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Leptin Stimulates Protein Synthesis-Activating Translation Machinery in Human Trophoblastic Cells[1]

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ABSTRACT

Leptin was originally considered as an adipocyte-derived signaling molecule for the central control of metabolism. However, pleiotropic effects of leptin have been identified in reproduction and pregnancy, particularly in placenta, where it may work as an autocrine hormone, mediating angiogenesis, growth, and immunomodulation. Leptin receptor (LEPR, also known as Ob-R) shows sequence homology to members of the class I cytokine receptor (gp130) superfamily. In fact, leptin may function as a proinflammatory cytokine. We have previously found that leptin is a trophic and mitogenic factor for trophoblastic cells. In order to further investigate the mechanism by which leptin stimulates cell growth in JEG-3 cells and trophoblastic cells, we studied the phosphorylation state of different proteins of the initiation stage of translation and the total protein synthesis by [^{3}H]leucine incorporation in JEG-3 cells. We have found that leptin dose-dependently stimulates the phosphorylation and activation of the translation initiation factor EIF4E as well as the phosphorylation of the EIF4E binding protein EIF4EBP1 (PHAS-I), which releases EIF4E to form active complexes. Moreover, leptin dose-dependently stimulates protein synthesis, and this effect can be partially prevented by blocking mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 kinase (PIK3) pathways. In conclusion, leptin stimulates protein synthesis, at least in part activating the translation machinery, via the activation of MAPK and PIK3 pathways.

leptin, leptin receptor, mechanisms of hormone action, placenta, trophoblast

INTRODUCTION

Leptin is a nonglycosylated hormone of 146 amino acids [1]. Leptin was originally considered an adipocyte-derived signaling molecule for the central control of metabolism [2]. Within the last few years, pleiotropic effects of leptin have been identified in reproduction and pregnancy [3], particularly in placenta, where it may work as an autocrine hormone [4]. Possible physiological effects of placenta-derived leptin include angiogenesis, growth, and immunomodulation. Virtually all known cytokines have been found to be expressed in the human placenta, though their temporal pattern of expression is still not completely understood [5, 6]. LEPR shows sequence homology to members of the gp130 superfamily [7]. In fact, we have found that leptin may function as a proinflammatory cytokine on human monocytes [8] and lymphocytes [9], promoting the production of Th1-type cytokines. Moreover, among the different hormones secreted by the placenta, human chorionic gonadotropin (hCG) and leptin are involved in an autocrine/paracrine loop regulating placental function principally during the first trimester of pregnancy. In this context, we reported that hCG up-regulates leptin expression most likely by using the MAPK signal transduction pathway [10]. Thus, leptin may function as a hormone and cytokine during gestation. Moreover, leptin is a trophic and mitogenic factor for trophoblastic cells in the sense that it inhibits apoptosis and promotes proliferation [11]. We have also demonstrated that leptin promotes the proliferation and cell survival of human peripheral blood mononuclear cells [8, 12] via MAPK activation. Moreover, deregulation of leptin metabolism and/or leptin function in the placenta may be implicated in the pathogenesis of various disorders during pregnancy, such as recurrent miscarriage, gestational diabetes, intrauterine growth retardation, and preeclampsia [13, 14]. However, the molecular mechanisms underlying these effects of leptin on trophoblastic cells are not completely understood [3].

On the other hand, protein synthesis is known to be regulated by insulin and other hormones by phosphorylation of different translation factors that are involved in the initiation and elongation stages of translation [15]. Thus, the initiation factor EIF4E binds to the cap structure at the 5′-end of the mRNA and mediates the assembly of the initiation-factor complex EIF4E. The assembly of this complex is inhibited by EIF4E-binding proteins (EIF4EBPs) such as EIF4EBP1 (PHAS-I) [16]. Phosphorylation of these EIF4EBPs releases EIF4E from inactive EIF4EBP-EIF4E complex, allowing EIF4E to bind to EIF4G, and EIF4A to form the active EIF4F complex [17]. The activity of this complex is also regulated by phosphorylation of EIF4E [16, 18]. Consistent with this observation, our group has demonstrated EIF4E and EIF4EBP1 phosphorylation in adipocytes [19] and hepatocytes as well as in the rat hepatoma cell line HTC [20]. Interestingly, the intracellular signaling cascade leading to EIF4EBP1 phosphorylation involves the activation of RPS6KB1 (also known as p70S6k). Moreover, RPS6KB1 is mainly activated by PIK3

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and protein kinase C pathways [21, 22]. In this line, we have also demonstrated that leptin stimulates MAPK and PIK3 pathways in JEG-3 cells as well as in trophoblastic cells obtained from placenta of healthy donors [23].

