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ER Stress in adipocytes inhibits insulin signalling, represses lipolysis and alters the secretion of adipokines without inhibiting glucose transport

Running title: ER stress in adipocytes

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ABSTRACT

The endoplasmic reticulum (ER) is the intra-cellular site, where secreted and membrane proteins are synthesized. ER stress and activation of the unfolded protein response (UPR) contribute to insulin resistance and the development of diabetes in obesity. It was shown previously in hepatocytes that the UPR activates c-jun N-terminal kinase (JNK) which phosphorylates insulin receptor substrate (IRS) proteins on serine residues thereby inhibiting insulin signal transduction. Here we describe how ER stress affects insulin signalling and the biological function of adipocytes. In addition to inhibition of IRS we found that ER stress downregulates the expression of the insulin receptor. Concomitantly, insulin-induced activation of Akt/PKB and of ERK1/2 was strongly inhibited. Ectopic expression of IRS1 or IRS2 strongly counteracted the inhibitory effect of ER stress on insulin signalling while pharmacological inhibition of JNK with SP600125 resulted only in a mild improvement. ER stress decreased the secretion of the adipokines adiponectin and leptin, but strongly increased secretion of IL-6. ER stress inhibited expression and insulin-induced phosphorylation of AS160, reduced lipolysis but did not inhibit glucose transport. Finally, supernatants collected from 3T3-L1 adipocytes undergoing ER stress improved or impaired proliferation when used to condition the culture medium of INS-1E β-cells dependent on the degree of ER stress. It appears that ER stress in adipocytes might initially lead to changes resembling early pre-diabetic stages which at least in part support the regulation of systemic energy homeostasis.

Key words: ER stress, insulin signalling, insulin resistance, adipocytes, adipokines

Introduction

The endoplasmic reticulum (ER) is a complex intra-cellular membranous network that forms the site of synthesis, processing and sorting for ER-resident, membrane-bound and secreted proteins. Deterioration of ER homeostasis can impair the regulation of blood glucose levels since insulin secretion from pancreatic β-cells [1] and insulin action both depend on the proper functioning of the ER. Importantly, it was shown that obesity which is associated with the pathogenesis of type 2 diabetes impairs proper function of the ER (ER stress) in fat and liver cells causing insulin resistance in hepatocytes [2]. ER stress occurs when unfolded proteins accumulating in the lumen of the ER activate a cellular emergency program to re-establish homeostasis called unfolded protein response (UPR). The presence of unfolded proteins in the ER is sensed and activation of the UPR is regulated via the ER chaperone Grp78/BiP and its interaction with the three proximal ER stress sensors inositol requiring ER-to-nucleus signal kinase (IRE1), double-stranded RNA-activated kinase (PKR)-like ER kinase (PERK) and activating transcription factor (ATF) 6. The UPR triggers complex intracellular events including upregulation of chaperones (e.g. Grp78/BiP) to increase folding capacity, selective downregulation of translation, protein degradation and regulation of apoptosis. Importantly, ER stress activates c-jun N-terminal kinase (JNK, [3]) which is a known inhibitor of insulin receptor substrate (IRS) proteins [4,5], and it was found that obesity-dependent ER stress in liver cells inhibits insulin action by downregulation of IRS1 [2]. IRS proteins bind to activated insulin receptors and relay the insulin signal to downstream pathways such as the phosphatidyl inositol-3 kinase (PI3K)/protein kinase B (Akt/PKB) and mitogen-activated protein kinase (MAPK p44/42 or ERK1/2) modules [6]. Activation of Akt/PKB increases glucose transport in muscle and fat tissue via phosphorylation/inhibition of AS160, a Rab GTPase activating protein that represses Glut4 translocation when in its non-phosphorylated form [7-9].

Factors synthesized and secreted by adipose cells are collectively called adipokines. They control systemic glucose and lipid metabolism, food intake, vascular homeostasis and immune response [10-12]. Among adipocyte secretory proteins and peptides are proinflammatory cytokines like IL-6 [13]. Chronically elevated levels of IL-6 are associated with the development of type 2 diabetes [14-16] and it has been found to inhibit insulin secretion from pancreatic islets [17] as well as insulin action in the liver [18-20]. Two other important products secreted into circulation by adipocytes are adiponectin and leptin [21,22] that either negatively or positively correlate with body fat mass, respectively.

The importance of ER homeostasis for systemic metabolic regulation is well established [23-27] but only few data are available on the metabolic consequences of ER stress and the UPR in adipocytes [28]. However, the molecular consequences of ER stress in adipocytes relating to insulin signal transduction and insulin action have not been described yet. Therefore, the aim of our study was to elucidate the effects of ER stress on insulin signalling and function in 3T3-L1 and primary mouse

adipocytes. We studied expression and activation of components of the insulin signalling pathway, lipolysis, insulin-induced 2-Deoxy-D-[1-^{14}C] glucose uptake, glucose incorporation, and the secretion of selected adipokines after chemical induction of ER stress or under homeostasis. We also used conditioned medium to assess if and how changes in secretion might affect proliferation of INS-1E β-cells. Our findings indicate that ER stress in adipocytes can strongly inhibit insulin signalling and disturb the secretory function. However, not all consequences of ER stress appear to negatively affect metabolic regulation as demonstrated by unaffected glucose transport, repression of lipolysis and the promotion of β-cell proliferation.

Experimental Procedures

Cell Culture

Human Embryonic Kidney (HEK) 293 cells (gift from the research laboratory for Calcium Metabolism, Klinik Balgrist, Zurich, Switzerland) and 3T3-L1 fibroblasts (purchased from ATCC, Manassas, USA) were cultured in DMEM (4.5 g/L D-glucose) medium (Invitrogen) containing 10% (v/v) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin. CHO-IR cells (obtained from J. E. Pessin, University of Lowa, USA) were maintained in F12 medium (Invitrogen) containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin. INS-1E cells [29] were obtained from Pierre Maechler and Claes Wollheim (University of Geneva, Switzerland) and cultured in RPMI 1640 medium containing 11 mmol/l D-glucose, 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamax, 1 mM sodium pyruvate, 10 mM HEPES and 50 μM β-mercaptoethanol (Invitrogen, Carlsbad, California, USA). Cells were incubated at 37 °C, under 5% CO_2 in a humidified atmosphere.

Differentiation of 3T3-L1 fibroblasts into adipocytes and ectopic expression

Fibroblasts were seeded on gelatin-coated plates and cultured in growth medium. Upon reaching confluence, differentiation was induced by incubating cells in differentiation medium (growth medium containing 0.5 mM isomethylbutylxanthine, 1 μM dexamethasone and 1.7 μM insulin) for 3 days. Differentiation medium was replaced with growth medium, supplemented with 240 nM insulin for an additional 3 days. Cells were kept in DMEM (4.5 g/L D-glucose) containing 2% FCS for 5-7 days followed by DMEM (1.0 g/L D-glucose), 2% FCS for 5 days prior to the start of an experiment.

Ectopic expression - Adenoviral constructs for ectopic expression of IRS1, IRS2 or GFP (as a control) have been previously described [30]. In brief, viral particles were purified from HEK293 cells 3-5 days post infection. Detached cells were harvested, lysed and infectious particles were purified by a centrifugation-based method. Ectopic expression was monitored with an antibody (4E10) against the myc tag.

Cell lysis and protein determination

Cells were washed three times in ice cold PBS and the last rinse was aspirated completely. Afterwards, lysis buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM PMSF, 0.5% Triton X-100, 10 mM NaF, 1 mM $Na_2H_2P_2O_7$, 1 mM Na_2O_4V, 3 μg/ml aprotinin, 3 μg/ml leupeptin) was added and cells were detached with a scraper. Lysates were transferred to Eppendorf tubes and incubated at 4 °C for 1 hour on a roller followed by centrifugation at 15,000 g for 20 min at 4 °C. Supernatants were transferred

into fresh tubes and protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce).

Immunoblotting

Equal amounts of protein were separated on SDS-PAGE gels (NuPAGE, Invitrogen) and transferred onto PVDF membranes. 2% non-fat milk in TBST (10 mM Tris-HCL, pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween 20) was used to block nonspecific binding of antibodies to membranes. Incubation with indicated primary and secondary antibodies was either at room temperature for 1 hour or overnight at 4 °C. Immuno-reactive proteins were visualized by the Lumi-Light Western Blotting Substrate (Roche) using the LAS-3000 imaging system (Fuji). Where appropriate, signal intensities of the respective immunoblots were quantified using the AIDA software package (Raytest). Equal loading and transfer was confirmed with an antibody against actin (MAB1501, Chemicon International).

Induction of ER stress and assessment of insulin signalling

To induce ER stress cells were washed and supplemented with fresh medium containing appropriate concentrations of either tunicamycin (Applichem) or thapsigargin (Calbiochem), respectively. Incubation time and concentrations used are specified for each experiment within the text or in figure legends. Tunicamycin was dissolved at 20 mg/ml in DMSO and thapsigargin at 5 mg/ml in ethanol. Induction of ER stress was monitored by immunoblotting using a polyclonal antibody raised against Grp78 (H129 from Santa Cruz). Insulin stimulation: Cells were starved (in culture medium containing 0.5% BSA but no FCS) for two hours, stimulated with insulin (Sigma), washed on ice, lysed and protein concentration was determined. Where required, tunicamycin or thapsigargin were included in the starvation medium and during stimulation. Expression and activation of signalling components was determined by immunoblotting. Antibodies used to detect expression and activation of signalling components: IR, C-19 (Santa Cruz) and anti-phosphotyrosine (mouse monoclonal IgG2bk, Upstate Biotechnology). IRS1, C-20 (Santa Cruz). IRS2, 06-605 (Upstate Biotechnology). Akt/PKB, monoclonal anti-Akt/PKB (BD transduction Laboratories) and anti-phospho-Akt (Ser 473, Cell Signalling). ERK1/2 (p44/42), anti-p44/42 Map Kinase (#9102) and anti-phospho-p44/p42 Map Kinase (Thr202/Tyr204, #9101S) both from Cell Signalling. Anti-phospho-c-Jun (Ser73, Cell Signalling). AS160, anti-AS160 (#07-741) and anti-phospho-AS160 (#07-802) both from Upstate. Anti-Glut4 and anti-Glut1 were kindly provided by Amira Klip (The Hospital for Sick Children, Toronto, Ontario, Canada). Inhibitors: JNK, SP600125 (ALX-270-339, Alexis) dissolved in DMSO. PI3K, wortmannin (Sigma) dissolved in DMSO.

