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Insulin Enhances Leptin Expression in Human Trophoblastic Cells[1]

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ABSTRACT

Leptin, one of the adipokines that controls energy metabolism via the central nervous system, also has pleiotropic peripheral effects, acting as a proinflammatory cytokine. Leptin is also produced by trophoblastic cells in the placenta, where leptin seems to function as a trophic autocrine hormone. Leptin expression is regulated by various tissue-specific factors, such as insulin, in the adipocyte. However, the complete regulation of leptin production in the placenta is still poorly understood. That is why we investigated the regulation of leptin expression by insulin in JEG-3 trophoblastic cells and human placental explants from normal pregnancies. Western blot analysis and quantitative real time RT-PCR was performed to determine the leptin expression level after treatment of cells or trophoblast explants with different concentrations of insulin (0.1–100 nM). Leptin promoter activity was evaluated by transient transfection with a plasmid construct containing different promoter regions and the reporter luciferase gene. We found a stimulatory, dose-dependent effect of insulin on endogenous leptin expression in human placental explants. Maximal effect was achieved at 10 nM insulin, and this effect can be totally prevented both by blocking phosphatidylinositol 3 kinase (PI3K) pathways and mitogen-activated protein kinase (MAPK). Moreover, insulin treatment significantly enhanced leptin promoter activity up to 40% in JEG-3 trophoblastic cells. Deletion analysis demonstrated that a minimal promoter region between −1951 and −1546 bp is necessary to achieve insulin effects. In conclusion, we provide evidence suggesting that insulin induces leptin expression in trophoblastic cells, enhancing the activity of leptin promoter region between −1951 and −1546 bp, via both PI3K- and MAPK-signaling pathways.

hormone action, leptin/leptin receptor, placenta, signal transduction, trophoblast cells

INTRODUCTION

The placenta is an important endocrine organ during pregnancy that locally releases a broad spectrum of hormones and growth factors, thus playing a key role in maintaining pregnancy. These molecules may facilitate enhanced compensatory changes to profoundly alter metabolism within the placenta. In this context, leptin as well as its receptors seem to play a possible role in this process [1].

Leptin, the product of the *ob* gene, mainly secreted by the adipose tissue, is best known as a regulator of food intake and energy expenditure via hypothalamic-mediated effects [2]. Nevertheless, placenta is the second highest producing tissue in humans, where leptin may be an important local factor acting in an autocrine or paracrine manner. In this line, leptin may have physiological effects, including angiogenesis, growth, and immunomodulation [3]. Leptin also exerts an antiapoptotic action in placenta, and this effect is mediated by the mitogen-activated protein kinase (MAPK) pathway [4]. Moreover, leptin stimulates protein synthesis by activating the translational machinery via both phosphatidylinositol 3 kinase (PI3K) and MAPK pathways [4–6].

In addition, previous studies have demonstrated that leptin also activates the release of proinflammatory cytokines and prostaglandins from human placental explants, further implicating leptin as a modulator of placental endocrine function [7]. Its deregulation in the placenta has been implicated in the pathogenesis of various disorders during pregnancy, such as gestational diabetes (GDM) [8–11], a state of greater insulin resistance than normal pregnancies. However, the complete regulation of leptin production in the placenta is still poorly understood [10]. Pregnancy is a state of insulin resistance and is associated with profound alterations in metabolism, directed toward supplying adequate nutrition for the fetus. Thus, because leptin serum concentration depends on ingestion and nutritional status, insulin is a key regulator of leptin expression in adipose tissue [12, 13]. It has also been shown that insulin leads to an increase in leptin concentration in cell culture supernatants of adipocytes [14]. However, in placenta, a better understanding of the specific mechanisms regulating leptin biosynthesis is required to elucidate its physiological and pathophysiological relevance during pregnancy.

Expression of leptin in placenta is highly regulated, suggesting that certain key pregnancy molecules participate in such regulation [15]. The leptin gene in placenta has a specific upstream enhancer, known as the placental leptin enhancer region (PLE) [16], implying that leptin gene expression is regulated differently in placenta and in adipose tissue. In placenta, leptin expression has been shown to be up-regulated by different pregnancy hormones such as chorionic gonadotrophin and 17beta-estradiol and by second messengers such as cyclic adenosine 5′-monophosphate, mediated through MAPK- and PI3K-signaling pathways [15, 17–21]. Moreover, placental leptin production is regulated at the transcriptional

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level through specific promoter sequences (from −2931 to +1) by these hormones [17, 19] as well as by hypoxia response elements [22, 23], glucocorticoid response elements, and CCATT/enhancer-binding sites [24].

The human leptin gene promoter also contains multiple transcription regulatory elements that can be activated by insulin. These include seven Sp1-binding sites (GC boxes), which are a known target of MAPK on upstream structures of gene regulatory elements [25]. Moreover, insulin is an inducer of leptin production in human placenta as shown in vivo [26]. In this context, it has been demonstrated that both insulin and hypoxia act as agonists on the human leptin transcription in BeWo trophoblastic cells, but on two different regulatory elements [26]. However, the nature of the leptin promoter elements responsible for the insulin effect in human placenta is unclear.

In addition to these observations, different pathophysiological situations are known to produce increased leptin and insulin levels in vivo, such as GDM, producing functional changes that include overgrowth [9, 27]. Thus, we sought to investigate the possible positive effect of regulation of leptin expression by insulin as well as the mechanisms whereby insulin up-regulates leptin expression in human trophoblastic cells.