These results led us to the assumption that leptin could promote cell growth and proliferation of trophoblastic cells triggering similar signaling pathways, as previously shown in hepatocytes by enhancing protein synthesis [24–29]. In vitro studies have demonstrated that leptin is capable of stimulating placental system A amino acid transport in a time-specific manner [30] and that this is dependent on activation of the JAK-STAT signaling pathway [31]. In order to further investigate the mechanism by which leptin stimulates cell proliferation, we analyzed protein synthesis in JEG-3 cells and trophoblastic cells. We studied the phosphorylation state of different proteins of the initiation stage of translation and [^{3}H]leucine incorporation in JEG-3 cells using pharmacological inhibitors of the major signaling pathways of the LEPR, PI3K, and MAPK. In this study, we show that leptin stimulates protein synthesis by activating the translation machinery via both PI3K and MAPK pathways.

MATERIALS AND METHODS

Reagents

Human recombinant leptin was from R&D Systems. Antibodies against phosphorylated EIF4E (Ser209) and phosphorylated EIF4EBP1 (PHAS-I) in Thr37 or Thr46 were from New England Biolabs. Monoclonal antibodies against EIF4E and EIF4EBP1 were from Santa Cruz Biotechnology. [^{3}H]leucine (162 Ci/mmol) was purchased from Amersham Pharmacia Biotech. MEK1 inhibitor (PD98059) and PIK3 inhibitor (Wortmannin) were purchased from Sigma-Aldrich.

Cell Culture and Treatments

The human choriocarcinoma cell line JEG-3 (generously provided by Susana Genti-Raimondi, Universidad Nacional de Córdoba, Córdoba, Argentina) was grown in DMEN-F-12 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS), 100 μU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (Invitrogen), and 1 mM sodium pyruvate at 37°C in 5% CO_2.

JEG-3 cells were treated for 10 min in the absence or presence of different concentrations of leptin, then washed with cold PBS and solubilized for 30 min at 4°C in lysis buffer containing 20 mM Tris (pH 8), 1% Nonidet P-40, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 0.4 mM sodium orthovanadate. After centrifugation, the soluble cell lysates were used for Western blot analysis. Total protein levels were determined by the bicinchoninic acid method (Thermo Scientific), using bovine serum albumin as standard.

Protein Synthesis

Cells were grown in 12-well plates (5×10^5 cells per well) in complete medium with 10% fetal bovine serum, and 24 h before leptin treatment, cells were starved in media supplemented with 1% FCS to lower the basal growth rate. Cells were treated with or without leptin for 4 h in the presence or absence of the pharmacological inhibitor wortmannin (50 nM) or PD98059 (100 μM). Next, [^{3}H]leucine (1 μCi/ml) was added, and incubation continued for 2 h. Cells were then washed and solubilized in 0.03% SDS. The lysate was precipitated with 5% trichloroacetic acid. The pellet was resuspended in 150 μl NaOH (1M). The incorporated radioactivity was quantified by scintillation counting. [^{3}H]leucine incorporation was estimated as percent of effect according to its basal protein synthesis rate.