2-Deoxy-D-[1-^{14}C] glucose uptake and D-[U-^{14}C] glucose incorporation

3T3-L1 fibroblasts were seeded on 24 well plates, differentiated as described and, if required, transfected with adenoviral constructs. Cells were starved in 3T3-L1 starvation medium (DMEM 1 g/L glucose and 0.5% BSA) for 2 hours. Uptake was initiated by the addition of 100 nCi/well of 2-Deoxy-D-[1-^{14}C] glucose (GE Healthcare) along with the indicated amounts of insulin. Following a 30 minute incubation period, reactions were stopped by replacing the medium with ice-cold PBS. The cells were immediately washed three times in ice cold PBS and lysed as described. Lysates were analyzed for the expression of transgenes by SDS-PAGE and Western blotting. For the determination of glucose uptake, the radioactivity in lysates was measured by liquid scintillation counting (Kontron Betamatic V).

Isolations of white adipocytes from 8-10 weeks old C57BL6 mice and glucose incorporation experiments were essentially performed as described previously [31,32]. Pooled adipocytes from 6-9 mice were incubated in Kreb's Ringer buffer containing 1% BSA in the presence of DMSO (control) or tunicamycin (to induce ER stress) for 8 hours. Wortmannin was added 30 min prior to insulin and D-[U-^{14}C]glucose. The radioactivity in lysates was measured by liquid scintillation counting (Kontron Betamatic V).

Lipolysis

Primary white adipocytes were isolated as described above and cultured in the presence of Tu (10 μg/ml) for eight hours. Lipolysis was assessed by measuring the concentration of glycerol in culture media for 1 hour, essentially as described previously [33]. Insulin (100 nM) or isoproterenol (1 μM) were added to the Kreb's Ringer buffer as indicated.

Conditioned medium and proliferation of INS-1E cells

Differentiated 3T3-L1 adipocytes were incubated in the presence or absence of Tu or Thap for 18 hours. After three washes with PBS cells were incubated in Kreb's Ringer buffer without substance but containing 1% BSA for two hours. Conditioned medium was prepared by diluting the Kreb's Ringer buffer into RPMI (2:3). Final concentrations of FCS and D-glucose were adjusted to 0.1% and 6 mM, respectively. INS-1E cells were starved in serum-free RPMI for 4 hours followed by incubation in conditioned medium for 18 hours. [^{3}H]-thymidine was added and incorporation was allowed to proceed for 4 hours. Finally, cells were washed in ice cold PBS and lysed in trichloric acid (TCA). Incorporated radioactivity was measured by liquid scintillation counting (Kontron Betamatic V).

Adipokines

3T3-L1 adipocytes were cultured and treated as described in the legend to Fig. 6. After each experiment, cells were lysed and the induction of ER stress was confirmed by Western blotting. Adiponectin concentrations in supernatants were assessed using the Mouse Adiponectin ELISA Kit from AdipoGen (Korea). Concentrations of Leptin and IL-6 were determined using multiplex panels from Linco Research (Labodia, Switzerland).

Statistical Analysis

Data are expressed as mean ± SEM unless indicated otherwise and were analyzed by unpaired two-sided Student's t-test or by ANOVA, where appropriate. $p < 0.05$ was considered significant.

Results

Induction of ER stress in 3T3-L1 adipocytes

The optimal conditions for the induction of ER stress in 3T3-L1 adipocytes were determined. Cells were cultured in the presence or absence of tunicamycin (Tu, 2-10 μg/ml, inhibits glycosylation) or thapsigargin (Thap, 300-1000 nM, disturbs Ca^{2+} homeostasis) for different time periods and the expression of the commonly used ER stress marker Grp78/BiP was assessed by Western blotting to monitor the induction of ER stress. Both substances induced ER stress in a concentration- and time-dependent manner with thapsigargin being more potent and faster acting as compared to tunicamycin. A representative time course experiment is shown in Fig. 1A. Cells incubated in the presence of 1 μM thapsigargin or 10 μg/ml tunicamycin displayed increased expression of Grp78/BiP already after 4 or 6 hours, respectively. Longer incubations at reduced concentrations (2 μg/ml tunicamycin or 300-600 nM thapsigargin) were also effective (not shown here but see Fig. 2C, 3 and 4B/C)).

Fig. 1B shows that c-jun which is a target of JNK was markedly upregulated in 3T3-L1 adipocytes after 6 hours culture in the presence of 10 μg/ml tunicamycin. Inclusion of the specific JNK inhibitor SP600125 prevented tunicamycin-dependent phosphorylation of c-Jun.

ER stress impairs insulin signalling in 3T3-L1 adipocytes

To determine if ER stress in 3T3-L1 adipocytes affects insulin signalling we assessed expression and activation of IR, IRS1, Akt/PKB and of ERK1/2. After 18 hours both Thap (Fig. 2A and 3B) and Tu (Fig. 3A) strongly reduced the expression and insulin-dependent phosporylation (Fig. 2A) of the IR. Only mild reduction in expression (Fig. 2B and 3A) but significantly reduced insulin-dependent tyrosine phosphorylation (Fig. 2B) of IRS1 was observed. Also, insulin-dependent phosphorylation/activation of Akt/PKB and ERK1/2 was strongly inhibited (Fig. 2C). Shorter incubations for 6 hours did not significantly reduce expression of the receptor but clearly reduced the activation of Akt/PKB and ERK1/2 (not shown). In all experiments expression of the ER stress marker Grp78/BiP was determined to control for induction of ER stress.

Constitutive ectopic expression of IRS1 or IRS2 and inhibition of JNK counteract ER stress-induced insulin resistance

Next we tested if overexpression of IRS1 or IRS2 under ER stress could rescue insulin-induced activation of Akt/PKB and ERK1/2. 3T3-L1 adipocytes overexpressing IRS1, IRS2 or GFP were incubated in the presence of tunicamycin or thapsigargin for 18 hours and stimulated with insulin for 5

or 20 min. Insulin-induced activation of Akt/PKB and of ERK1/2 under ER stress was strongly reduced in cells overexpressing GFP while activation of Akt/PKB was restored in cells overexpressing IRS1 or IRS2 (Fig. 3A). Activation of ERK1/2 was also improved significantly by overexpression of IRS1 or IRS2 but not to the same extend as Akt/PKB. In contrast, both IRS1 and IRS2 could not significantly improve expression and activation of the IR, although in some of our experiments a mild increase was observed (Fig. 3A, results for IRS2). Similar results were obtained with CHO-IR cells (not shown). In contrast to overexpression of insulin receptor substrates, inhibition of JNK by SP600125 could only partially counteract Tu- or Thap-induced downregulation of Akt/PKB after five min of insulin stimulation (Fig. 3B, results for Tu not shown). Inclusion of SP600125 did not affect expression of the IR.

ER stress in adipocytes does not inhibit insulin-induced glucose transport

Expression and insulin-dependent phosphorylation of AS160 was assessed by Western blotting and glucose transport by measuring the intra-cellular accumulation of 2-Deoxy-D-[1-^{14}C] glucose in 3T3-L1 adipocytes under ER stress or homeostasis. In several series of independent experiments neither Tu nor Thap did significantly impair insulin-dependent glucose transport. Two representative series of experiments are summarized in Fig. 4A. A series of experiments with increasing concentrations (1, 10 and 100 nM) of insulin was also performed but neither Thap nor Tu inhibited glucose transport at any of the insulin concentrations used. However, the PI3K inhibitor wortmannin consistently reduced uptake by 40-50%. A series of four independent experiments is summarized in Fig. 4B. After the uptake experiment cells were lysed and phosphorylation of Akt/PKB was analysed by Western blotting. While both, wortmannin and Tu, strongly reduced insulin-dependent activation of Akt/PKB, only wortmannin, but not Tu, inhibited glucose uptake. Insulin-induced phosphorylation of AS160 was strongly reduced but not abolished by Tu (Fig. 4C). Total expression levels of AS160 were slightly lower after incubation in the presence of Tu, however, expression levels of Glut1 and Glut4 were slightly increased under ER stress (Fig. 4C). Incubation of 3T3-L1 adipocytes in the presence of the PI3K inhibitor wortmannin resulted in strong inhibition of both, insulin-induced activation of Akt/PKB and uptake of 2-Deoxy-D-[1-^{14}C] glucose (Fig. 4B).

ER stress downregulates lipolysis and does not inhibit glucose incorporation in primary adipocytes

Insulin-induced glucose incorporation into white adipocytes isolated from epididymal fat pads of mice was measured. Fig. 5A shows that insulin-dependent glucose incorporation was not changed after eight hours of incubation in the presence of tunicamycin. Pre incubation for 30 min in the presence of wortmannin resulted in reduced glucose incorporation.

To assess lipolysis, the concentration of glycerol was determined in culture media of primary adipocytes under homeostasis or ER stress. Isoproterenol (10 μM) was used as a positive control to induce lipolysis. Isolated primary adipocytes from mice were incubated in the presence of Tu (10 μg/ml) for eight hours to induce ER stress. Tu and insulin both significantly reduced the accumulation of glycerol to around 80% ($p < 0.05$, Fig. 5B). Tu also significantly inhibited isoproteronol-induced accumulation of glycerol.

ER stress changes the secretion of adipokines from 3T3-L1 adipocytes

To assess if and how ER stress affects the secretion of adipokines from 3T3-L1 adipocytes the accumulation of adiponectin, leptin and IL-6 was measured in supernatants. Fig. 6A summarises the results of seven experiments in which we measured by ELISA adiponectin after incubation in the presence of tunicamycin. ER stress significantly lowered (to 48.9 ± 7%, $p < 0.05$) the accumulation of adiponectin compared to controls. Leptin and IL-6 were assessed in supernatants of cultured cells by multiplex analysis after short-term (5 hours) or long-term (20 hours) induction of ER stress with either tunicamycin or thapsigargin. Results are shown in Fig. 6B. Tunicamycin and thapsigargin both increased significantly the accumulation of IL-6 after 20 hours (1.91 ± 0.26 fold $p < 0.05$ and 40.7 ± 14.5 fold $p < 0.05$, respectively). After 5 hours, however, no significant increase was found for tunicamycin while thapsigargin did significantly upregulate the secretion of IL-6 (1.47 ± 0.26 fold n.s. and 3.7 ± 1.26 fold $p < 0.05$, respectively). Leptin accumulation was strongly decreased in the presence of tunicamycin after 20 hours (to 30 ± 4% $p < 0.05$) and thapsigargin after 5 hours (to 76 ± 12% n.s.) and 20 hours (to 31 ± 16% $p < 0.05$), respectively, while incubation with tunicamycin for only 5 hours did not significantly change leptin secretion (1.36 ± 0.22 fold n.s.).