MATERIALS AND METHODS

Cell Culture and Treatments

The human choriocarcinoma cell line JEG-3 was grown in Dulbecco modified Eagle medium/F-12 (DMEM/F-12) (Invitrogen) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (Invitrogen), and 1 mM sodium pyruvate (Sigma Chemical Co.) at 37°C in 5% CO_2. The effect of recombinant human insulin (Sigma Chemical Co.) was tested at different doses (0.1–100 nM). Once the insulin maximal effect on leptin expression was determined in JEG-3 cells (see Figure 2, A and B). Subsequent experiments used 100 nM insulin. All the treatments were performed in DMEM/F-12 supplemented with 1% FCS unless indicated otherwise. Serum present in the incubation medium was reduced from 10% to 1% to lower nonspecific effects.

Placental Explants Collection and Processing

Term placentas from uncomplicated pregnancies (n = 9) were obtained after cesarean section delivery following normal term pregnancies in the Virgen Macarena University Hospital. None of the patients had previous history of diabetes mellitus or any known endocrinopathy. Subject characteristics were maternal age at delivery (25.0 ± 7.0 yr), infant birth weight (3105 ± 72 g), and placenta weight (534 ± 85 g). Subject characteristics were similar with regards to gestational age (39.5 ± 0.7 wk).

Human placentas were obtained after cesarean section and immediately suspended in ice-cold PBS and transported to the laboratory, where they were washed two to three times in sterile PBS to remove excess blood. Villous tissue free of visible infarct, calcification, or hematoma was sampled from at least five cotyledons at a distance midway between the chorionic and basal plates. These core parts of cotyledons were cut into multiple cubic segments (10- to 15-mg wet weight) and thoroughly rinsed with cold Hanks medium (137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, and 4 mM $NaHCO_3$, pH 7.4). None of the donor patients suffered from anomalous pregnancy. This study was approved by the local ethical committee (Comité Local de Etica e Investigación del Hospital Universitario Virgen Macarena) and included obtaining the patients' written consent. The reported investigations have been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000.

Treatments of Placental Explants

Placental explants were randomly distributed in tubes containing 1 ml of DMEM/F-12 medium (one explant/tube, four replicates per treatment). Placental explants were maintained in a shaking water bath at 37°C for 5 min to equilibrate their temperature and incubated for 5 h in the same medium supplemented with no insulin or 0.1–100 nM insulin (Sigma Chemical Co.). In experiments designed to analyze the insulin-dependent signal transduction pathway in the placenta, the selective inhibitor of MAPK kinase (MEK) inhibitor (50 μM PD98059; Sigma Chemical Co.) and the inhibitor of PI3K (0.1 μM Wortmannin; Sigma Chemical Co.) were used. Inhibitors were added 10 min before insulin treatment. The explants were removed from the bath, centrifuged for 2 min at 2000 × *g* at 4°C, and resuspended in 500 μl of lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 50 mM NaF, 10 mM pyrophosphate, and protease inhibitor cocktail) for 30 min at 4°C on an orbital shaker and later centrifuged at 10 000 × *g* for 20 min. The supernatants were analyzed by Western blot analysis. For real-time PCR, after thoroughly washing with phosphate-buffered saline, the tissues were immediately frozen at −80°C and stored until extraction of total RNA.

Western Blot Analysis

Each treatment was performed in medium without FCS for 5 h for leptin immunoblots or for 10 min for protein phosphorylation determination. Total cell lysates were prepared in lysis buffer. The lysates were centrifuged at 10 000 × *g* for 10 min to remove cellular debris. Total protein levels were determined by the bicinchoninic acid method (Thermo Scientific), using bovine serum albumin as the standard; 50 μg protein were loaded in each lane. Lysates were mixed with Laemmli sample buffer containing 2% SDS and 30 mM β-mercaptoethanol, boiled for 5 min, resolved by SDS-PAGE on a 12% gel, and electrophoretically transferred to a nitrocellulose membrane (Hybond; Amersham Pharmacia Biotech) thereafter. Membranes were equilibrated in 1× PBS, and nonspecific binding sites were blocked with 5% nonfat milk in PBS at room temperature for 1 h. The membranes were then immunoblotted with polyclonal rabbit anti-human leptin Y20 (1:1000; Santa Cruz Biotechnology), with polyclonal rabbit anti-phospho-MAPK1/3 (Thr202/Tyr204) (1:3000; Cell Signaling Technologies), or with polyclonal rabbit anti-phospho-AKT (Ser 472/Ser473) (1:3000; BD Biosciences Pharmingen). Loading controls were performed by immunoblotting the same membranes with monoclonal anti-β-tubulin (1:5000; Sigma Chemical Co.), with monoclonal mouse anti-total MAPK1/3 (1:3000; Sigma Chemical Co.) or polyclonal rabbit anti-total-AKT (1:3000; Sigma Chemical Co.). The antibodies were detected using horseradish peroxidase-linked anti-rabbit/anti-mouse immunoglobulin (1:12 000; Promega Corp.) and visualized using a highly sensitive chemiluminescence system (Supersignal; Pierce). Quantification of protein bands was determined by densitometry using Image Gauge version 3.12 software (Science Lab; Fuji Photo Film Co., Ltd.).

Plasmids

The luciferase reporter constructs based on the PGL-3 basic vector and PGL-3 promoter vector are shown schematically in Figure 2. They were all kindly provided by Dr. Oksana Gavrilova (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health) [28]. To simplify the notation, we renamed several of them, indicating the number of base pairs upstream of transcription initiation as follows: pL1951 (p1775), pL1546 (p1776), pL948 (p1777), pL805 (p1778), and pL218 (p1779). An empty vector was used as the control in the reporter experiments. To normalize the efficiency of individual transfections, pRSV-βgal containing the β-galactosidase gene under the control of the Rous sarcoma virus (RSV) was used. In experiments using expression plasmids, the empty vectors were used as controls. To perform transient transfection assays, plasmids were purified using the Maxipreps kit (Sigma Chemical Co.), and the concentration of DNA was estimated spectrophotometrically.