Placental Explant Processing and Treatment

Human placentas (n = 9) were obtained after Caesarean section or vaginal delivery following normal term pregnancies in the Virgen Macarena University Hospital. The Institutional Ethical Review Board approval was obtained for the use of the human placenta. Each placenta was immediately suspended in ice-cold PBS and transported to the laboratory, where it was washed two to three times in sterile PBS to remove excess blood. Villous tissue free of visible infarct or calcification was sampled from at least five cotyledons at a distance midway between the chorionic and basal plates. Placental explants (50 mg) were randomly distributed in the tubes containing 1 ml of a Ham F-12 medium (four replicates per treatment). Incubation of placental explants started in a shaking water bath at 37°C for an equilibration period of 5 min. Then, explants were incubated during 10 min in the same incubation medium supplemented with or without recombinant leptin (0.1–10 nM). Then they were washed twice in PBS by centrifugation for 2 min at $2000 \times g$ and 4°C, resuspended in 500 μl of lysis buffer, incubated at 4°C for 30 min on an orbital shaker, and then centrifuged at $10\,000 \times g$ for 20 min. The supernatants were transferred to new tubes and treated as cellular lysates for Western blot assays. Total protein levels were determined as described above. Assessment of explant viability was routinely monitored by measuring the release of lactate dehydrogenase into medium relative to a 1% Triton X-100 (Sigma)-lysed positive control [32].

Western Blot Analysis

Samples were denatured by adding loading buffer 5× containing 100 mM dithiothreitol and boiled for 5 min. Samples were resolved by 8%–16% SDS-PAGE and then electrophoretically transferred to a nitrocellulose membrane (Amersham). Membranes were blocked by 5% nonfat milk in PBS at room temperature for 1 h, washed, and then incubated using the appropriate antibodies. The antibodies were detected using horseradish peroxidase-linked anti-rabbit/anti-mouse immunoglobulin (Santa Cruz) and visualized using a highly sensitive chemiluminescence system (Supersignal; Pierce).

Statistical Analysis

Results are reported as mean ± SEM and were compared by Student *t*-test or ANOVA for paired data, followed by a post hoc Bonferroni multiple-comparisons test. $P < 0.05$ was considered significant.

RESULTS

Leptin Stimulates the Phosphorylation of EIF4EBP1 and EIF4E in JEG-3 Trophoblastic Cells

To study the stimulation of protein synthesis by the LEPR in JEG-3 trophoblastic cells, we stimulated them with human leptin and analyzed the phosphorylation of EIF4EBP1 by Western blot using antibodies that specifically recognize the tyrosine-phosphorylated form of EIF4EBP1 (Fig. 1A). The amount of EIF4EBP1 in every sample was controlled by anti-EIF4EBP1 immunoblot (Fig. 1A). The effect of leptin on EIF4EBP1 phosphorylation was dose dependent. Although 0.1 nM leptin was sufficient to activate the phosphorylation of EIF4EBP1, maximal phosphorylation of EIF4EBP1 was observed at 10 nM leptin, the dose at which the phosphorylation level of EIF4EBP1 increased almost 10-fold.

Phosphorylation of EIF4EBP1 releases EF4E from the inactive EIF4EBP-EIF4E complex, allowing EIF4E to bind to the cap structure at the 5′ end of the mRNA and activate the translation machinery. Thus, we next decided to study the possible activation of EIF4E by human leptin in JEG-3 cells. As shown in Figure 1B, leptin stimulated EIF4E phosphorylation in a dose-dependent manner. Maximal phosphorylation was observed at 10 nM leptin, but effects were also observed at 0.1 nM leptin. The amount of EIF4E in every sample was controlled by anti-EIF4E immunoblot (Fig. 1B).

Leptin Stimulates the Phosphorylation of EIF4EBP1 and EIF4E in Human Trophoblastic Cells

In order to check whether leptin may activate the same proteins in the initiation stages of translation in normal trophoblast cells as those that we have found in the JEG-3 cells, we performed dose-response experiments using normal placental trophoblast explants. As shown in Figure 2, A and B, leptin stimulated the phosphorylation of EIF4EBP1 and EIF4E in a dose-dependent manner. Maximal phosphorylation of EIF4EBP1 by leptin was obtained at 1 nM leptin, but a