Conditioned medium derived from 3T3-L1 adipocytes under ER stress promotes or represses proliferation of INS-1E β-cells

We assessed proliferation of INS-1E β-cells after conditioning their culture medium with supernatants collected from 3T3-L1 adipocytes under homeostasis or ER stress. 3T3-L1 adipocytes were incubated in the presence of increasing concentrations of Tu (0.2, 2 and 10 μg/ml) or Thap (6, 60 and 600 nM) for 18 hours to induce ER stress. After thorough washing to prevent carry over of Tu/Thap, cells were incubated in Kreb's ringer buffer for 2 hours. INS-1E β-cells were cultured over night in medium conditioned with these supernatants followed by incorporation of [^{3}H]-thymidine for 4 hours. Supernatants from adipocytes under homeostasis stimulated proliferation of INS-1E β-cells (Fig. 7A). Conditioned medium derived from adipocytes cultured in the presence of 0.2 μg/ml of Tu further increased (8 ± 2.6%, $p < 0.05$) the incorporation of [^{3}H]-thymidine into INS-1E β-cells compared to controls incubated with medium from adipocytes under homeostasis. However, conditioned medium

from adipocytes incubated in the presence of 2 μg/ml or 10 μg/ml of Tu inhibited (by 12 ± 8% and 32 ± 15%, respectively, $p < 0.05$) the incorporation of [^{3}H]-thymidine into INS-1E β-cells (Fig. 7B). Similar results were obtained when Thap was used instead of Tu (Fig. 7C). Conditioned medium from cells exposed to 6 or 60 nM Thap enhanced the incorporation of [^{3}H]-thymidine into INS-1E β-cells by 3 ± 1% and 12 ± 7% ($p < 0.05$), respectively. Conditioned medium derived from adipocytes incubated in the presence of 600 nM Thap inhibited the incorporation of radioactivity into INS-1E β-cells by 92 ± 1% ($p < 0.05$).

Discussion

Repression of insulin signalling by ER stress was described previously in hepatocytes [2] and it was mainly attributed to activation of JNK leading to serine phosphorylation and downregulation of IRS1. Our findings suggest cell-specific differences in the effects of ER stress. Although ER stress represses insulin signalling also in adipocytes, the underlying molecular mechanisms seem to be different. ER stress only mildly repressed expression levels of IRS1 but in the long term (18 hours) strongly reduced expression of the IR. This reduction in the expression of the IR is well in line with repression of translation downstream of the UPR. However, this effect was not described in hepatocytes.

Ectopic expression of either IRS1 or IRS2 potently counteracted ER stress-induced repression of insulin signalling in 3T3-L1 adipocytes. This observation was surprising in light of the pronounced reduction of IR and also of IGFR (not shown) expression levels. However, remaining expression might be sufficient to initiate insulin signalling. On the other hand, ectopic expression of receptor substrates might compensate for loss of receptor expression. As shown previously [30,34] both IRS1 and IRS2 can efficiently activate insulin signalling independent of insulin. JNK has been implicated in negative regulation of insulin signalling [4,5] and was shown to cause insulin resistance downstream of the UPR in liver cells [2]. Our results suggest a minor role for JNK in the induction of ER stress-dependent insulin resistance in adipocytes since inhibition of JNK could only partially restore insulin signalling under ER stress.

Despite strongly inhibiting insulin signalling, ER stress did not repress insulin-induced uptake of 2-Deoxy-D-[1-^{14}C] glucose. This surprising finding is at odds with the observed strong impairment of insulin-induced activation of Akt/PKB under ER stress. Glucose uptake to a large extend depends on Akt/PKB [35] and incubation of adipocytes in the presence of the PI3K inhibitor wortmannin resulted indeed in significantly reduced insulin-induced glucose uptake. At this point, there are no data to conclusively explain why glucose uptake was not affected under ER stress despite strong inhibition of insulin signal transduction from receptor down to Akt/PKB. However, reduced expression of the repressor AS160 might contribute to the phenomenon. Moreover, others have shown previously that insulin-stimulated Glut4 translocation is not necessarily proportional to Akt/PKB phosphorylation [36] and residual activity of Akt/PKB as observed in our experiments under ER stress might allow for sufficient translocation of Glut4 to the plasma membrane. Changes in the expression of glucose transporters under ER stress have been described. Miller and co-workers [37] found around 50% lower expression of *glut4* mRNA in 3T3-L1 adipocytes after incubation with either thapsigarin or tunicamycin. However, such a small reduction in mRNA expression might not lead to significantly less Glut4 protein, which was not determined in the study. Another report even showed increased expression of *glut1* mRNA and protein levels, but no change in *glut4* expression under ER stress in L6 myocytes [38]. We find that Tu- and Thap-induced ER stress in 3T3-L1 adipocytes is not associated

with reduced but possibly even increased expression of Glut4 and Glut1. Higher levels of Glut1 might increase basal glucose uptake under ER stress as observed in our experiments. However, such small upward changes in Glut1 and Glut4 are unlikely to account for the unaffected glucose transport under ER stress. Because glucose deposition as measured in our experiments with primary mouse adipocytes is mostly due to incorporation into lipids [39] insulin-dependent lipid synthesis also appears unaffected under ER stress. This notion is in line with our finding that ER stress downregulated lipolysis and also with recent reports showing that the UPR directly regulates genes responsible for lipid homeostasis in liver [40-42]. Since primary adipocytes are not viable for longer periods of time when cultured *in vitro* it was not possible to assess glucose incorporation or lipolysis after longer incubations in the presence of Tu or Thap. Our results hence apply to primary adipocytes undergoing ER stress for several hours only.

ER stress induced remarkable changes in adipokine secretion. In all of the cases these changes were qualitatively the same for Tu and Thap. However, for IL-6 Thap was a far more potent inducer than Tu, possibly a consequence of changes in the distribution of in intra-cellular Ca^{2+}. Increased production of IL-6 appears to be at odds with downregulation of overall translation by the UPR but fits well to the previously described upregulation of TNF-α [43] linking ER stress to inflammatory processes. Reduction of adiponectin protein secretion under ER stress in our experiments is consistent with published evidence showing reduced adiponectin mRNA accumulation under hypoxia-induced ER stress [44]. Given the generally observed interdependence of cytokine production and secretion it is likely that ER stress leads to global changes in adipokine production and secretion beyond adiponectin, leptin and IL-6. As a consequence obesity-induced ER stress in adipocytes *in vivo* will most likely impact on systemic metabolic regulation. Our experiments with conditioned medium indicate, however, that the severity of ER stress might determine how other cell types are affected. We found that proliferation of INS-1E β-cells was increased after conditioning their culture medium with supernatants from adipocytes incubated at lower concentrations of Tu or Thap while the use of higher concentrations of either compound resulted in reduced proliferation and in some experiments even in cell death. Proliferation of β-cells in the face of insulin resistance is key to prevent transition to overt hyperglycemia [45,46]. In this respect, ER stress-induced changes in adipocytes, specifically the observed increase in IL-6 secretion, might help to establish the systemic compensatory state often observed in early, pre-diabetic but insulin resistant stages. Indeed, a recent report indicates that IL-6 might be required for compensatory β-cell function in mice [47]. On the other hand, prolonged and severe ER stress might change the pattern of adipokine production from beneficial to detrimental thereby accelerating β-cell loss.

In conclusion, we show that adipocytes are severely insulin resistant at the signalling level under ER stress. At the functional level, however, insulin-responsiveness with respect to glucose uptake and lipid synthesis is retained, while the secretion of adipokines is deregulated. As a consequence, obesity-

induced ER stress might allow for further increase in fat mass while contributing to the changes in humoral milieu associated with the development of the metabolic syndrome.

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Figure Legends

Figure 1 Induction of ER stress in 3T3-L1 adipocytes. A, Time course experiment showing the induction of ER stress in 3T3-L1 adipocytes by tunicamycin or thapsigargin. Cells were incubated in the presence of tunicamycin or thapsigargin as indicated, lysed and expression of Grp78/BiP was analysed by Western blotting. B, Tunicamycin induces JNK in 3T3-L1 adipocytes. Cells were incubated for 6 hours in the presence of tunicamycin (10 μg/ml) with or without the specific JNK inhibitor SP600125. Activation of JNK was assessed in lysates by Western blotting using an antibody against the phosphorylated form of c-jun. Expression of actin was assessed as a control.

Figure 2 ER stress represses insulin signalling at different levels in 3T3-L1 adipocytes. ER stress was induced for 18 hours using either tunicamycin or thapsigargin followed by starvation for 2 hours and stimulation with insulin, as indicated. Cells were lysed and insulin signalling was analysed by Western blotting. A, Expression and tyrosine phosphorylation of the IR after 5 or 10 min stimulation with insulin. B, Expression (top and bottom) and tyrosine phosphorylation (bottom) of IRS1 in 3T3-L1 adipocytes undergoing ER stress for 16 hours. Where indicated cells were stimulated with insulin for 5 min. C, Time course experiment showing insulin-dependent phosphorylation and expression of Akt/PKB and ERK1/2. Insulin was added to a final concentration of 100 nM for 5, 10, 15, 20 or 30 min. In A, B and C expression of Grp78/BiP was assessed as a control for the induction of ER stress (not shown in A and B). See supplemental Figures 1 and 2 for quantification of signal intensities.

Figure 3 A, Ectopic expression of IRS1 or IRS2 efficiently counteracts ER stress-induced repression of insulin signalling in 3T3-L1 adipocytes. 3T3-L1 adipocytes were transfected with adenoviral constructs encoding IRS1, IRS2 or GFP and cultured for 48 h. Tunicamycin was added to a final concentration of 2 μg/ml and incubation was continued for 18 h. Cells were starved for 2 h prior to the addition of insulin for 5 or 20 min. Expression and activity state of the IR, Akt/PKB and ERK1/2 were analysed in lysates by Western blotting. Phosphorylation of the IR was visualized using an antibody against phospho-tyrosine. Endogenous and ectopic expression of IRS1 and IRS2 was visualized with specific antibodies or with an antibody against the myc tag. Expression of Grp78/BiP was assessed to monitor the induction of ER stress and actin to confirm that equal amounts of protein were loaded in each lane. B, Inhibition of JNK partially restores insulin signalling under ER stress. ER stress was induced with thapsigargin (300 nM) for 18 hours. The JNK inhibitor SP600125 was used at a final concentration of 30 μM and was added 30 min prior to insulin. After starvation for 2 h and stimulation with insulin (10 nM) for 5 min cells were lysed and insulin signalling was assessed with antibodies against the IR, Akt/PKB phosphor-Akt/PKB. Phosphorylation of c-Jun was assessed to

confirm that JNK was indeed repressed in the presence of the SP600125 compound (not shown). See supplemental Figures 3 and 4 for quantification of signal intensities.

