Insulin and Leptin Signaling in Placenta from Gestational Diabetic Subjects

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Abstract
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Insulin and leptin receptors are known to share signaling pathways, such as JAK2/STAT-3 (Janus kinase2/signal transduction and activator of transcription3), MAPK (Mitogen activated protein kinase), and PI3K (phosphoinositide 3-kinase). Both positive and negative cross-talk have been previously found in different cellular systems. Gestational diabetes (GDM) is a pathophysiological state with high circulating levels of both insulin and leptin. We have previously found that these 3 signaling pathways are activated in placenta from GDM patients to promote translation, involving the activation of leptin receptor. Now, we have tested the hypothesis that both leptin and insulin receptors might contribute to this activation in a positive way that may become negative when the system is overactivated. We studied the activation of leptin and insulin receptors in placenta from GDM and healthy pregnancies. We have also performed in vitro studies with insulin and leptin stimulation of trophoblast explants from healthy placenta. We have found that both leptin and insulin receptors are activated in placenta from GDM. In vitro stimulation of trophoblast explants with both leptin and insulin at submaximal doses (0.1 nM) potentiated the activation of signaling, whereas preincubation with maximal concentrations of insulin (10 nM) and further stimulation with leptin showed negative effect. Trophoblastic explants from GDM placenta, which presented high signaling levels, had a negative signaling effect when further incubated in vitro with leptin. In conclusion, insulin and leptin receptors have positive effects on signaling, contributing to high signaling levels in GDM placenta, but insulin and leptin have negative effects upon overstimulation.

Introduction
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The placenta is a fetal organ that plays pleiotropic roles during fetal growth. Because of this unique position, the placenta is exposed to the regulatory influence of hormones, cytokines, growth factors, and substrates present in both circulatory system and the placenta. In turn, it can produce molecules that will affect both the mother and the fetus independently. In this sense, leptin, the product of the (*ob*) gene and regulator of food intake and energy expenditure, is an important placental regulator that may act through paracrine or autocrine mechanisms [1,2]. Previous studies have demonstrated that leptin stimulates the process of cell proliferation and protein synthesis, as well as the inhibition of apoptosis in human trophoblastic cells [3–6]. Increases of leptin in the placenta has been implicated in the pathogenesis of various disorders during pregnancy, such as gestational diabetes mellitus (GDM) [7–10] – a common complication of pregnancy (3–5 % of pregnancies) associated with a high perinatal morbidity and mortality as well as insulin resistance, hyperinsulinemia, and hyperleptinemia. Insulin is an important growth hormone in utero. Its deficiency causes growth retardation. In contrast, induction of fetal hyperinsulinemia produces weight gain in human subjects [11,12]. Insulin stimulates fetal growth, in part by its anabolic effects on glucose and amino acid metabolism. It increases the cellular uptake of these metabolites and enhances the rates of protein synthesis and glucose utilization [13]. The current view is that the abnormal maternal metabolic environment may generate stimuli within the placenta resulting in the increased production of inflammatory cytokines whose expression is minimal during a healthy pregnancy [11]. In this way, hyperinsulinemia in the

pregnancy, probably may regulate placental leptin production, perhaps acting as a circulating signal to control fetal homeostasis [14]. In fact, we have recently reported that insulin induces leptin expression in trophoblastic cells, enhancing the activity of leptin promoter region [15]. Moreover overexpression of both leptin and leptin receptor have been reported in placenta from GDM compared with placenta from healthy pregnancy, which might contribute to the observed increase in the placenta weight in GDM [8,14]. Therefore, since it is known that increased leptin concentrations are associated with insulin-resistant states, it has been suggested that leptin mediated insulin resistance might be the major factor in the pathogenesis of the GDM [16]. However, insulin resistance does not develop uniformly throughout the organism but it may affect specific downstream pathways within a cell or it may occur in specific cell types and organs, whereas sensitivity is still maintained in others, including placenta [11]. In fact, it has been suggested that only the central actions of leptin are required to increase insulin sensitivity [17].

Human placenta not only expresses endogenous LEPR (leptin receptor) but also IR (insulin receptors), and it responds to either leptin or insulin with stimulation of signaling cascades, such as JAK2/STAT-3 (Janus kinase 2/signal transduction and activator of transcription 3), MAPK (mitogen-activated protein kinase), and PI3K (phosphoinositide3-kinase) [4,18]. Both positive and negative effects of the 2 systems have been previously found in different cellular types. However, comprehensive details on the cellular mechanisms underlying insulin and leptin signaling are limited, particularly in the placenta. Given the evidence supporting the overlap of insulin and leptin signal transduction, it has been postulated that inhibitory effect between these pathways is an adaptive mechanism when the system is overactivated, in situations of either hyperinsulinemia or hyperleptinemia, to ensure regulation. That is why, in the present study, we aimed to dissect the possible relation between LEPR and IR at the level of 3 major signaling pathways, STAT-3, MAPK and PI3K in explants form human placenta of GDM pregnancies, which is characterized by hyperinsulinemia and hyperleptinemia. We tested the hypothesis that both leptin and insulin receptors might contribute to this activation in a positive way that may become negative when the system is overactivated. Therefore, we also aimed to determine whether in vitro exposure of trophoblast explants to insulin could alter leptin signal transduction in human trophoblastic cells from normal placenta.