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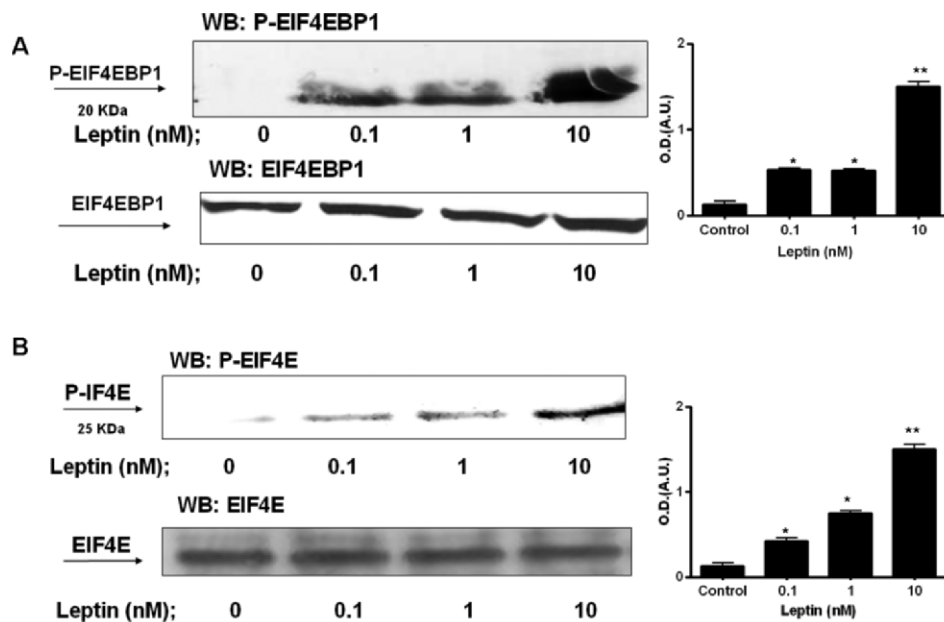

FIG. 1. Leptin stimulates phosphorylation of EIF4EBP1 (**A**) and EIF4E (**B**) in JEG-3 cells. JEG-3 cells were incubated for 10 min with or without leptin. Cells were lysed, denatured, and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose and analyzed by immunoblot with antibodies that recognize phosphorylated EIF4EBP1 in either Thr37 or Thr46 and phosphorylated EIF4E in Ser209. The amount of protein was controlled by immunoblotting with anti-EIF4EBP1 and anti-EIF4E. Leptin increases the phosphorylation level of EIF4EBP1 and EIF4E in JEG-3 cells in a dose-dependent manner. Each experiment shown is representative of four independent experiments. Densitograms with standard error are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. KDa, kilodalton.

significant effect was observed at 0.1 nM leptin (Fig. 2A). Leptin also stimulates the phosphorylation of EIF4E (Fig. 2B), with maximal effect at 10 nM leptin. Nevertheless, 0.1 nM exerted an inductive effect similar to the results obtained with JEG-3 cells. We analyzed the same samples by immunoblot with anti-EIF4EBP1 and anti-EIF4E antibodies for loading control.

Leptin Stimulates Protein Synthesis in JEG-3 and Human Trophoblastic Cells

Leptin effect on protein synthesis was investigated in JEG-3 cells and human villous explants by [^{3}H]leucine incorporation. Cells were seeded at 50%–60% confluence in complete Dulbecco modified Eagle medium-F12 medium (10% FCS), and 24 h before leptin treatment, cells were starved in media supplemented with 1% FCS. Leptin treatment was performed in the same media for 4 h. As can be seen in Figure 3, A and B, leptin enhanced protein synthesis in JEG-3 cells and human trophoblastic cells in a dose-dependent manner. Maximal effect was achieved at 1 nM leptin, which increased twice the basal protein synthesis rate. This effect of leptin on protein synthesis was partially (50%) prevented by pretreatment with wortmannin, an inhibitor from PIK3 pathway. The pretreatment with PD98059, an inhibitor of MAPK pathway, led to 85% inhibition of the leptin effect. As seen in Figure 4, A and B, both the MAPK and PIK3 pathways are required to elicit the effect of leptin on protein synthesis in JEG-3 cells and villous explants, although the major pathway whereby leptin exerts this effect seems to be the MAPK one.