Transient Transfection Experiments

For transient transfection experiments, JEG-3 cells were used and plated at a density of 2.5×10^5 cells/ml onto six-well dishes containing 2 ml of DMEM/F-12 plus 10% FCS. The JEG-3 cell line from trophoblastic cells is a good known model used in transfection studies. Cells were incubated for 24 h. The medium was replaced, and transfection of cells was performed according to the standard liposome-mediated method. To determine the sensitivity of the method in this cell type, a standard dose of reporter plasmid versus light emission was performed (data not shown). Typically, 5 μg of the luciferase (Luc) reporter and 5 μg of pRSV-βgal internal control construct were transfected using 5 μl of Lipofectamine (Life Technologies, Inc.). The medium was replaced after 5 h with DMEM/F-12 containing 1% FCS with the addition of the different effectors. Transfection analysis was performed by duplicate in each of at least three independent experiments.

Assays for Luc and β-Galactosidase Activities

Luciferase activity in cell lysates was measured using the Luc Assay System (Promega Corp.). Cells were washed with PBS and harvested 72 h after transfection procedure using 50 μl of lysis buffer. Cell extracts were centrifuged, and 30 μl of the supernatant was mixed with 50 μl of Luc assay buffer (Promega Corp.). Luciferase activity was measured with a junior luminometer (Hidex Ltd.). β-Galactosidase activity was assayed using 1 mg of o-nitrophenyl-β-D-galactopyranoside (AmResco) as the substrate in 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 0.07% β-mercaptoethanol, and incubated at 37°C until the appearance of yellow staining. The product was determined by absorption at 420 nm. This value was used to correct variations in transfection efficiency. Luciferase results were calculated as the ratio of Luc activity per unit of β-galactosidase activity. Duplicate samples were analyzed for each data point.

Quantitative Real-Time RT-PCR Assay

Abundance of leptin mRNA was determined by quantitative real-time RT-PCR (qRT-PCR). Total RNA was extracted from placental explants and JEG-3 cells using TRI-SURE reagent, according to the manufacturer's instructions (Bioline Co.). Concentration and purity of the isolated RNA were estimated spectrophotometrically at 260 and 280 nm. For cDNA synthesis, 5 μg of total RNA was reverse transcribed at 50°C for 1 h using the Transcriptor First Strand cDNA Synthesis Kit (Roche). The qRT-PCR was performed using the following primers based on the sequences of the National Center for Biotechnology Information GenBank database: leptin (GenBank accession: NM_000230; product length = 294 bp; forward, 5′ GAA CCC TGT GCG GAT TCT T 3′; reverse, 5′ CCA GGT CGT TGG ATA TTT GG 3′) and cyclophilin (GenBank accession: NM_000942; product length = 194 bp; forward, 5′ TCT TGG TGC TCT CCA CCT TC 3′; reverse, 5′ TCT TGG TGC TAC CTC 3′). Quantitative RT-PCR Master Mix Reagent kit was obtained from Roche (Fast Start Universal SYBR Green), and PCRs were performed on a Chromo 4 DNA Engine (Bio-Rad). A typical reaction contained 10 μM of forward and reverse primer, 3 μl of cDNA, and a final reaction volume of 25 μl. The reaction was initiated by preheating at 50°C for 2 min, followed by heating at 95°C for 10 min. Subsequently, 41 amplification cycles were carried out as follows: denaturation 15 sec at 95°C and 1 min annealing and extension at 58°C. The threshold cycle (CT) from each well was determined by the Opticon Monitor 3 Program. Relative quantification was calculated using the $2-\Delta\Delta CT$ method. For the treated samples, evaluation of $2-\Delta\Delta CT$ indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophylin) and relative to the untreated control.

Data Analysis

For the placental explants, a representative immunoblot was chosen from the experiments on the nine placentas studied. For the qRT-PCR, each experiment was run in triplicate, and data are the mean from the nine placentas studied.

For the JEG-3 cells, Western blot analysis of a representative image of three independent experiments along with quantification of immunoreactive bands are shown. The qRT-PCR experiments were repeated separately at least three times to ensure reproducible results. Transient transfection experiments were repeated at least three times, and each treatment was performed in duplicate. The results are expressed as the mean ± SD. The statistical significance was assessed by ANOVA followed by Bonferroni multiple comparison post hoc test and was calculated using the GraphPad Instat computer program (GraphPad); a P value less than 0.05 was considered statistically significant.

RESULTS

Insulin Increased Endogenous Leptin Expression in Placental Cells

In this study, we investigated insulin modulation of leptin in human placental explants from healthy donors, which is a more physiological system than cell lines and are known to express leptin and leptin receptor as well [29–31]. Explants were incubated for 5 h in medium with or without different insulin concentrations (0.1–100 nM). As seen in Figure 1A, insulin enhanced leptin expression in human placental explants, as determined by Western blot analysis. This effect was dose dependent, and the maximal effect was achieved at 10 nM insulin.

In order to further study the effects of insulin on leptin expression, qRT-PCR analysis was carried out. As shown in Figure 1B, placental expression level of leptin gene was increased in placentas from healthy donors. This effect was dose dependent, and a maximal effect was achieved at 10 nM insulin.