Figure 4 Analysis of glucose transport under ER stress. A, Insulin-induced glucose transport is not affected under ER stress. For the induction of ER stress 3T3-L1 adipocytes were incubated in the presence of 10 μg/ml tunicamycin (n=6) or 600 nM thapsigargin (n=5) for 20 hours. DMSO or ethanol were used as control, respectively. After starvation for 2 hours uptake of 2-Deoxy-D-[1-^{14}C] glucose was stimulated with 100 nM insulin for 30 min (Tu or Thap were included during starvation and uptake). Protein concentrations in cell lysates were determined and uptake expressed as cpm/mg protein. B, the PI3K inhibitor wortmannin but not ER stress inhibits insulin-induced glucose transport in 3T3-L1 adipocytes. ER stress was induced in adipocytes with tunicamycin (2 μg/ml) for 20 hours. Wortmannin (W) was used at a concentration of 100 nM. Uptake of 2-Deoxy-D-[1-^{14}C] glucose was stimulated with insulin as indicated for 30 min and results are presented as cpm/mg protein (n=4). Induction of ER stress and activation of Akt/PKB were assessed by immunoblotting using antibodies against Grp78/BiP and the phosphorylated form of Akt/PKB, respectively. * $p < 0.05$. C, ER stress partially inhibits insulin-induced phosphorylation of AS160 but does not downregulate expression of Glut4 or Glut1. ER stress was induced as indicated for 20 hours. Where indicated cells were stimulated with 100 nM insulin for 30 min. The occurrence of ER stress and inhibition of insulin signalling were confirmed with antibodies against Grp78/BiP and phospho-Akt/PKB, respectively. Actin was visualized as loading control. Grp78/BiP, phospho-Akt/PKB, AS160, Glut4, Glut1 and actin were detected in total lysates. phospho-AS160 was immunoprecipitated from the same lysates prior to immunodetection.

Figure 5 A, D-[^{14}C] Glucose incorporation into primary adipocytes isolated from mice. Adipocytes from 6 mice were incubated (in triplicates) for 8 hours in Kreb's Ringer buffer containing either DMSO (control) or tunicamycin (Tu, 10 μg/ml). Wortmannin (W, 100 nM) was added to cells incubated in DMSO 30 min prior to the start of incorporation. Incorporation was stimulated with 100 nM insulin for 60 min in the presence of D-[^{14}C] glucose. * $p < 0.05$. The Western blot on the right shows expression of the ER stress marker Grp78/BiP after incubation of cells for 8 hours in the presence of either Thap (600 nM) or Tu (10 μg/ml). B, Glycerol concentrations were measured in supernatants of primary adipocytes to assess lipolysis. Epididymal white adipocytes were isolated from nine mice and cultured for eight hours in the presence of DMSO (c) or 10 μg/ml Tu. Insulin (100 nM) and isoproterenol (iso, 1 μM) were included as indicated for 1 hour in Kreb's buffer. * $p < 0.05$.

Figure 6 Analysis of adipokine secretion under ER stress. A, ER stress represses the production of adiponectin from 3T3-L1 adipocytes. Cells were incubated in the presence of 2 μg/ml tunicamycin or DMSO (as a control) for 20 hours, medium was collected and the concentration of adiponectin was

determined by ELISA. Washed cells were lysed and protein content was determined. The graph represents a summary of seven independent experiments. Adiponectin secretion was significantly lower after incubation with tuncamycin (* $p < 0.05$). Induction of ER stress was confirmed by Western blotting with an antibody against Grp78/BiP (not shown). B, ER stress represses leptin but increases the secretion of IL-6 from 3T3-L1 adipocytes. Adipocytes were incubated in the presence of tunicamycin (10 μg/ml) or thapsigargin (600 nM) for 5 hours to induce ER stress. Cells were washed and incubated in Krebs buffer containing BSA and tunicamycin or thapsisgargin for 90 min. Krebs buffer was removed, cells were washed and incubated over night in normal medium containing tunicamycin or thapsigargin. Afterwards medium was collected, cells were washed and lysed for protein determination. Levels of IL-6 and leptin were determined in the Krebs buffer (black bars) and the medium from the over night incubation (white bars) using multiplex panels. Results are expressed as fold compared to levels determined from controls.

Figure 7 Analysis of proliferation of INS-1E cells. To induce ER stress 3T3-L1 adipocytes were incubated in the presence of Tu or Thap as indicated. Induction of ER stress was monitored by Western blotting (not shown). INS-1E cells were incubated for 18 hours in conditioned medium prior to the addition of [^{3}H]-thymidine. A, Medium conditioned with supernatants form 3T3-L1 adipocytes increases the incorporation of [^{3}H]-thymidine into INS-1E β-cells. Basal, conditioned and FCS-induced proliferation of INS-1E β-cells was assessed by conditioning culture medium with Kreb's Ringer, Kreb's Ringer supernatant (2 hours collection) from 3T3-L1 adipocytes and Kreb's Ringer supplemented with FCS to yield a final concentration of 10%, respectively. Basal incorporation of [3H]-thymidine was set to 100%. Four independent experiments were performed (* $p < 0.05$). B and C, Culture medium conditioned with supernatants from 3T3-L1 adipocytes under ER stress can increase or decrease the incorporation of [^{3}H]-thymidine into INS-1E β-cells. Results are presented as % of control (DMSO or EtOH for Tu or Thap-induced ER stress, respectively). n=6, * $p < 0.05$.

Supplemental Figure 1 A, quantification of expression and phosphorylation of the IR (n=4, * $p < 0.05$). Representative Western blot is shown in Fig. 2A. B, quantification of expression and phosphorylation of IRS1, as shown in Fig. 2B (n=5, * $p < 0.05$).

Supplemental Figure 2 Quantification of results described in Fig. 2C. Five independent experiments were densitometrically analysed (* $p < 0.05$)

Supplemental Figure 3 quantification of the phosphorylation of Akt/PKB and ERK1/2 under ER stress with and without overexpression of IRS1, as shown in Fig. 3A. Similar results were obtained for IRS2 (not shown). n=4, * $p < 0.05$.

Supplemental Figure 4 Effect of inhibition of JNK on phosphorylation of Akt/PKB under ER stress. Quantification of results described in Fig. 3B, n=3, * $p < 0.05$.

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Figure 1

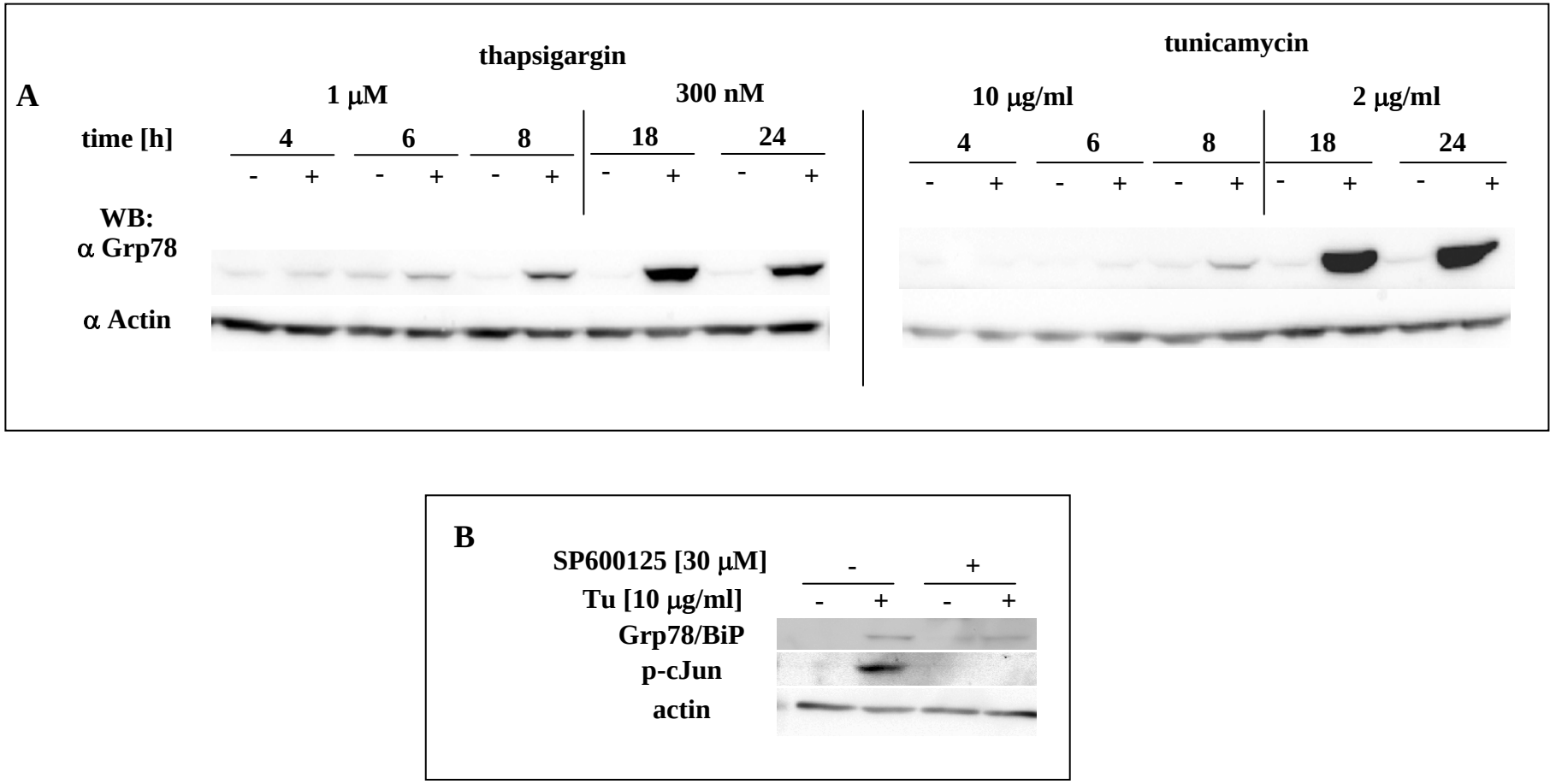

A
thapsigargin
1 μM
300 nM
tunicamycin
10 μg/ml
2 μg/ml
time [h]
4
6
8
18
24
- + - + - + - + - + - + - + - + - + - +
WB:
α Grp78
α Actin
B
SP600125 [30 μM]
Tu [10 μg/ml]
- + - +
Grp78/BiP
p-cJun
actin