In order to directly check the effect of the PIK3 and MAPK pathways in the leptin activation of protein translation, we confirmed the inhibition of the leptin-mediated phosphorylation of the translation initiation factor EIF4E and its regulatory binding protein (EIF4EBP1) in the presence of the pharmacological inhibitors. As seen in Figure 5, PD98059 and to a lesser

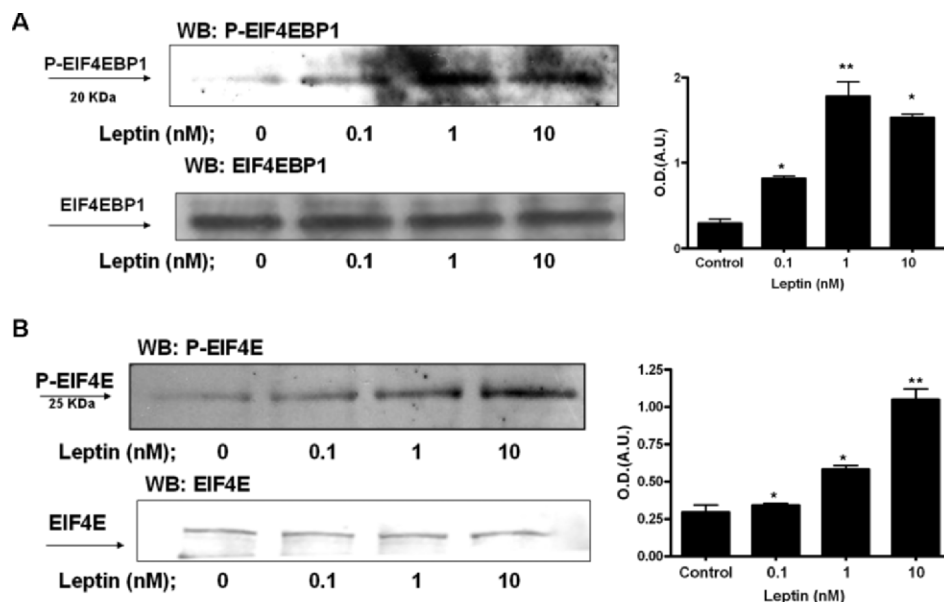

FIG. 2. Leptin stimulates phosphorylation of EIF4EBP1 (**A**) and EIF4E (**B**) in placenta trophoblastic cells. Placenta trophoblastic cells were incubated for 10 min with or without leptin. Cells were lysed, denatured, and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose and analyzed by immunoblot with antibodies that recognize phosphorylated EIF4EBP1 in either Thr37 or Thr46 and phosphorylated EIF4E in Ser209. The amount of protein was controlled by immunoblotting with anti-EIF4EBP1 and anti-EIF4E. Leptin increases the phosphorylation level of EIF4EBP1 and EIF4E in trophoblastic cells in a dose-dependent manner. Each experiment shown is representative of three independent experiments. Densitograms with standard error are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. KDa, kilodalton.

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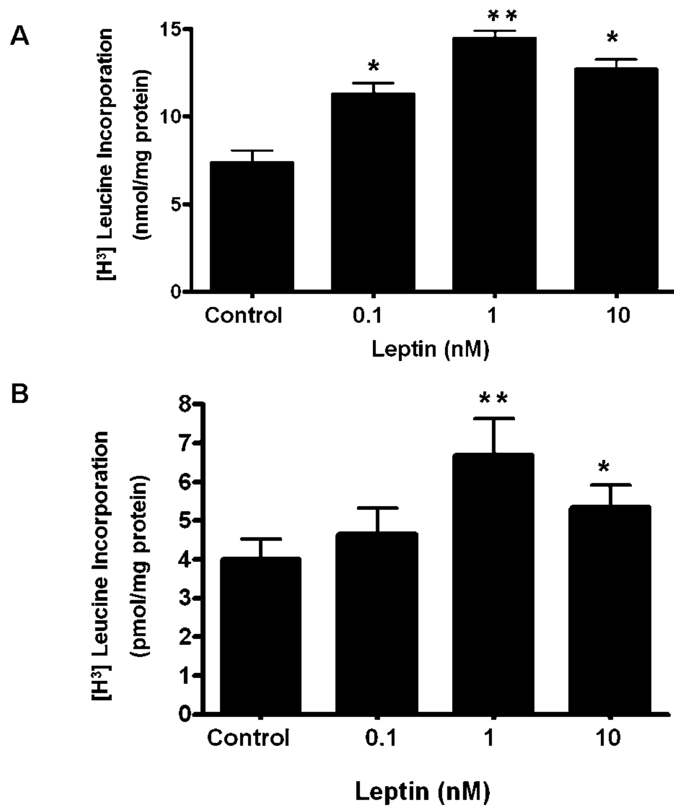