Materials and Methods
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Placental samples
Term placentas from pregnancies complicated with GDM (n=7, 4 patients who were diet-controlled and 3 patients who required insulin therapy) and from healthy pregnancies (n=7) were obtained after planned Cesarean delivery. The patients were diagnosed with GDM based on either (1) a value > 200 mg/dl on a 50 g 1-h post-glucose blood sugar or (2) two or more elevated values on a 100 g 3-h glucose tolerance test. None of the patients had previous history of diabetes mellitus or any known endocrinopathy. Clinical data of the subject are shown in ○ Table 1. All patients delivered at term. The placental tissues were collected at delivery and immediately transferred on ice to the laboratory for preparations of RNA isolation and protein lysate.

All samples were obtained from the Department of Obstetrics and Gynecology from the Virgen Macarena University Hospital.

Table 1 Subject characteristics of GDM cases and controls.

Characteristics	Controls (n=7) Mean±SD	GDM (n=7) Mean±SD
Maternal age at delivery (years)	27.1±6.0	34.8±5.0
Gestational age at delivery (weeks)	39.5±0.7	37.6±1.4
Body mass index	26.7±3.6	29.8±3.2
Ethnicity	white	white
Infant birth weight (g)	3 170.4±87.2	3 245.8±467.2
Fetal sex (male/female)	4/3	3/4
Placental weight (g)	564±105	670±122
Plasma leptin concentration (ng/ml)	25.0±14	54.0±31
Plasma insulin concentration (mU/ml)	5.4±2.0	12.3±10

This study was approved by the local ethical committee; informed written consent was obtained from all subjects.

Placental villous explants culture
Human placentas were immediately suspended in ice-cold phosphate-buffered saline (PBS) and transported to the laboratory (within 20 min), where they were washed 2–3 times in sterile PBS to remove excess blood. Villous tissue free of visible infarct, calcification, or hematoma was sampled from at least 5 cotyledons at a distance midway between the chorionic and basal plates. These core parts of cotyledons were cut into multiple cubic segments (10–15 mg wet weight) and washed with phosphate buffer saline.

Treatments of placental explants
Placental explants were randomly distributed in tubes containing 1 ml of DMEM-F12 medium. Placental explants were maintained in a shaking water bath at 37 °C during 5 min to equilibrate temperature. Placental explants from GDM were incubated for 10 min in the same medium supplemented or not with 0.1, 1, and 10 nM leptin. Placental explants from healthy pregnancies were preincubated or not with high insulin concentration (10 nM) (Sigma Chemical Co.) for 4 h and stimulated or not with 0.1, 1, and 10 nM leptin for 10 min. Hormone concentrations are based on previous published studies of dose-response curves of insulin and leptin in trophoblastic cells [8,15], which overlap physiological plasma levels. Moreover, placental explants from healthy pregnancies also were stimulated with submaximal doses of insulin (0.1 nM), leptin (0.1 nM) or with the combination of both hormones for 10 min. After all these manipulations, explants were removed from the bath, centrifuged for 2 min at 2 000 g at 4 °C and resuspended in 500 μl of lysis buffer [1×PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 10 mg/ml phenylmethanesulfonyl fluoride (PMSF)] during 30 min at 4 °C on an orbital shaker and later centrifuged at 10 000 g for 20 min. Supernatants were analyzed by Western blot.

Western blot analysis
Total protein levels were determined by the bicinchoninic acid method (Thermo Scientific), using bovine serum albumin as standard. Supernatants were mixed with Laemmli's sample buffer containing 2% SDS and 30 mM β-mercaptoethanol, boiled for 5 min, resolved by SDS- PAGE on a 12% gel (60 μg of protein per lane), and thereafter electrophoretically transferred to a nitrocellulose membrane (Hybond, Amersham Pharmacia) by means of a semidry system. Membranes were blocked by 5% nonfat milk in PBS at room temperature for 1 h. Membranes

were then immunoblotted with an antibody that detects the phosphorylation level of LEPR (Tyr1077) (1:1 000, Millipore). We also employed polyclonal rabbit anti-phospho-STAT-3 (Tyr705) (1:1 000, Cell Signaling), polyclonal rabbit anti-phospho-insulin receptor (Tyr972) (1:1 000, Sigma), polyclonal rabbit anti-phospho-insulin receptor substrate-1 (Tyr612) (1:1 000, Sigma), polyclonal rabbit anti-phospho-mitogen-activated protein kinases 1 and 3 (MAPK1/3; Thr202/Tyr204, 1:3 000, Cell Signaling technology), and polyclonal rabbit anti-phospho-PKB (Ser472/473, 1:1 500, Cell Signaling technology). Loading controls were performed by immunoblotting the same membranes with monoclonal mouse anti-β-tubulin (1:1 000, Santa Cruz), with polyclonal rabbit anti-LEPR (1:1 000, Santa Cruz), with polyclonal rabbit anti-total-STAT-3 antibody (C-20, 1:1 000; Santa Cruz Biotechnology), with polyclonal rabbit anti-total-insulin receptor (IR) (1:2 000, Santa Cruz), with polyclonal rabbit anti-total-Insulin receptor substrate-1 (1:2 000, Santa Cruz), with monoclonal mouse anti-total-MAPK1/3 (1:3 000; Sigma) and with polyclonal rabbit anti-total-PKB antibody (1:3 000; Sigma). The antibodies were detected using horseradish peroxidase-linked anti-rabbit/anti-mouse immunoglobulin (1:12 000, Promega) and visualized using a highly sensitive chemiluminescence system (Supersignal; Pierce). Quantification of protein bands was determined by densitometry (referred to as phosporylated protein vs. non-phosphorylated protein) using Image Gauge version 3.12 software (Science Lab, Fuji Photo Film Co., Ltd.).