Figure 2

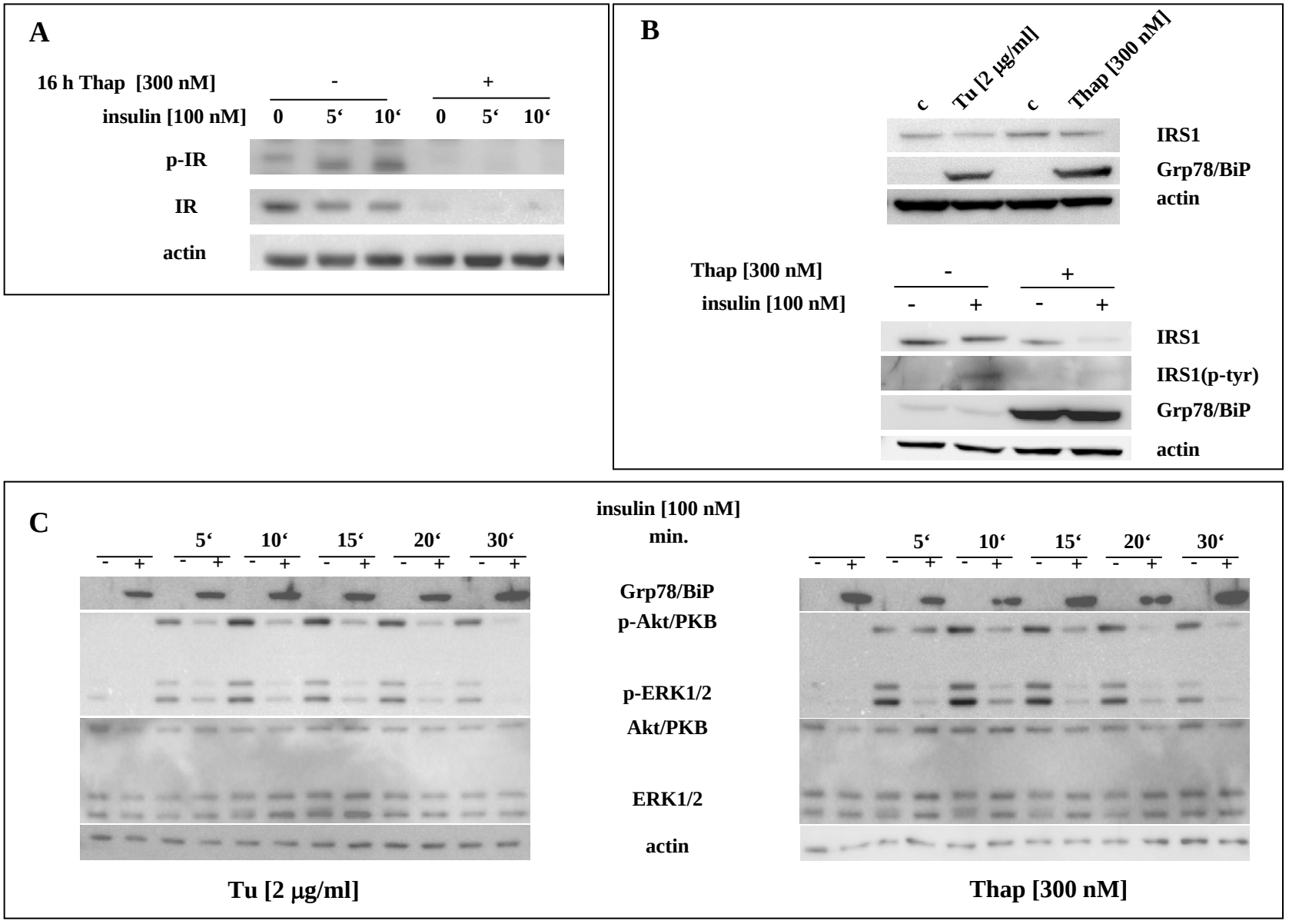
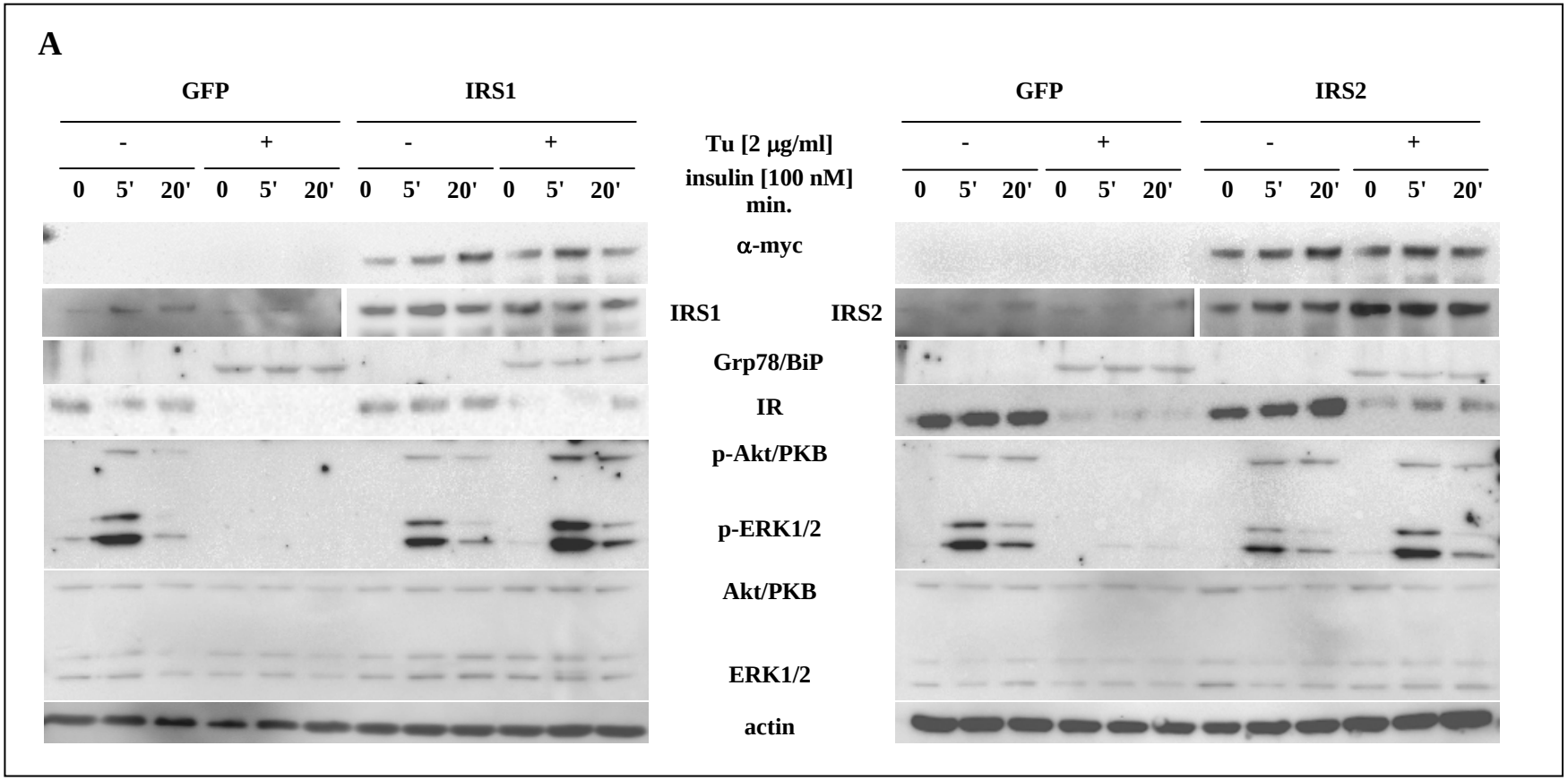

A
16 h Thap [300 nM]
-
+
insulin [100 nM]
0
5‘
10‘
0
5‘
10‘
p-IR
IR
actin
B
c
Tu [2 µg/ml]
c
Thap [300 nM]
IRS1
Grp78/BiP
actin
Thap [300 nM]
-
+
insulin [100 nM]
-
+
-
+
IRS1
IRS1(p-tyr)
Grp78/BiP
actin
C
insulin [100 nM]
min.
5‘
10‘
15‘
20‘
30‘
Grp78/BiP
p-Akt/PKB
p-ERK1/2
Akt/PKB
ERK1/2
actin
Tu [2 µg/ml]
Thap [300 nM]

Figure 3


A
GFP
IRS1
GFP
IRS2
Tu [2 μg/ml]
insulin [100 nM]
min.
0 5' 20' 0 5' 20' 0 5' 20' 0 5' 20'
α-myc
IRS1
IRS2
Grp78/BiP
IR
p-Akt/PKB
p-ERK1/2
Akt/PKB
ERK1/2
actin