FIG. 3. Leptin stimulates protein synthesis in JEG-3 and human villous explants. **A**) JEG-3 cells grown in DMEN-F12 medium were deprived of serum for 24 h prior to stimulation with leptin. Cells were treated with or without leptin for 4 h and immediately after were treated with [^{3}H]leucine for 2 h. [^{3}H]leucine incorporation was determined as indicated in *Materials and Methods*. Leptin increases the protein synthesis rate in JEG-3 cells in a dose-dependent manner. Maximal effect was achieved at 1 nM leptin. Results are mean ± SEM of three independent experiments run in triplicate. Standard errors are shown. $^*P < 0.05$, $^{**}P < 0.01$ versus control. Protein synthesis is estimated as absolute units. **B**) Villous explants (50 mg), which were randomly distributed in the tubes containing 1 ml of a Ham F-12 medium, were treated with or without leptin for 4 h, and immediately after were treated with [^{3}H]leucine for 2 h. [^{3}H]leucine incorporation was determined as indicated in *Materials and Methods*. Leptin increases the protein synthesis rate in villous explants in a dose-dependent manner. Maximal effect was achieved at 1 nM leptin. Results are mean ± SEM of three independent experiments run in triplicate. Standard errors are shown. $^*P < 0.05$, $^{**}P < 0.01$ versus control. Protein synthesis is estimated as absolute units.

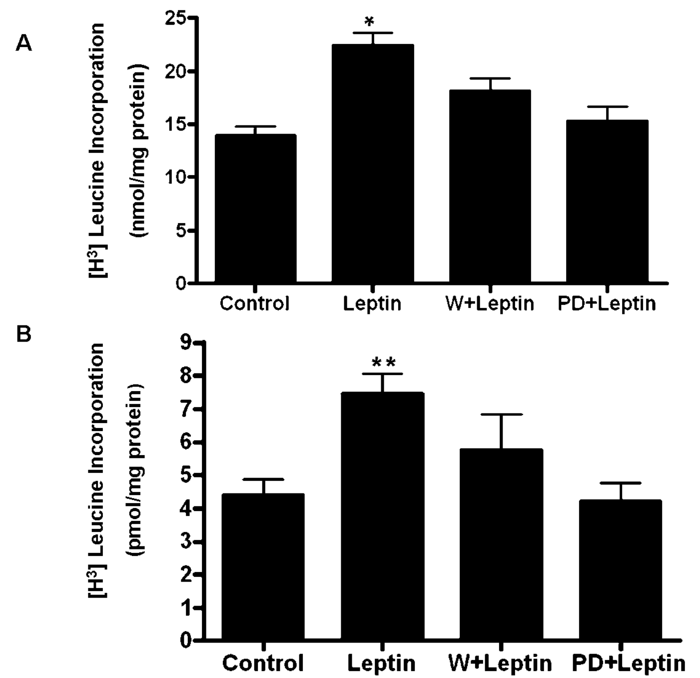

FIG. 4. Inhibition of leptin-mediated protein synthesis in the presence of the pharmacological inhibitors wortmannin (W) and PD98059 (PD) in JEG-3 cells and villous explants. **A**) JEG-3 cells grown in DMEN-F12 medium were deprived of serum for 24 h prior to stimulation with leptin. Cells were treated with or without leptin (10nM) for 4 h in the presence or absence of the pharmacological inhibitor wortmannin (50 nM) or PD98059 (100 μM), and then cells were processed as described in *Materials and Methods*. The effect of leptin on protein synthesis was partially (50%) prevented by pretreatment with wortmannin. The pretreatment with PD98059 led to 85% inhibition of the leptin effect. Standard errors are shown. $^*P < 0.05$ versus control. Results are mean ± SEM of three independent experiments run in triplicate. Protein synthesis is estimated as absolute units. **B**) Villous explants (50 mg) were randomly distributed in the tubes containing 1 ml of a Ham F-12 medium and were treated with or without leptin (1 nM) for 4 h in the presence or absence of the pharmacological inhibitor wortmannin (50 nM) or PD98059 (100 μM), and then explants were processed as described in *Materials and Methods*. The effect of leptin on protein synthesis was partially prevented by pretreatment with wortmannin. The pretreatment with PD98059 led to inhibition of the leptin effect. Standard errors are shown. $^{**}P < 0.01$ versus control. Results are mean ± SEM of three independent experiments ran in triplicate. Protein synthesis is estimated as absolute units.