Leptin Promoter Activity Is Induced by Insulin

Because insulin was able to induce leptin expression, we decided to study whether this effect occurred at the transcriptional regulation level. In JEG-3 trophoblastic cells, an insulin dose-response curve was performed both by Western blot and qRT-PCR analyses. In this model, the effect was dose dependent, and the maximal effect was achieved at 100 nM insulin (Fig. 2, A and B). Next, we performed transient transfection assays with plasmid pL1951, which contains the regulatory region of leptin gene, from −1951 to +42 bp, fused to the Luc reporter gene. JEG-3 trophoblastic cells transfected with pL1951 and treated with 100 nM insulin for 48 h showed significantly increased LUC expression (Fig. 2C). This result suggests that insulin enhances leptin promoter activity and regulates leptin gene expression at the transcriptional level. In order to identify the minimal promoter region necessary for insulin induction of leptin expression, JEG-3 cells were transfected with plasmids containing serial deletions of leptin promoter. The stimulatory effect of insulin on LUC activity was not observed to be significant when constructions containing regions below 1546 bp were used (Fig. 2C). Moreover, in addition to the stimulatory effect of insulin, basal LUC activity was also diminished in the region below 1546 bp, suggesting the importance of these fragments in the regulation of leptin expression in trophoblastic cells. These results suggest that the inducing effect of insulin may involve the promoter region from 1951 to 1546 bp and that this region contains strong positive elements that regulate leptin expression in human placenta.

MAPK1/3 (ERK) and AKT Are Activated by Insulin and Are Involved in Placental Leptin Expression

Insulin receptor-mediated action rapidly activates several protein kinase pathways. To determine whether insulin could stimulate MAPK- and PI3K-signaling pathways in trophoblastic cells, placental explants were first incubated for 10 min in the presence or absence of the pharmacological inhibitors PD98059 (50 μM) and Wortmannin (0.1 μM) to block MAPK and PI3K pathways, respectively, and then treated with or without 10 nM insulin for 10 min. MAPK1/3 and AKT phosphorylation were determined by Western blot analysis. Figure 3 shows that 10 nM insulin increased both MAPK1/3 and AKT phosphorylation. However, treatment with PD98059 and Wortmannin showed a significant effect on phosphorylation of both MAPK and PI3K pathways, suggesting that the pharmacological inhibitors blocked each pathway. Next, in order to analyze the role of these signaling pathways on insulin-induced leptin expression, placental explants were pretreated for 10 min in the presence or absence of the pharmacological inhibitors PD98059 (50 μM) and Wortmannin (0.1 μM) to block MAPK and PI3K pathways, respectively. Subsequently, 10 nM insulin was added, and explants were further incubated for 5 h. Both Western blot analysis (Fig. 4A) and qRT-PCR analysis (Fig. 4B) showed that treatment with pharmacological inhibitors, PD98059 (50 μM) and Wortmannin (0.1 μM) significantly blocked insulin-mediated up-regulation of leptin expression, suggesting that both MAPK

