HS, Martin F, Brady HR.** Suppression subtractive hybridization identifies high glucose levels as a stimulus for expression of connective tissue growth factor and other genes in human mesangial cells. *J Biol Chem* 274: 5830–5834, 1999.
32. **Naito T, Masaki T, Nikolic-Paterson DJ, Tanji C, Yorioka N, Kohno N.** Angiotensin II induces thrombospondin-1 production in human mesangial cells via p38 MAPK and JNK: a mechanism for activation of latent TGF-beta1. *Am J Physiol Renal Physiol* 286: F278–F287, 2004.
33. **Oda A, Taniguchi T, Yokoyama M.** Leptin stimulates rat aortic smooth muscle cell proliferation and migration. *Kobe J Med Sci* 47: 141–150, 2001.
34. **Olson BA, Day JR, Laping NJ.** Age-related expression of renal thrombospondin 1 mRNA in F344 rats: resemblance to diabetes-induced expression in obese Zucker rats. *Pharmacology* 58: 200–208, 1999.
35. **Parhami F, Tintut Y, Ballard A, Fogelman AM, Demer LL.** Leptin enhances the calcification of vascular cells: artery wall as a target of leptin. *Circ Res* 88: 954–960, 2001.
36. **Park HY, Kwon HM, Lim HJ, Hong BK, Lee JY, Park BE, Jang Y, Cho SY, Kim HS.** Potential role of leptin in angiogenesis: leptin induces endothelial cell proliferation and expression of matrix metalloproteinases in vivo and in vitro. *Exp Mol Med* 33: 95–102, 2001.
37. **Quehenberger P, Exner M, Sunder-Plassmann R, Ruzicka K, Bieglmayer C, Endler G, Muellner C, Speiser W, Wagner O.** Leptin induces endothelin-1 in endothelial cells in vitro. *Circ Res* 90: 711–718, 2002.
38. **Raman P, Harry C, Weber M, Krukovets I, Stenina OI.** A novel transcriptional mechanism of cell type-specific regulation of vascular gene expression by glucose. *Arterioscler Thromb Vasc Biol* 31: 634–642, 2011.
39. **Raman P, Krukovets I, Marinic TE, Bornstein P, Stenina OI.** Glycosylation mediates up-regulation of a potent antiangiogenic and proatherogenic protein, thrombospondin-1, by glucose in vascular smooth muscle cells. *J Biol Chem* 282: 5704–5714, 2007.
40. **Ramis JM, Franssen-van Hal NL, Kramer E, Llado I, Bouillaud F, Palou A, Keijer J.** Carboxypeptidase E and thrombospondin-1 are differently expressed in subcutaneous and visceral fat of obese subjects. *Cell Mol Life Sci* 59: 1960–1971, 2002.
41. **Raso GM, Pacilio M, Esposito E, Coppola A, Di Carlo R, Meli R.** Leptin potentiates IFN-gamma-induced expression of nitric oxide synthase and cyclo-oxygenase-2 in murine macrophage J774A.1. *Br J Pharmacol* 137: 799–804, 2002.
42. **Raugi GJ, Mullen JS, Bark DH, Okada T, Mayberg MR.** Thrombospondin deposition in rat carotid artery injury. *Am J Pathol* 137: 179–185, 1990.
43. **Riessen R, Kearney M, Lawler J, Isner JM.** Immunolocalization of thrombospondin-1 in human atherosclerotic and restenotic arteries. *Am Heart J* 135: 357–364, 1998.
44. **Roth JJ, Gahtan V, Brown JL, Gerhard C, Swami VK, Rothman VL, Tulenko TN, Tuszynski GP.** Thrombospondin-1 is elevated with both intimal hyperplasia and hypercholesterolemia. *J Surg Res* 74: 11–16, 1998.
45. **Sajid M, Hu Z, Guo H, Li H, Stouffer GA.** Vascular expression of integrin-associated protein and thrombospondin increase after mechanical injury. *J Investig Med* 49: 398–406, 2001.
46. **Schafer K, Halle M, Goeschen C, Dellas C, Pynn M, Loskutoff DJ, Konstantinides S.** Leptin promotes vascular remodeling and neointimal growth in mice. *Arterioscler Thromb Vasc Biol* 24: 112–117, 2004.
48. **Stenina OI.** Regulation of vascular genes by glucose. *Curr Pharm Des* 11: 2367–2381, 2005.
49. **Stenina OI, Krukovets I, Wang K, Zhou Z, Forudi F, Penn MS, Topol EJ, Plow EF.** Increased expression of thrombospondin-1 in vessel wall of diabetic Zucker rat. *Circulation* 107: 3209–3215, 2003.
50. **Stenina OI, Plow EF.** Counterbalancing forces: what is thrombospondin-1 doing in atherosclerotic lesions? *Circ Res* 103: 1053–1055, 2008.
51. **Sundell J, Huupponen R, Raitakari OT, Nuutila P, Knuuti J.** High serum leptin is associated with attenuated coronary vasoreactivity. *Obes Res* 11: 776–782, 2003.
53. **Takekawa M, Tatebayashi K, Itoh F, Adachi M, Imai K, Saito H.** Smad-dependent GADD45beta expression mediates delayed activation of p38 MAP kinase by TGF-beta. *EMBO J* 21: 6473–6482, 2002.
54. **Topol EJ, McCarthy J, Gabriel S, Moliterno DJ, Rogers WJ, Newby LK, Freedman M, Metivier J, Cannata R, O'Donnell CJ, Kottke-Marchant K, Murugesan G, Plow EF, Stenina O, Daley GQ.** Single nucleotide polymorphisms in multiple novel thrombospondin genes may be associated with familial premature myocardial infarction. *Circulation* 104: 2641–2644, 2001.

55. **Urano Y, Shirai K, Watanabe H, Miyashita Y, Hashiguchi S.** Vascular smooth muscle cell outgrowth, proliferation, and apoptosis in young and old rats. *Atherosclerosis* 146: 101–105, 1999.
56. **Varma V, Yao-Borengasser A, Bodles AM, Rasouli N, Phanavanh B, Nolen GT, Kern EM, Nagarajan R, Spencer HJ 3rd, Lee MJ, Fried SK, McGehee RE Jr, Peterson CA, Kern PA.** Thrombospondin-1 is an adipokine associated with obesity, adipose inflammation, and insulin resistance. *Diabetes* 57: 432–439, 2008.
57. **Yamagishi SI, Edelstein D, Du XL, Kaneda Y, Guzman M, Brownlee M.** Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A. *J Biol Chem* 276: 25096–25100, 2001.
58. **Zeadin M, Butcher M, Werstuck G, Khan M, Yee CK, Shaughnessy SG.** Effect of leptin on vascular calcification in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 29: 2069–2075, 2009.
59. **Zhan Y, Kim S, Izumi Y, Izumiya Y, Nakao T, Miyazaki H, Iwao H.** Role of JNK, p38, and ERK in platelet-derived growth factor-induced vascular proliferation, migration, and gene expression. *Arterioscler Thromb Vasc Biol* 23: 795–801, 2003.