extent wortmannin prevented the effect of leptin on phosphorylation of EIF4E and EIGF4EBP1. We analyzed the same samples by immunoblot with anti-EIF4E antibodies for loading control.

DISCUSSION

Previously published results have suggested that leptin might play a role in reproduction, particularly in fetoplacental physiology. In addition to white adipose tissue, the placenta is the second place of leptin synthesis in human pregnancy [33, 34]. The presence of LEPR in trophoblast cells indicates that placental leptin may also have a role as an autocrine hormone [35]. Many observations suggest that leptin could be a key player in the regulation of the embryo implantation as well as its maintenance. The altered leptin concentrations associated with certain complications of gestation, such as choriocarcinoma, hydatidiform mole, gestational diabetes, and preeclampsia, suggest the physiological and pathophysiological significance of leptin in pregnancy and in the maintenance of the physiology of the fetoplacental unit [14, 36–38]. The present work describes the effects of leptin on protein synthesis in JEG-3 human choriocarcinoma cells. These cells maintain many characteristics of human trophoblast cells and have been widely used to study placental cellular signaling [39, 40]. Normal trophoblastic explants from healthy donors were also studied to confirm the physiological relevance of the activation of protein synthesis by leptin.

Leptin has been shown to induce proliferative activity in monocytes, lymphocytes, hematopoietic progenitors, and osteoblasts, among others [8, 9, 41, 42]. Insulin also has growth-promoting effects in JAR placental cells [43, 44], suggesting potential cross-talks between insulin and leptin signaling pathways. This may be relevant for the development of excessive placental growth in situations of hyperleptinemia and hyperinsulinemia, such as in pregnancies complicated by diabetes. Thus, to further understand the mechanism of action of leptin in trophoblastic cells, we decided to assess the effect

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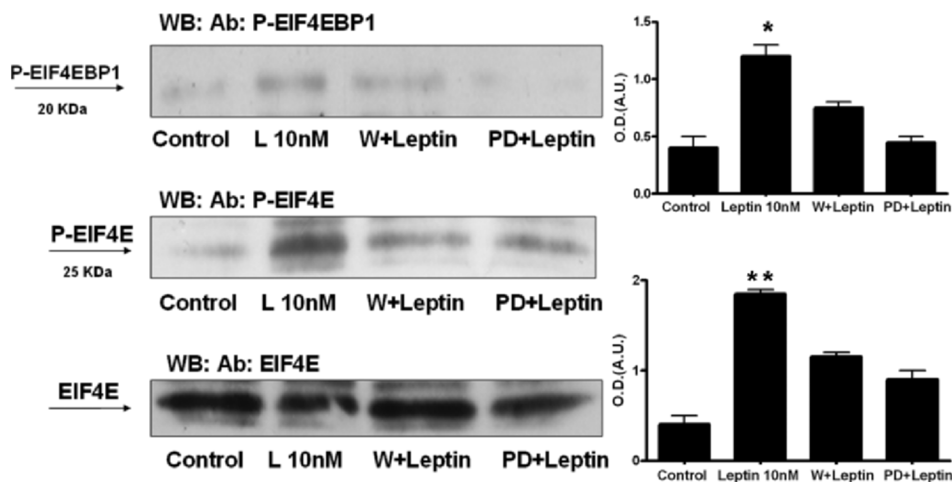