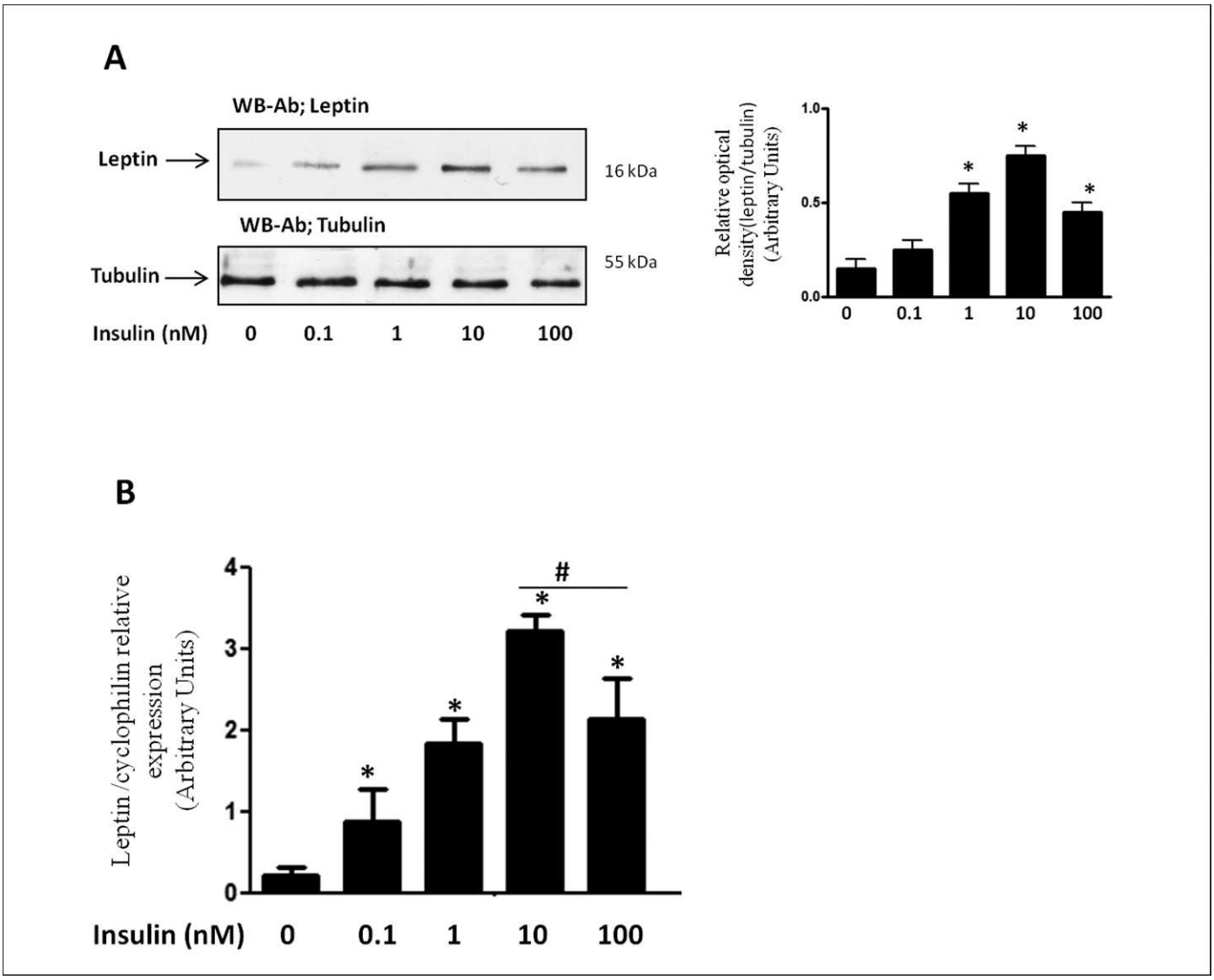

FIG. 1. Insulin enhances leptin expression in human placental explants. **A**) Human placental samples were obtained from control placentas (n = 9) and were incubated for 5 h with different insulin doses (0.1–100 nM), as indicated, in DMEM/F-12 medium without FCS. They were then lysed and analyzed by Western blots to determine leptin expression. Loading controls were performed by immunoblotting the same membranes with anti-β-tubulin. The results shown in the immunoblot are from a representative experiment. Densitograms show the mean ± SD from placental samples and are expressed as arbitrary units. Statistical analyses were performed by ANOVA; $^{*}P < 0.05$ indicates significant differences from the control. **B**) Human placental samples were obtained from control placentas and were incubated for 5 h with different insulin doses (0.1–100 nM), as indicated, in DMEM/F-12 medium without FCS. Next, leptin mRNA was quantified with qRT-PCR, and cyclophilin was used as the internal standard. Data represent fold change from the mean values of control. The results shown are the mean from the nine placentas studied and are expressed as means ± SD. $^{*}P < 0.05$ indicates significant differences from the control. $^{\#}P < 0.05$ indicates significant differences from the 10 nM insulin condition.

and PI3K are involved in the signaling pathway of insulin on leptin expression in placenta.

DISCUSSION

Leptin controls the functional integrity of the feto-placental unit, thereby maintaining pregnancy. Moreover, leptin has a physiological effect on the development of the placenta. Placenta is a source of as well as a target for the action of leptin, as evidenced by the fact that leptin receptor has been detected in placental trophoblast [32, 33]. During gestation, leptin plasma levels are greater than in nongravid women, and it increases with gestational age [34].

Several studies have shown that leptin induces trophoblast cell proliferation by inhibiting apoptosis [4, 30], stimulates protein synthesis [5], stimulates hormone and cytokine production in trophoblastic cells [35], and regulates fetal growth and development [36]. Deregulation of the autocrine/paracrine function of leptin at the feto-placentomaternal interface may be implicated in the pathogenesis of gestational diabetes, preeclampsia, and intrauterine fetal growth restriction [8, 37]. That is why it is important to have a better understanding of the specific mechanisms regulating leptin expression and biosynthesis and to elucidate its physiological and pathophysiological relevance during pregnancy. It has been demonstrated that the human leptin gene is actively engaged by hypoxia through mechanisms that are common to other hypoxia-inducible genes [38].

Human placental leptin is identical to that derived from adipose tissue in terms of size, charge, and immunoreactivity [39]. Nevertheless, the leptin gene has a specific upstream enhancer, known as PLE [16], implying that leptin gene