Inmunohistochemistry

The placental explants were fixed in 10% formalin for at least 24 h and then embedded in paraffin and processed routinely. A series of 5 μm sections from each tissue sample were cut. Pilot studies were undertaken to establish the optimum dilution of the antibody as well as confirming concentration and application times for the chemicals used during the immunohistochemistry (IHC) procedure. Placental explants were picked up onto a slide, deparaffined in xylene, rehydrated by sequential immersion in a graded series of alcohols and transferred into water for 10 min. Then, specimens were heat treated in sodium citrate buffer (pH 6.0), for 40 min at 96 °C, to unmask antigens. The sections were cooled and washed in phosphate-buffered saline (PBS, pH 7.2). Specimens were incubated in blocking agent (5% albumin bovine serum in PBS) for 1 h. Endogenous peroxidase activity was quenched by incubating the sections in a solution of 3% H_2O_2 in 10% methanol and in PBS for 15 min. Second sections were incubated with rabbit antihuman LEPR monoclonal primary antibodies, respectively, at 1:100 dilutions overnight at 4 °C. The sections were then washed in PBS (3 ×, 5 min each) and incubated with their respective secondary antibodies. The manufacturer's instructions were followed for the sequential incubation and durations for the exposure to the secondary antibodies. After washing with PBS, the sections were incubated with diaminobenzidine substrate kit (Dako, Carpinteria, CA, USA) for 5 min that resulted in a brown-colored precipitate at the antigen-antibody binding sites and the reaction was stopped in distilled water. After removing the slides from water, all the sections were dehydrated and one drop of aqueous mounting medium (Dako Faramount) was applied, and the sections were cover-slipped. Finally, the immunohistochemical specimens were examined using a Leica Laborlux S Microscope (Leica Microsystem GmbH, Wetzlar, Germany) with a Nikon DSL2 photo digital system (Nikon Corp, Tokyo, Japan). Representative sites in each sample were photographed at and captured with a software system (CS3, version 10.0.1; Adobe Photoshop, San Jose, CA, USA).

Data analysis

Experiments were repeated separately at least 3 times to assure reproducible results. Results are expressed as the mean ± standard deviation (S.D.). The Statistical significance was assessed by one-way ANOVA followed by Bonferroni's multiple comparison
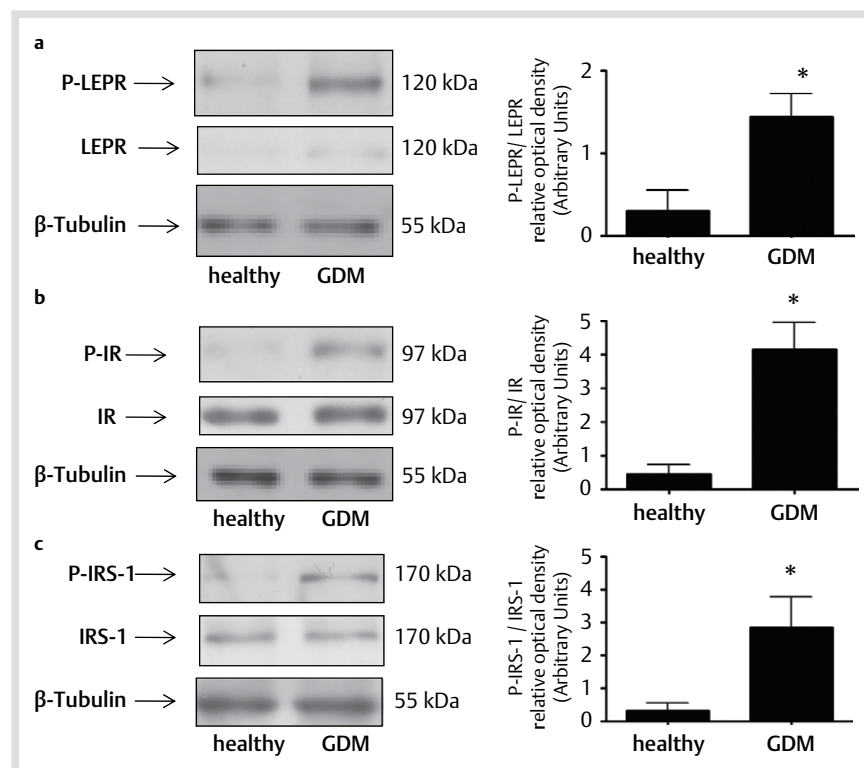

Fig. 1 Increased basal activation of leptin receptor (LEPR), insulin receptor (IR) and insulin receptor substrate 1 (IRS-1) in placentas from pregnancies with gestational diabetes mellitus (GDM) compared with placentas from healthy pregnancies. Placental samples (7 from healthy pregnancies, 7 from gestational diabetic pregnancies) were lysed and analyzed by Western blot to determine the basal phosphorylation of LEPR, IR, and IRS-1. Loading controls were performed by immunoblotting the same membranes with anti-total leptin receptor, anti-total insulin receptor, and anti-total IRS-1 as well as with anti-β-tubulin. Results shown in the immunoblot are from a representative experiment. Densitograms show mean ± SD and expressed as arbitrary units (referred to nonphosphorylated protein). Statistical analyses were performed by one-way ANOVA followed by Bonferroni's multiple comparison post hoc test. * p < 0.05 indicates significant differences from the healthy samples.

post hoc test and was calculated using the Graph Pad Instat computer program (San Diego, CA). A p-value < 0.05 was considered statistically significant. (GraphPad Software, San Diego, CA, USA).