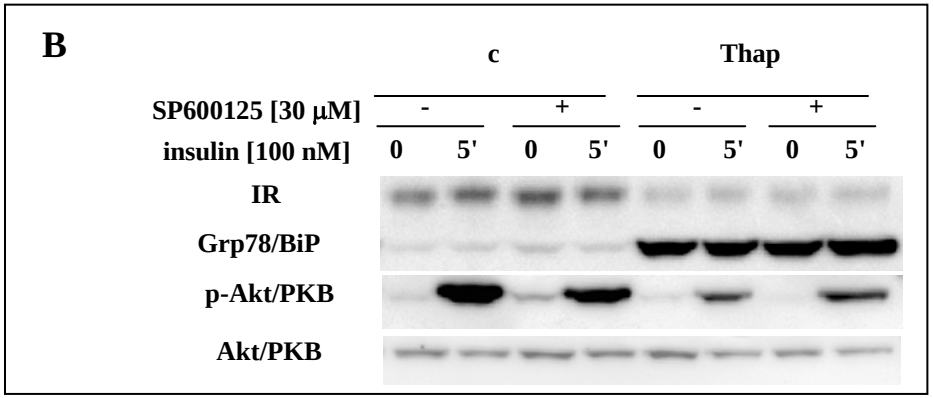
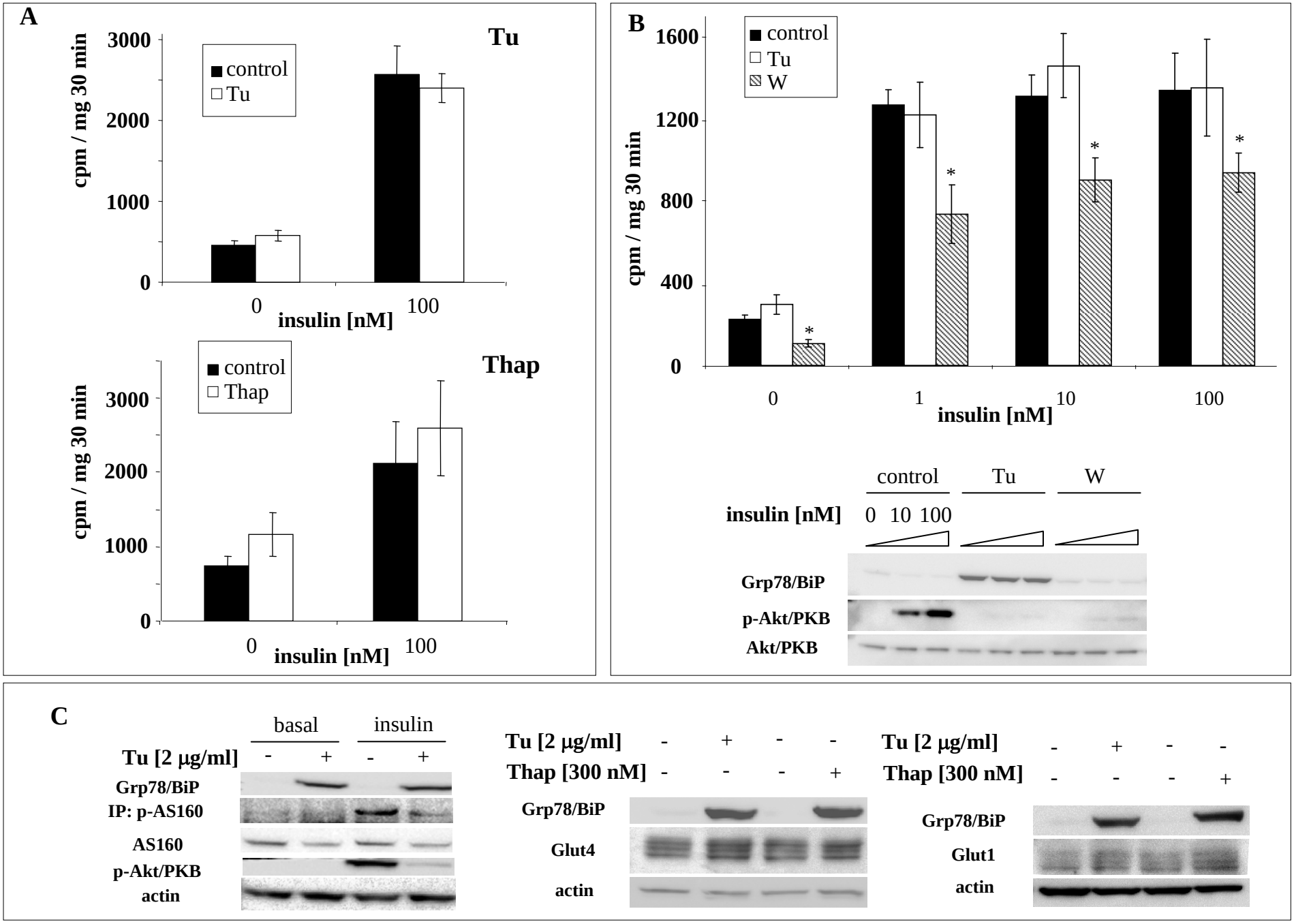
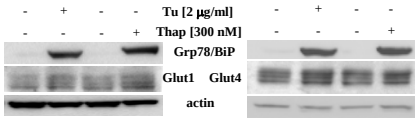

B
c
Thap
SP600125 [30 μM]
insulin [100 nM]
0 5' 0 5' 0 5' 0 5'
IR
Grp78/BiP
p-Akt/PKB
Akt/PKB

Figure 4

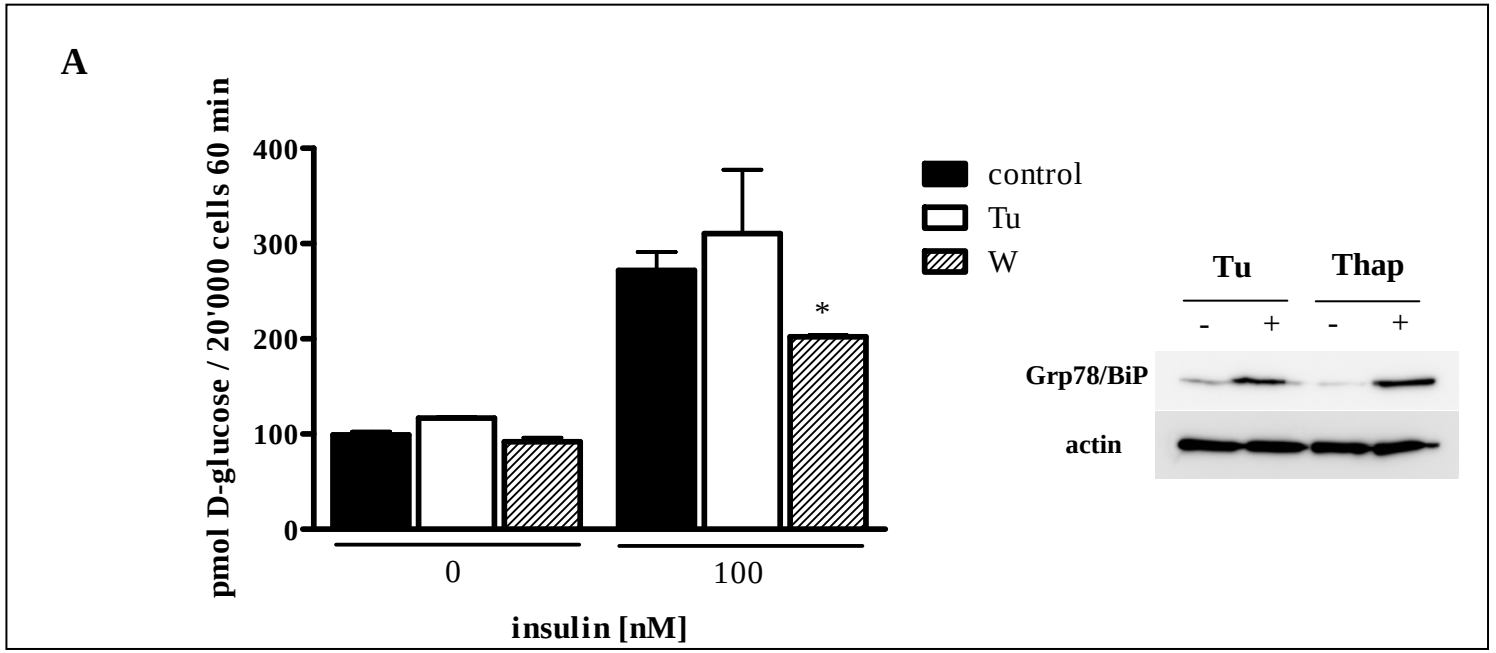

A
Tu
control
Tu
cpm / mg 30 min
3000
2000
1000
0
0
100
insulin [nM]
Thap
control
Thap
cpm / mg 30 min
3000
2000
1000
0
0
100
insulin [nM]
B
control
Tu
W
cpm / mg 30 min
1600
1200
800
400
0
0
1
10
100
insulin [nM]
control
Tu
W
insulin [nM]
0 10 100
Grp78/BiP
p-Akt/PKB
Akt/PKB
C
basal
insulin
Tu [2 µg/ml]
- + - +
Grp78/BiP
IP: p-AS160
AS160
p-Akt/PKB
actin
Tu [2 µg/ml]
Thap [300 nM]
- + - -
- - - +
Grp78/BiP
Glut4
actin
Tu [2 µg/ml]
Thap [300 nM]
- + - -
- - - +
Grp78/BiP
Glut1
actin



Glut4

actin

Figure 5


A
pmol D-glucose / 20'000 cells 60 min
400
300
200
100
0
*
0
100
insulin [nM]
control
Tu
W
Tu
Thap
- + - +
Grp78/BiP
actin

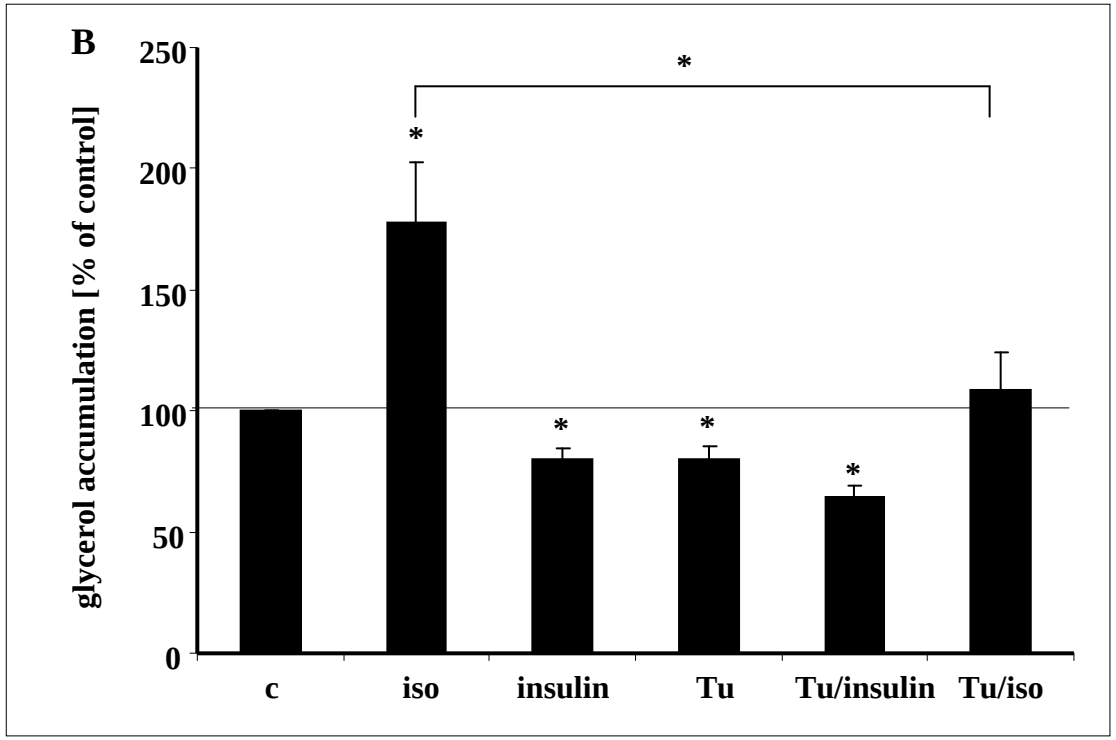

B
glycerol accumulation [% of control]
250
200
150
100
50
0
*
*
*
*
*
c
iso
insulin
Tu
Tu/insulin
Tu/iso