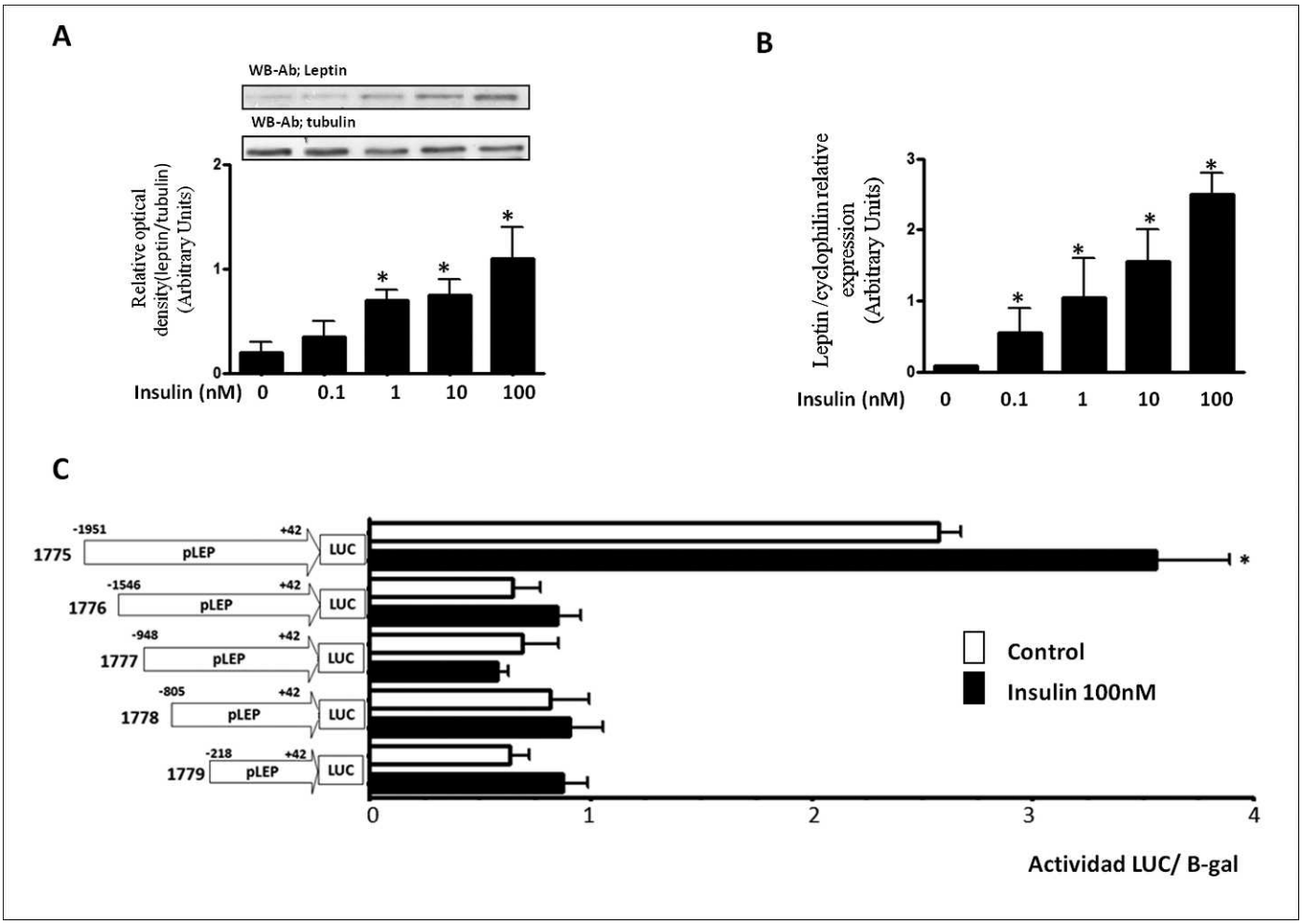

FIG. 2. Leptin promoter activity is induced by insulin in JEG-3 cells. **A**) Trophoblastic JEG-3 cells, starved for 24h, were incubated for 5 h with different insulin doses (0.1–100 nM), as indicated, in DMEM/F-12 medium. The cells were then lysed and analyzed by Western blots to determine leptin expression. Loading controls were performed by immunoblotting the same membranes with anti-β-tubulin antibodies. The results shown in the immunoblot are representative of three independent experiments. Densitograms show the mean ± SD from the placental samples and are expressed as arbitrary units. Statistical analyses were performed by ANOVA; $*P < 0.05$ indicates significant differences from the control. **B**) Trophoblastic JEG-3 cells, starved for 24h, were incubated for 5 h with different insulin doses (0.1–100 nM), as indicated, in DMEM/F-12 medium. Leptin mRNA was then quantified using qRT-PCR with cyclophilin being used as the internal standard. Data represent fold change from the mean values of control. The results shown are the mean from three independent experiments and are expressed as means ± SD. $*P < 0.05$ indicates significant differences from the control. **C**) Cells were transiently transfected with plasmids containing different leptin promoter deletions and treated with (black bars) or without (white bars) 100 nM insulin as indicated. The numbers at the right show basal promoter activity of each construct relative to the empty vector. In all the cases, after transfection, cells were incubated for 48 h in DMEM/F-12 medium supplemented with 1% FCS. Luciferase activity was measured in cellular extracts and normalized to β-galactosidase activity. Activity obtained in the absence of insulin was set as the control. The results are expressed as mean ± SD for three independent experiments performed in duplicate. Statistical analysis was performed by ANOVA. Significant differences from control were determined by Dunnett multiple comparison post hoc test. $*P < 0.05$ versus the control; a.u., arbitrary units.

expression is regulated differently in the placenta than in adipose tissue.

In this regard, it has been shown that insulin is a regulator of leptin expression both in adipose tissue and placenta. Insulin administration enhances the expression of leptin mRNA transcripts and protein secretion by adipocytes in vitro. However, little is known about the regulation of leptin expression by insulin in the placenta. Chardonnens et al. [40] observed that insulin increased leptin production in cultured human trophoblastic cells from placenta, suggesting that insulin regulates leptin gene expression and could be an important regulator of leptin expression in GDM. In this study, we investigated the regulation of leptin expression by insulin in human trophoblastic cells, which are a physiological system that express leptin and leptin receptor [36, 41] as well as insulin receptor. When the effect of insulin on leptin expression in human placental explants was analyzed, a significant up-regulation was observed. This effect was obtained with 10 nM insulin and is in agreement with some in vivo studies in which a positive correlation between insulin and serological levels of leptin in pregnant women was found [34, 42]. However, we observed that the highest doses of insulin tested (100 nM) had a down-regulatory effect on leptin expression in trophoblastic cells from placenta, but not in JEG-3 cell line. This might be explained by an increase in insulin receptors in JEG-3 trophoblastic cells. That is why we used insulin doses of 10 nM in the other experiments using trophoblastic explants, but 100 nM in the studies using JEG-3 trophoblastic cells.

Initially, we have found that both MAPK and PI3K signal transduction pathways can be activated in placental explants following insulin treatment, as assessed by Western blot analysis. Furthermore, the stimulatory effect of insulin on