Results
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Activated LEPR and insulin receptor (IR) signaling pathways in placenta from GDM

Since leptin, LEPR and IR have been found to be over-expressed in placenta from GDM [8, 19], we evaluated whether LEPR and also IR were activated in basal conditions in GDM placenta compared to healthy placenta. We investigated the activation of the LEPR and IR by analyzing the phosphorylation state of LEPR and IR as well as insulin receptor substrate 1 (IRS-1). As shown in ◉ **Fig. 1**, LEPR (◉ **Fig. 1a**), IR (◉ **Fig. 1b**) as well as IRS-1 (◉ **Fig. 1c**) phosphorylation are increased in placenta from GDM, compared with healthy placenta. Moreover, analysis of the immunohisto-chemical expression of leptin receptor by using anti-leptin

receptor antibody showed the presence of positive LEPR cells in syncytiotrophoblast of placenta, and apparent increased expression in placenta from GDM, compared with healthy placenta, which was previously described both by Western blot and qRT-PCR analysis by our group [8] (◉ **Fig. 2**).

To confirm a positive effect between insulin and leptin signaling, we next examined whether simultaneous stimulation with leptin and insulin could affect the early steps of the leptin and insulin signal transduction pathways. We measured the phosphorylation of STAT-3, MAPK1/3 and PKB in healthy placenta that was stimulated with submaximal doses of insulin, leptin or insulin and leptin together. As shown in ◉ **Fig. 3**, leptin (0.1 nM) or insulin (0.1 nM) produced similar submaximal effect in STAT-3 (◉ **Fig. 3a**), MAPK1/3 (◉ **Fig. 3b**) and PKB (◉ **Fig. 3c**) phosphorylation. The stimulation with both leptin and insulin further increased ~2–3 fold the effect of any of them on the 3 signaling pathways. These results suggest that the positive effect between insulin and leptin signaling is due to additive effects of submaximal doses of both insulin and leptin.
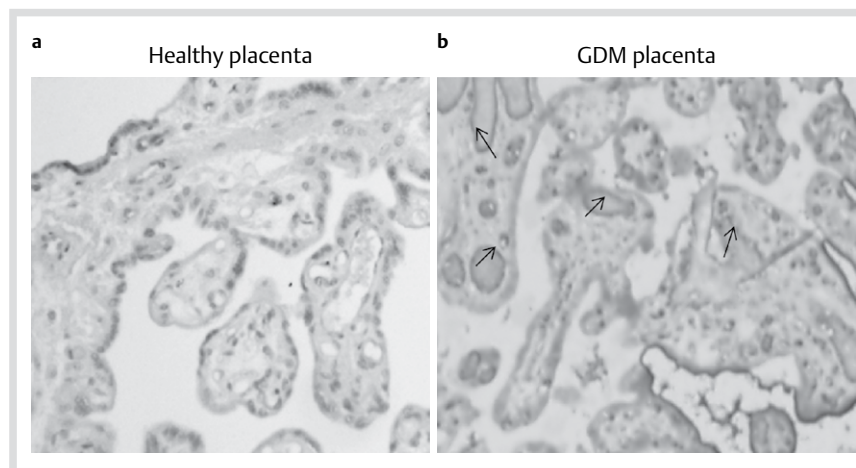

Fig. 2 Immunohistochemical study. Representative sections of 3 independent experiments from placental explant samples showing the increased expression of the LEPR in syncytiotrophoblast from GDM placenta. Leptin receptor (LEPR) expression by immunohistochemistry (40×) are shown in healthy placenta **a** and in GDM placenta **b**.
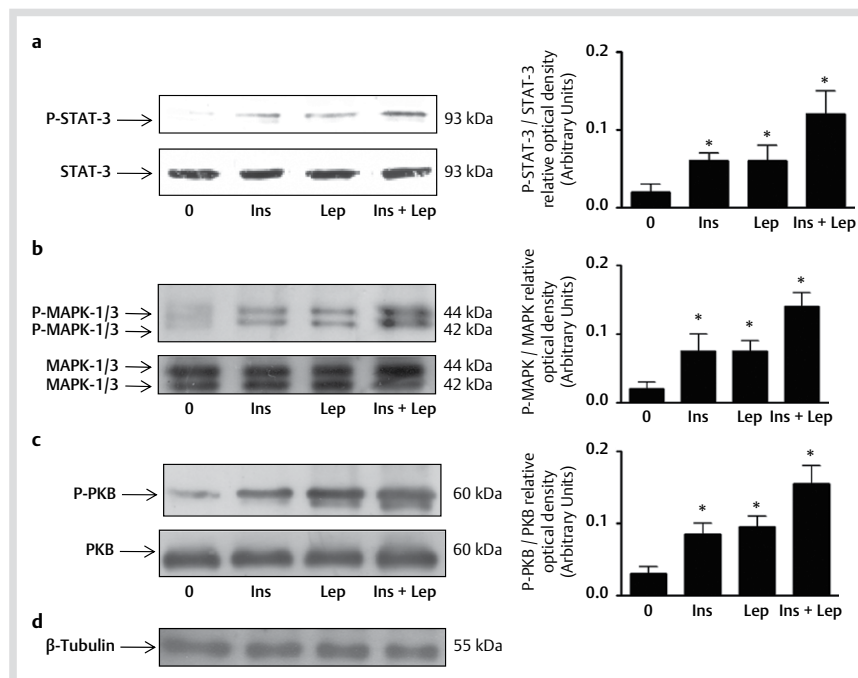