Figure 6

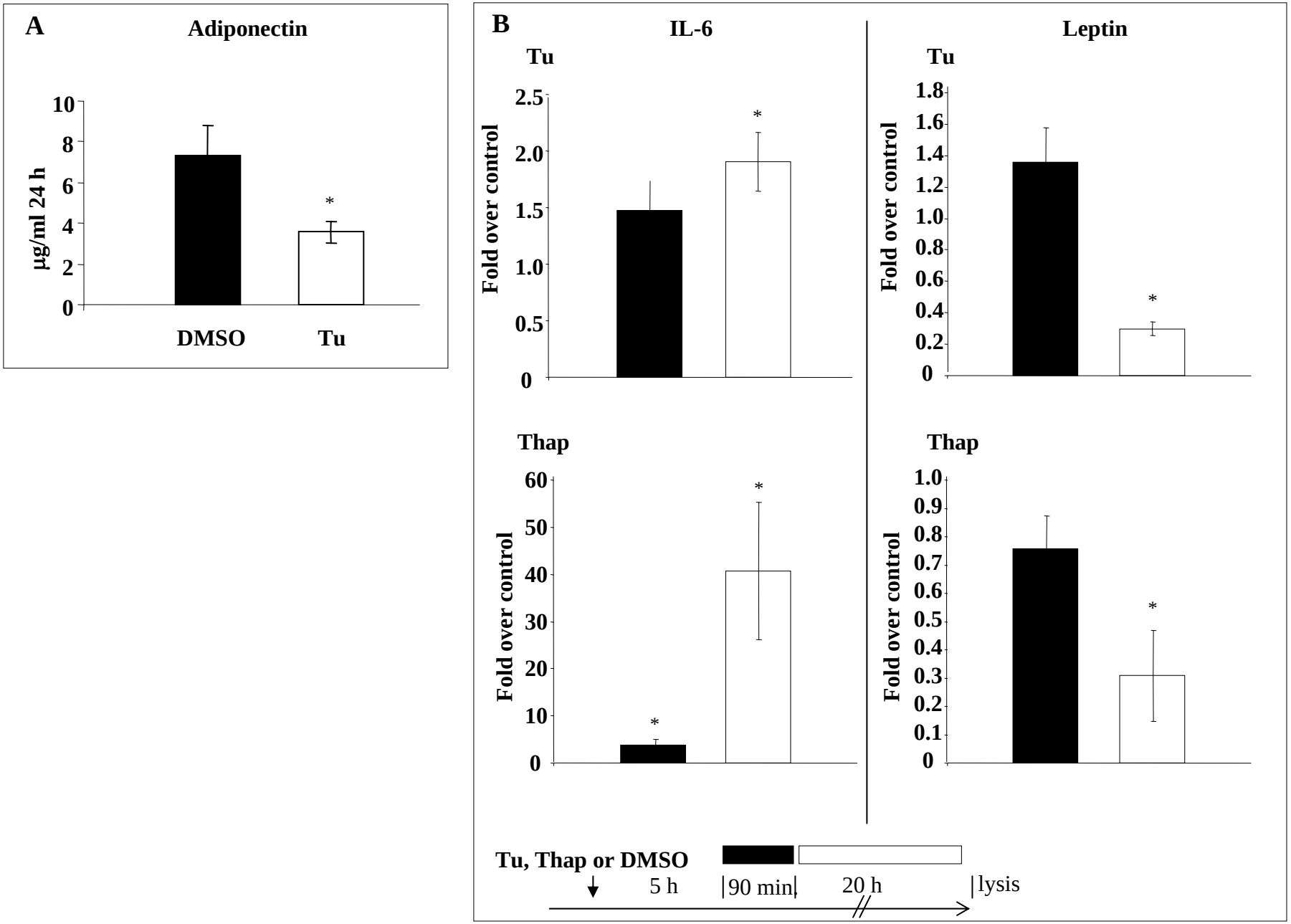
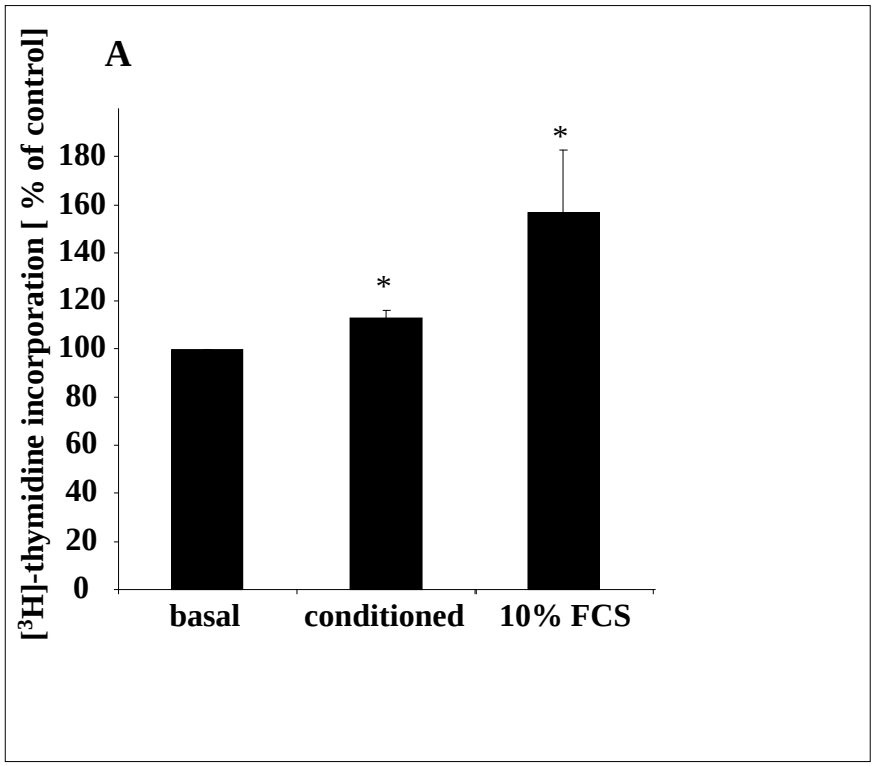
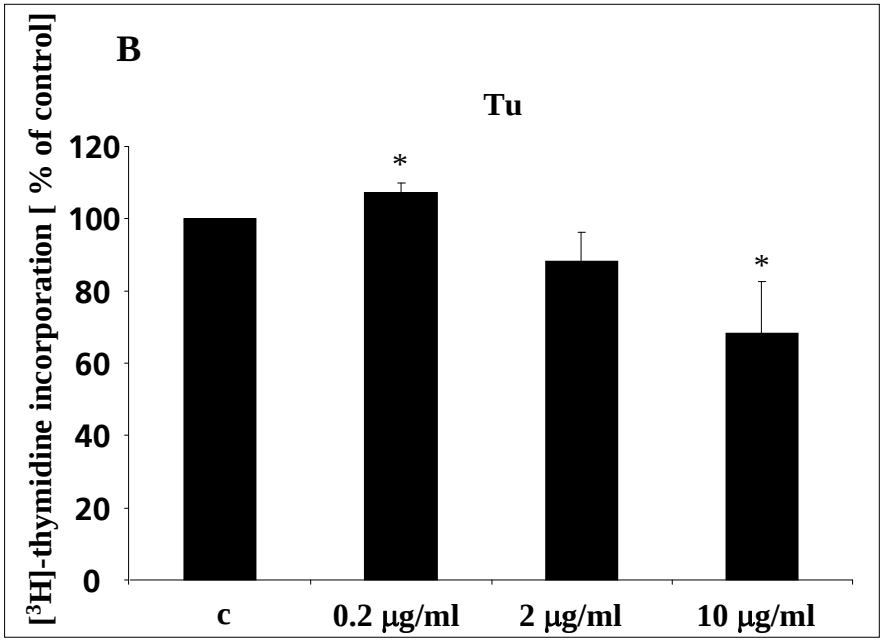
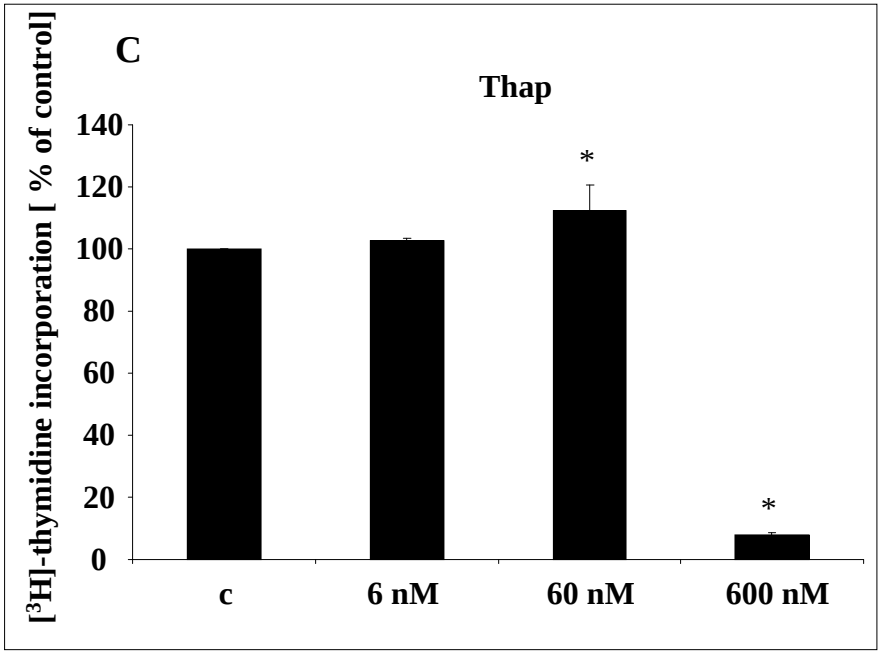

A
Adiponectin
µg/ml 24 h
10
8
6
4
2
0
*
DMSO
Tu
B
IL-6
Leptin
Tu
Fold over control
2.5
2.0
1.5
1.0
0.5
0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Thap
60
50
40
30
20
10
1.0
0.9
0.7
0.6
0.5
0.3
0.1
Tu, Thap or DMSO
5 h
90 min
20 h
lysis