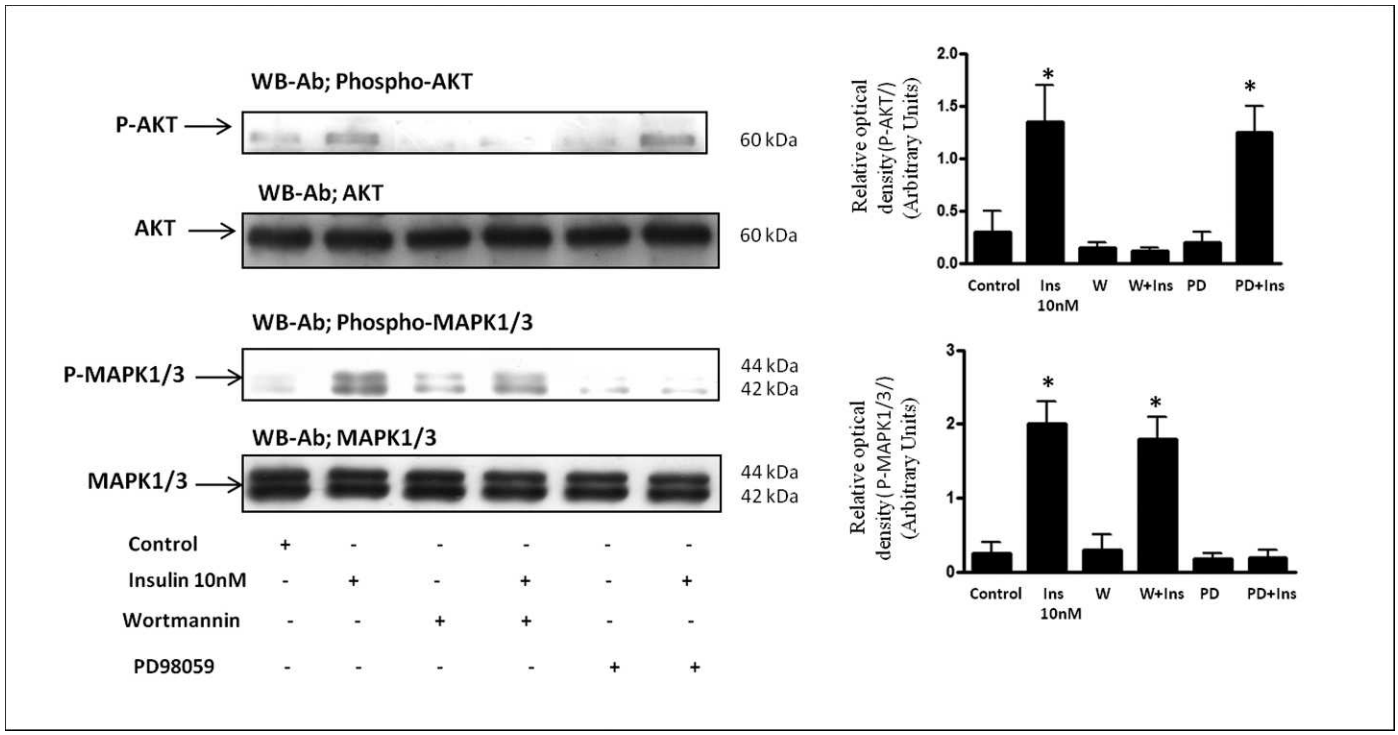

FIG. 3. MAPK1/3- and PI3K-signaling pathways are blocked respectively by PD98059 and Wortmannin in the presence of insulin. Placental explants were processed as previously described. The cells were pretreated with or without PD98059 (50 μM) and Wortmannin (0 1 μM) for 10 min and then were treated with or without 10 nM insulin for 10 min. MAPK1/3 and AKT phosphorylation was determined by Western blot analysis as indicated in *Materials and Methods*. Total MAPK1/3 or AKT protein levels in the cell extracts were determined as loading controls and are expressed as mean ± SD of two independent experiments. Statistical analyses were performed by one-way ANOVA followed by Bonferroni multiple comparison post hoc test.*$P < 0.05$ indicates significant differences from the control.

leptin expression was blocked both by PD98059 and Wortmannin, which are MEK and PI3K pharmacological inhibitors, respectively. We found that both signaling pathways are necessary to allow for the effect of insulin on leptin expression, suggesting a permissive role for each pathway, in addition to providing a mechanism for the insulin stimulation of leptin expression. This kind of dual (redundant) dependency has been previously described in other systems [43]. The Ras/MAPK and PI3K pathways have been implicated in insulin-induced gene transcription [44, 45]. In the rat leptin promoter, it has been found that a GC-rich region located between −101 and −83 bp containing a binding site for SP1 might be responsible for insulin-mediated effects on leptin transcription [46]. Moreover, it was reported that Sp1/Sp3 compound heterozygous mice are not viable and manifest severe placental defects [47]. From a pathophysiological point of view, this is important because different pathological situations are known to produce increased leptin and insulin levels in vivo, such as GDM, producing functional changes in the placenta, including overgrowth [27]. In order to elucidate the specific effects of insulin on leptin expression and to determine its molecular mechanism of action, we employed the luciferase reporter gene system in human trophoblastic cells to investigate leptin promoter activation directly. Deletion analysis demonstrated that a minimal promoter region between −1951 and −1546 bp is, at least, both necessary and sufficient to achieve insulin effects. Interestingly, it has been shown that the region between −1951 and −1546 bp is up-regulated by different pregnancy hormones such as chorionic gonadotrophin and 17beta-estradiol, which induce leptin expression through pathways that include activating MAPK and PI3K [15, 17–21], suggesting a possible region for insulin's effect. However, the insulin-responsive promoter region identified in the current study appears to be far upstream of that identified by Meissner et al. [26]. In fact, they studied a shorter sequence of the promoter region (−722 to +28 bp), responsive to both hypoxia and insulin in HEK 293 cells, but strikingly, the sequence with the insulin response element (−722 to −150 bp) did not contain the putative SP1-binding site, whereas the region from −1951 to −150 bp do contain a SP1-binding site [17]. These discrepancies might be explained by the different cell lines employed. Therefore, we can now provide evidence that the placental human leptin promoter has a potential insulin response element, located in the region from −1951 and −1546 bp, which involves a SP1-binding site [19]. We have also tested fragments containing others leptin promoter regions. We found no significant evidence concerning an additional regulatory element, which is important for insulin-mediated activation of leptin transcription, at least in human trophoblast cells.