Fig. 3 Effects of submaximal doses of insulin (Ins) and leptin (Lep) on STAT-3, MAPK1/3 and PKB phosphorylation in explants from healthy placenta. Placental explants were processed as previously described and explants were incubated in the absence or presence of insulin (Ins) 0.1 nM, leptin (Lep) 0.1 nM, or insulin plus leptin (Ins+Lep) 0.1 nM for 10 min. Following solubilization, explants lysates were subjected to Western blotting, and blots were incubated with anti-phospho-STAT-3 **a**, anti-phospho-MAPK 1/3 **b** and anti-phospho-PKB antibodies **c**. The amount of protein was controlled by immunoblotting with anti-total STAT-3, anti-total MAPK1/3, and anti-total PKB as well as with a representative western blotting of monoclonal mouse anti-β-tubulin. Results shown in the immunoblot are from a representative experiment from a total of 7 placentas. Densitograms show mean ± SD and expressed as arbitrary units (referred to nonphosphorylated protein). Statistical analyses were performed by one-way ANOVA followed by Bonferroni's multiple comparison post hoc test. * p < 0.05 indicates significant differences from the basal.
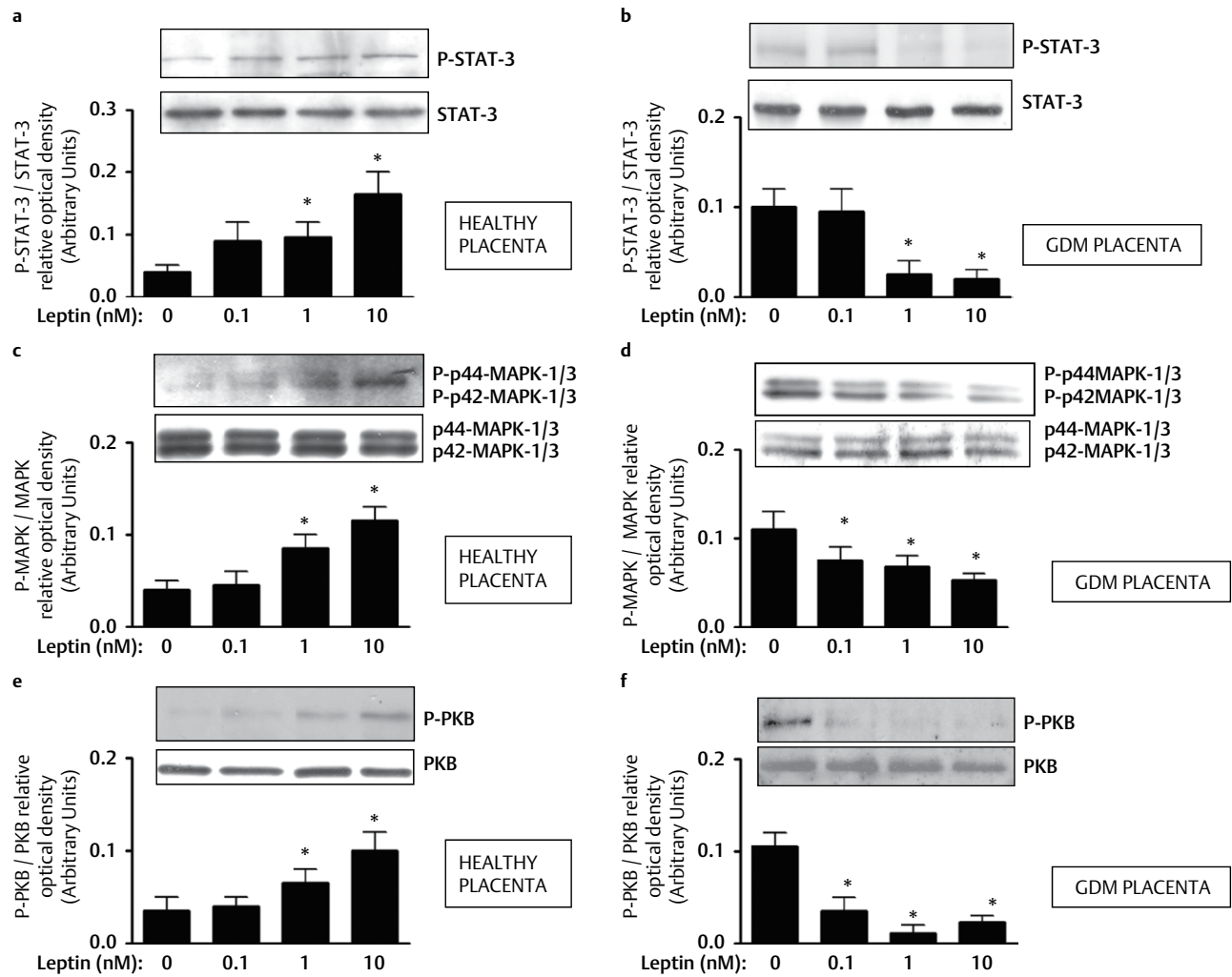
Fig. 4 Increased basal phosphorylation of STAT-3, MAPK1/3 and PKB in placentas from gestational diabetes (GDM) compared with placentas from healthy control pregnancies. Placental samples obtained from healthy placenta (n = 7) and placenta from GDM (n = 7) were stimulated in the absence or presence of different leptin concentrations (0.1, 1, 10 nM) for 10 min as described under the Materials and Methods section and analyzed by immunoblot with antibodies that specifically recognize STAT-3 (Tyr705) phosphorylated **a**, **b**, MAPK1/3 (Thr202/Tyr204) phos-

phorylated **c**, **d** and PKB (Ser472/473) phosphorylated **e**, **f**. The amount of protein was controlled by immunoblotting with polyclonal rabbit anti-total-STAT-3, anti-total-MAPK1/3, and anti-total PKB, respectively. Results shown in the immunoblot are from a representative experiment. Densitograms show mean ± SD and expressed as arbitrary units (referred to nonphosphorylated protein). Statistical analyses were performed by one-way ANOVA followed by Bonferroni's multiple comparison post hoc test. * $p < 0.05$ indicates significant differences from the basal.