FIG. 5. Inhibition of the leptin-mediated phosphorylation of the translation initiation factor EIF4E and its regulatory binding protein (EIF4EBP1) in the presence of the pharmacological inhibitors wortmannin (W) and PD98059 (PD) in JEG-3 cells and placental explants. JEG-3 cells grown in DMEN-F12 medium were deprived of serum for 24 h prior to stimulation with leptin. Cells were treated with or without leptin (10 nM) for 4 h in the presence or absence of the pharmacological inhibitor wortmannin (50 nM) or PD98059 (100 μM), and then cells were processed as described in *Materials and Methods*. The effect of leptin on phosphorylation of both EIF4EBP1 and EIF4-E was almost completely prevented by pretreatment with PD98059, whereas it was partially inhibited by wortmannin. The amount of protein was controlled by immunoblotting with anti-EIF4E. Results are mean ± SEM of three independent experiments run in triplicate. Each experiment shown is representative of two independent experiments. Densitograms with standard error are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. KDa, kilodalton.

of leptin on protein synthesis that is known to be activated by leptin and insulin in other systems. The effect of insulin in the stimulation of protein synthesis is known to be mediated by activation of RPS6KB1 and phosphorylation of the EIF4EBP1 [45]. Along these lines, we have recently found that leptin stimulates phosphorylation of RPS6KB1in JEG-3 cell lines and in human trophoblast cells [23]. In the present study, we have found that leptin stimulates phosphorylation of EIF4EBP1 and EIF4E in both JEG-3 and trophoblastic cells in a dose-dependent manner. Moreover, this effect correlates with the protein synthesis rate.

On the other hand, it has been extensively reported that MAPK is one of the known kinases that phosphorylates RPS6KB1, EIF4EBP1, and EIF4E [46]. RPS6KB1 also has been shown to be activated by the PIK3-PDK pathway [21, 47]. The PIK3 pathway has been implicated in the regulation of many cellular processes, including resistance to apoptosis, cell motility, differentiation, and proliferation [48]. In this context, we have recently demonstrated the leptin activation of PIK3 and MAPK in human trophoblatic cells [23]. Thus, the activation of translation by leptin may be mediated by MAPK and PIK3 pathway. In this regard, we have demonstrated in the present study that the stimulation of protein synthesis by leptin was almost completely prevented by pre-treatment with PD98059, an inhibitor of MAPK pathway, whereas it was partially inhibited by wortmannin, an inhibitor of PIK3 pathway. These results strongly suggest that both the MAPK and the PIK3 pathways might be involved in the stimulation of protein synthesis by leptin. Our results further support the possible role of leptin in EIF4EBP1 and EIF4E phosphorylation and suggest that this mechanism might contribute to the leptin stimulation of protein synthesis. We can not discard the possibility that the activation of translation and the activation of total protein synthesis may be stimulated by leptin independently. Depending on the predominant pathway activated by leptin, either growth or proliferation effects could be exerted by leptin in placenta cells. On the other hand, wortmannin inhibits all three classes of PIK3, and since PIK3C3 (also known as hVps34, class III PI3K) was recently reported to be critical in mediating the effects of nutrients on MTOR signaling and protein synthesis [49], the wortmannin-dependent component of leptin-stimulated protein synthesis observed in the current study may be mediated not only by classical PIK3 pathways but also by class III PI3K. Moreover, PD 98059 is also known to inhibit cyclooxygenases 1 and 2, the rate-limiting enzymes in the production of prostaglandins and leukotrienes [50]. This is important since prostaglandins activate MTOR signaling [51] and, therefore, protein synthesis.

Therefore, we have provided some evidence for the possible role of leptin, produced by trophoblastic cells, in the physiology of the placenta, describing the effect of leptin on protein synthesis in trophoblastic cells, and we have pointed to some of the signaling pathways underlying this effect.

This is the first time that leptin activation of the translation machinery in placenta has been reported. This may be relevant both physiologically and pathophysiologically since a decrease in EIF4EBP1 phosphorylation has been recently found in intrauterine growth restriction of the fetus, resulting from impaired placental development [52].

In conclusion, leptin effect on protein synthesis in JEG-3 human choriocarcinoma cells and in human placenta seems to be mediated mainly by MAPK, but also in part by PIK3, and both pathways might be necessary for leptin regulation of protein synthesis, at least in part modulating translation machinery, i.e., the phosphorylation of the initiation factor EIF4E and the phosphorylation of the EIF4E-binding protein, EIF4EBP1.

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