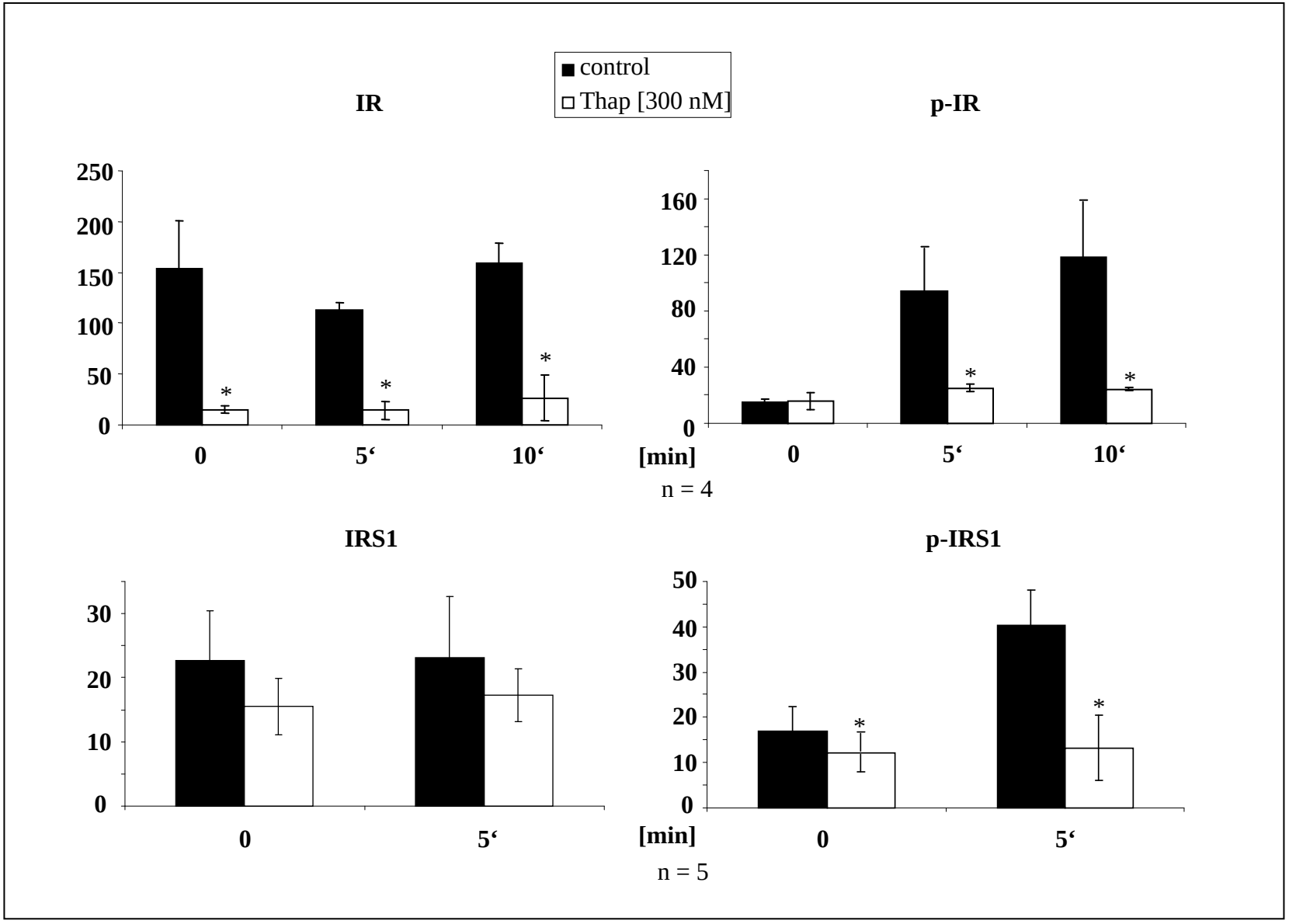
Figure 7


A
[3H]-thymidine incorporation [% of control]
0
20
40
60
80
100
120
140
160
180
*
*
basal
conditioned
10% FCS

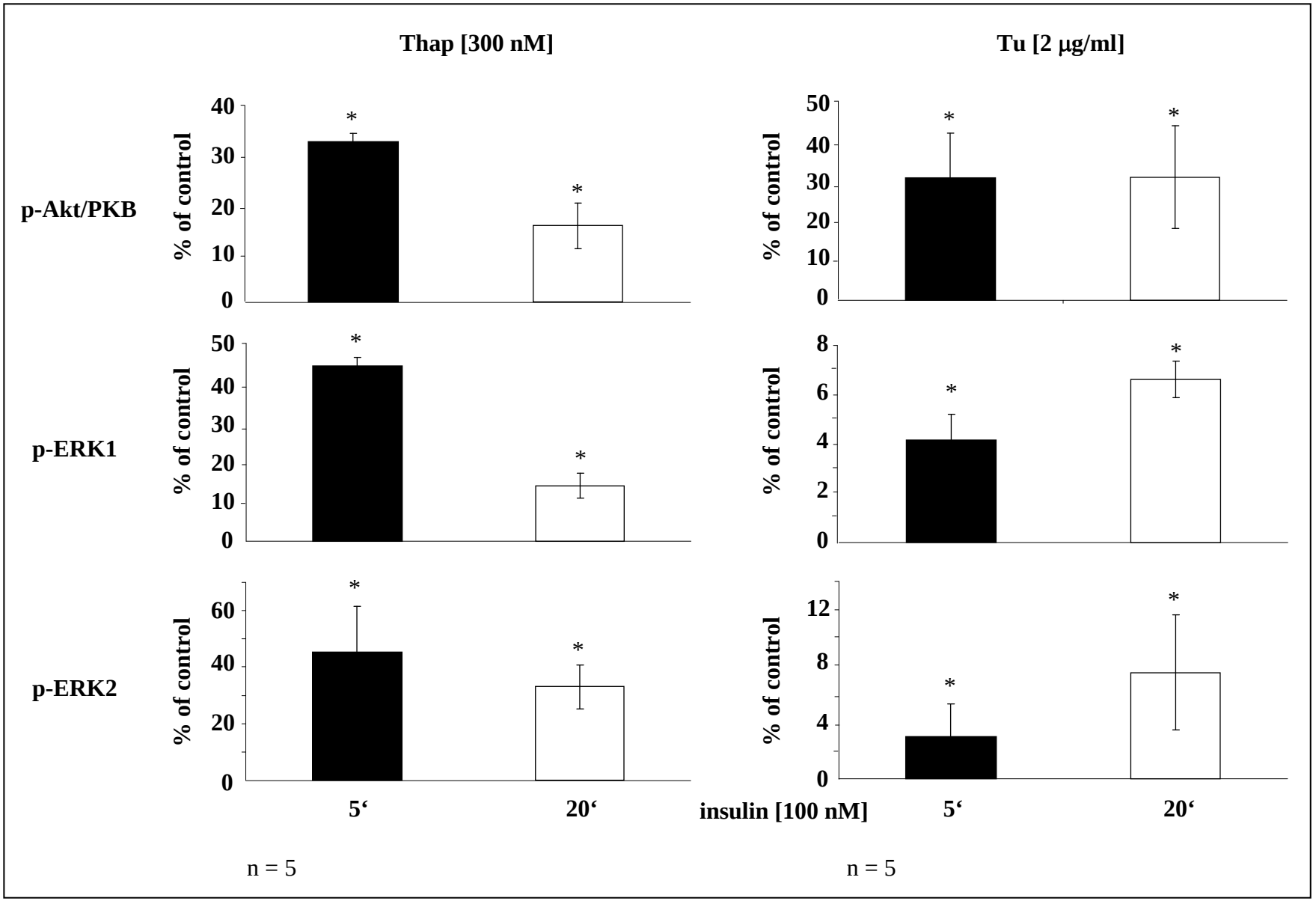

B
Tu
[3H]-thymidine incorporation [% of control]
0
20
40
60
80
100
120
*
*
c
0.2 μg/ml
2 μg/ml
10 μg/ml


C
Thap
[3H]-thymidine incorporation [% of control]
0
20
40
60
80
100
120
140
*
*
c
6 nM
60 nM
600 nM

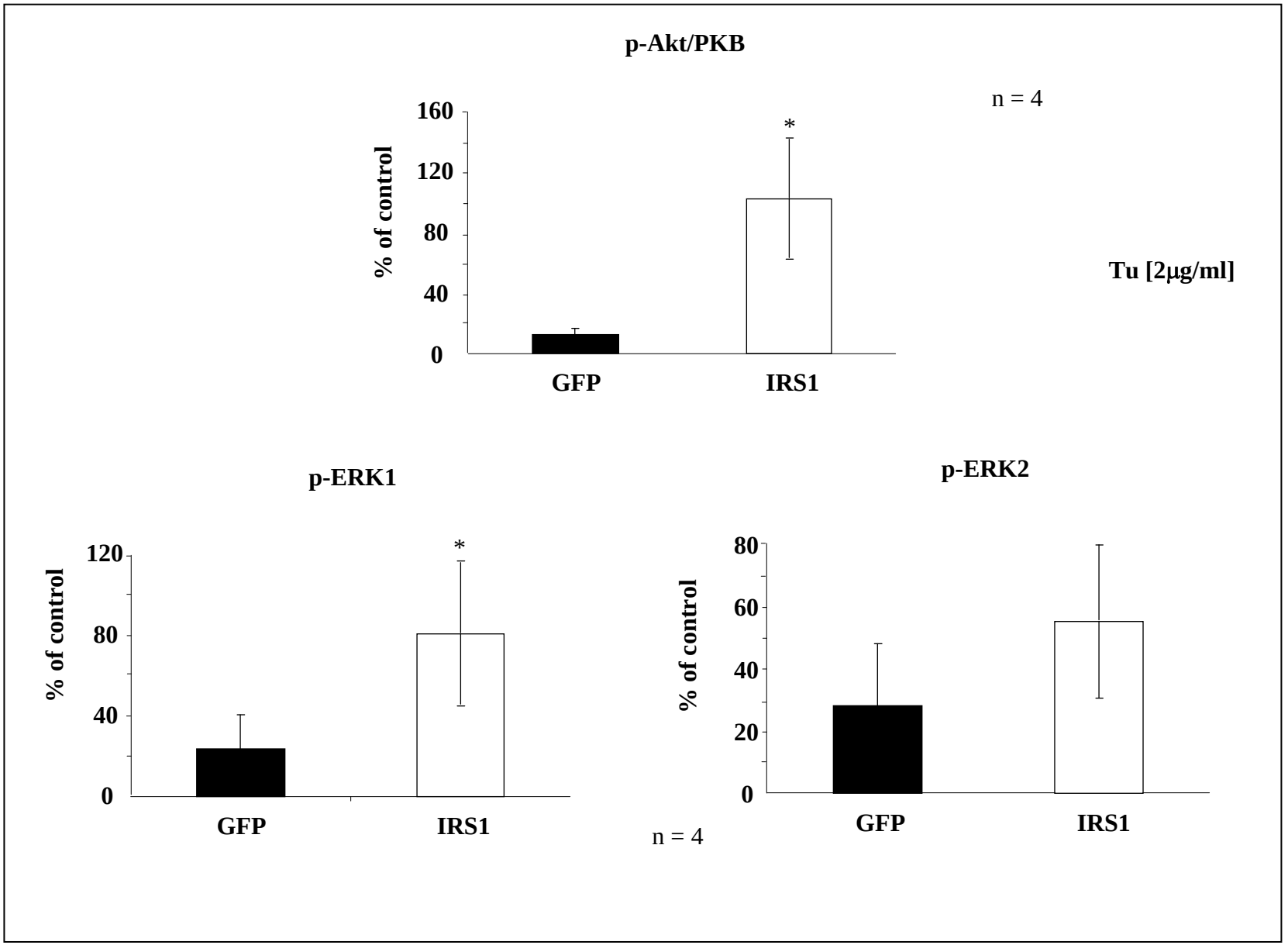
Supplemental
Figure 1



Supplemental
Figure 2



Supplemental
Figure 3



Supplemental
Figure 4