Downregulation of LEPR signaling pathways in placentas from GDM, and in placentas from healthy pregnancies pretreated with high insulin concentration

We next evaluated the effect of leptin on the activation of the signaling pathways downstream of leptin receptor, that is, STAT-3, MAPK1/3 and PKB. We performed dose-response experiments using GDM and healthy placenta trophoblast explants. As shown in ⊙ **Fig. 4**, leptin dose-dependently stimulates the phosphorylation of STAT-3 (⊙ **Fig. 4a**), MAPK1/3 (⊙ **Fig. 4c**), and PKB (⊙ **Fig. 4e**) in trophoblastic explants from healthy placenta. However, these signaling pathways were found to be activated under basal conditions in placentas from GDM which showed increased basal phosphorylation of STAT-3 (⊙ **Fig. 4b**), MAPK1/3 (⊙ **Fig. 4d**) and PKB (⊙ **Fig. 4f**), compared to normal placenta, whereas they showed a dose-dependent downregulation when the explants were incubated with increasing concentrations of

leptin. These results suggest an adaptive response of the placenta to the diabetic environment in response to leptin and a possible interplay between the LEPR and IR systems which becomes more evident in GDM.

In order to investigate the possible cross-regulation of these signaling pathways, we wanted to simulate a diabetic environment by preincubating explants from the same healthy placentas with 10 nM insulin for 4 h and then incubated for 10 min in the absence or presence of different leptin concentrations (0.1, 1, 10 nM) as described under the Materials and Methods section. In healthy placental explants, pretreated with 10 nM insulin during 4 h, STAT-3 (⊙ **Fig. 5a**), MAPK1/3 (⊙ **Fig. 5b**), and PKB (⊙ **Fig. 5c**) phosphorylations were found to be increased and acute incubation with leptin did not further increase the phosphorylation level of those signaling proteins, but a dose-dependent inhibition was observed.
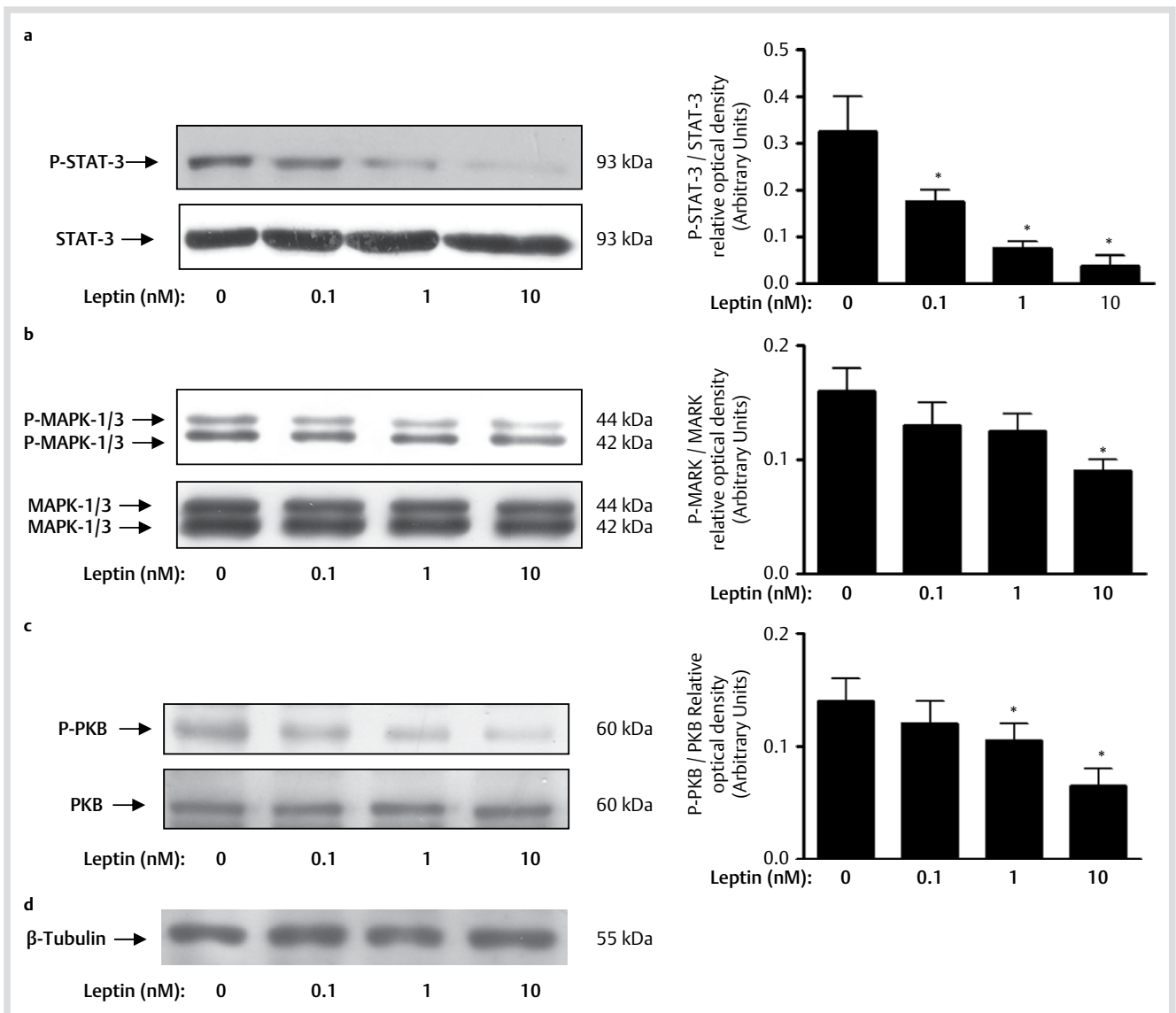
Fig. 5 Increased basal phosphorylation of STAT-3, MAPK 1/3, and PKB in healthy placenta pretreated with maximal doses of insulin (10 nM) for 4 h and dose-dependent inhibition of leptin stimulation for 10 min. Trophoblasts explants (from the 7 healthy placentas) were processed as described in ⊙ **Fig. 3**, and explants were pretreated with insulin (10 nM) for 4 h and then were treated with leptin (0, 0.1, 1 and 10 nM) for 10 min. Phosphorylated STAT-3 **a**, MAPK1/3 **b** and PKB **c** were determined by Western blot as indicated in Materials and Methods. Total STAT-3,