Nevertheless, it should be pointed out that constitutive expression was also based on the same promoter region responsive to insulin (between −1951 and 1546 bp), and therefore, we cannot discard the presence of other insulin response elements in the leptin promoter from human trophoblastic cells. The identification of the exact part of the leptin promoter is under current investigation. Further analysis should also reveal whether there is a synergistic effect of insulin and others hormones, sharing the ability to induce leptin expression by the same promoter region.

In conclusion, we provide evidence that insulin may induce leptin expression through pathways that involve the activation of both MAPK and PI3K in trophoblastic cells. Our results support a possible mechanism of action of insulin through an

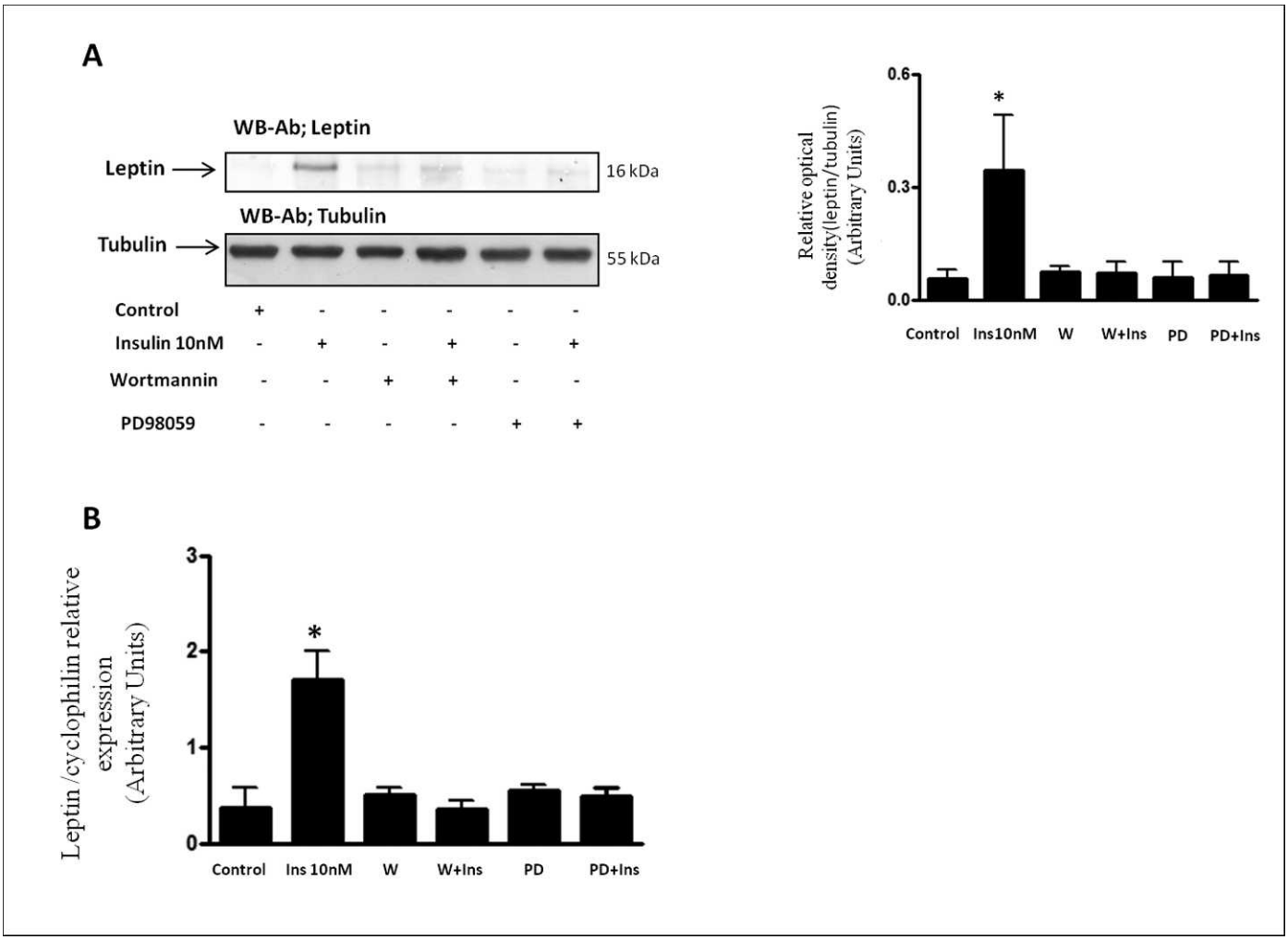

FIG. 4. MAPK1/3 and AKT are activated by insulin and are involved in placental leptin expression. **A**) Placental explants were processed as previously described and were pretreated with or without PD98059 and Wortmannin for 10 min and then were treated with or without 10 nM insulin for 5 h. Leptin expression was determined by Western blot analysis as indicated in *Materials and Methods*. Beta-tubulin protein levels in trophoblast explants were determined as the loading controls and are expressed as mean ± SD of two independent experiments. Statistical analyses were performed by one-way ANOVA followed by Bonferroni multiple comparison post hoc test. $*P < 0.05$ indicates significant differences from the control. **B**) Human placental explants were pretreated with or without PD98059 (50 μM) and Wortmannin (0 1 μM) for 10 min and then were incubated for 5 h with or without 10 nM insulin. Leptin expression was determined by qRT-PCR analysis. The results from a representative experiment are shown. They are expressed as mean ± SD of two independent experiments. Statistical analyses were performed by one-way ANOVA followed by Bonferroni multiple comparison post hoc test. $*P < 0.05$ indicates significant differences from the control.

insulin-response-element between −1951 and −1546 bp in the human leptin promoter in trophoblastic cells.

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