MAPK1/3 or PKB protein levels in explants were determined as loading controls. Moreover, a representative Western blotting of monoclonal mouse anti-β-tubulin was also used as loading control. Results from a representative experiment (of a total of 7) are shown. Densitograms show mean ± SD and expressed as arbitrary units (referred to nonphosphorylated protein). Statistical analyses were performed by one-way ANOVA followed by Bonferroni's multiple comparison post hoc test. * $p < 0.05$ indicates significant differences from the basal.

Discussion

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In the present study, we have investigated the possibility of direct interactions between insulin and leptin action in placenta, focusing on some key intermediate steps in these signaling pathways, STAT-3, MAPK, and PI3K. Whereas the effect of leptin on these signaling pathways is well documented in healthy placenta [3–6], the role of leptin in the microenvironment from GDM (characterized by hyperinsulinemia and hyperleptinemia) placenta is poorly understood. In many different systems it is known that both leptin and insulin share the same signaling cascades, such as JAK2/STAT-3, MAPK and PI3K [20–23] (⊙ **Fig. 6**). However, in placenta, a better understanding of the relationship between insulin and leptin is also required in order to elucidate its physiological and pathophysiological rele-

vance during pregnancy. Intracellular interactions between different signaling systems may function as mechanisms for enhancing or counter-regulating hormone action.

We have demonstrated in GDM placenta a significant increase in basal phosphorylation of STAT-3, MAPK 1/3 and PKB, suggesting a positive interaction between insulin and leptin signaling. We performed in vitro experiments with trophoblast explants from healthy placentas. We have found that in vitro simultaneous stimulation with submaximal doses of both hormones led to increased phosphorylation of STAT-3, MAPK1/3 and PKB, suggesting the additive effect of both signals. Therefore, the simultaneous activation of insulin and leptin receptors results in positive interactions at the level of STAT-3, MAPK1/3, and PKB signaling pathways.
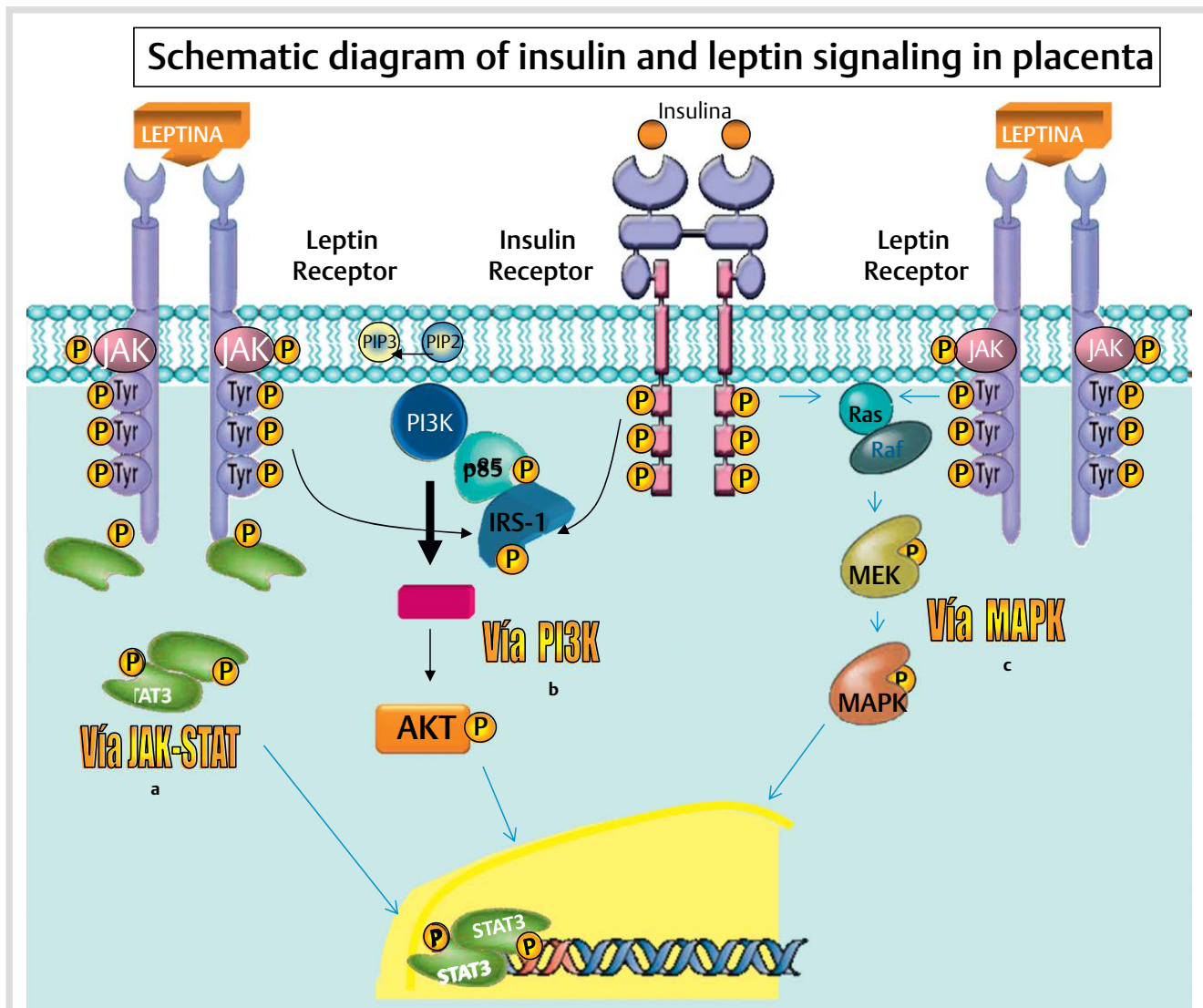
Fig. 6 Schematic picture of the shared insulin and leptin signaling in placenta. The figure shows the 3 major signaling pathways shared by insulin and leptin receptors **a** JAK-STAT; **b** IRS/PI3K/Akt; **c** MAPK pathways. IRS: Insulin receptor substrate; PIP2: Phosphatidylinositol-4,5-biphosphate; PIP3: Phosphatidylinositol-3,4,5-triphosphate; PI3K: Phosphatidylinositol 3-OH kinase; PDK: Phosphoinositide-dependent kinase; Akt: Protein kinase B; JAK: Janus kinase; STAT3: Signal transducer and activator of transcription 3; MAPK: Mitogen-Activated Protein Kinases; MEK: Mitogen-activated protein kinase kinase; RAS: Small GTPase; RAF: Raf quinasa.

On the other hand, a decreased effect on STAT3, MAPK1/3, and PKB phosphorylation was observed when GDM trophoblast explants were challenged with increasing concentration of leptin in vitro. These results are in contrast with those observed in healthy placenta which showed a dose-dependent increase in STAT-3, MAPK1/3, and PKB phosphorylation in response to leptin, suggesting a downregulation of an over-activated system. Similarly, when healthy placenta was preincubated with high insulin concentration (10 nM) [15], basal phosphorylation level of STAT-3, MAPK1/3 and PKB was significantly increased and leptin acute stimulation not only did not further increase phosphorylation levels but a dose-dependent inhibition was observed. The absence of leptin acute stimulation, and the inhibitory effect observed may be the consequence of the downregulation of leptin signaling because of the insulin pretreatment in pretreated trophoblast explants, similarly to previous results obtained in neuronal cells [20]. Thus, the stimulation by leptin of the already insulin-activated signaling pathways may lead to an inhibitory

effect on the phosphorylation of STAT-3, MAPK1/3 and PKB. Therefore, the effects of insulin and leptin in human placenta seem to be synergistic as demonstrated in other systems [24]. However, the insulin dose employed for preincubating the trophoblast cells in vitro was higher than plasma insulin levels. High insulin concentration (10 nM) employed in the present work was not based on basal plasma insulin levels, which range from 0.3–0.5 nM in GDM. We do not know the plasma insulin levels in pregnant patients after feeding nor the concentration of insulin in the trophoblast in vivo, which are limitations to this study. For this reason, maximal insulin concentration employed in the present work was based on previous in vitro studies in trophoblast explants [15]. Anyhow, the decrease in leptin signaling from diabetic placenta upon leptin stimulation observed in vitro should be expected in vivo. We do not know the pathophysiological consequences of this effect, but we may speculate that it may be a protective mechanism whereby the trophoblast is avoiding an overactivation. Alternatively, this inhibitory effect

may have deleterious consequences in the leptin-mediated function of the trophoblast, and therefore it may affect the nutrient exchange in case of morbid obesity.

Nevertheless, this pilot study provided initial evidence for activated JAK/STAT, MAPK, and PI3K signaling pathways in GDM pregnancies, even though the limited number of patients is a potential limitation. In the present work, we have not studied whether GDM affects placental endocrine signaling pathways in relation to fetal sex and it also remains to be elucidated. Our results will have to be confirmed by further studies with larger number of patients, in order to assess the full impact of our studies.

In conclusion, our results indicate a positive interaction between insulin and leptin signaling in human placental explants at submaximal doses of both hormones at the level of STAT-3, MAPK1/3, and PKB phosphorylation. Even though this is a descriptive study, the mechanism presented may serve to potentiate the activity of both insulin and leptin pathways and to increase stimulation in physiological processes such as the regulation of carbohydrate, protein, and lipid metabolism, which are under the combined control of both insulin and leptin. On the other hand, further studies are needed to understand the mechanisms underlying the downregulation effect of leptin on signaling in overstimulated trophoblast cells, as in GDM.

Conflict of Interest
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The authors declare no conflict of interest.

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