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MAPK and PI3K activities are required for leptin stimulation of protein synthesis in human trophoblastic cells

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ABSTRACT

Leptin, the LEP gene product, is produced in placenta where it has been found to be an important autocrine signal for trophoblastic growth during pregnancy. Thus, we have recently described the antiapoptotic and trophic effect of leptin on choriocarcinoma cell line JEG-3, stimulating DNA and protein synthesis. We have also demonstrated the presence of leptin receptor and leptin signaling in normal human trophoblastic cells, activating JAK-STAT, PI3K and MAPK pathways. In the present work we have employed dominant negative forms of MAPK and PKB constructs to find out the signaling pathways that specifically mediates the effect of leptin on protein synthesis. As previously shown, leptin stimulates protein synthesis as assessed by ^{3}H-leucine incorporation. However, both dominant negative forms of MAPK and PKB inhibited protein synthesis in JEG-3 choriocarcinoma cells. The inhibition of PKB and MAPK activity by transfection with the dominant negative kinases prevented the leptin stimulation of p70 S6K, which is known to be an important kinase in the regulation of protein synthesis. Moreover, leptin stimulation of phosphorylation of EIF4EBP1 and EIF4E, which allows the initiation of translation was also prevented by MAPK and PI3K dominant negative constructs. Therefore, these results demonstrate that both PI3K and MAPK are necessary to observe the effect of leptin signaling that mediates protein synthesis in choriocarcinoma cells JEG-3.

1. Introduction

Leptin is one of the best-characterized adipokines that modulates carbohydrate, lipid and protein metabolism. This hormone has a broad pattern of expression in the organism and it has been implicated in many different functions, including reproduction. Leptin and its receptor have been shown to be expressed in ovary [1–5] and testis [6–8], as well as in several other tissues of the reproductive tract, where the most important source of leptin is placenta [9]. Circulating leptin levels are increased during pregnancy [10]. Possible physiological effects of placenta-derived leptin include angiogenesis, growth, and immunomodulation [11,12]. Compelling evidence in recent years also implicated different leptin functions, such as the regulation of embryo development and implantation [13–15]. In this process molecular interactions between leptin and other cytokines are crucial for the establishment of pregnancy [13], although knowledge of the cytokine functions in this system is limited [16]. We have previously provided evidence that point to the role of leptin as a trophic and mitogenic factor for human peripheral blood mononuclear cells [13,17], as well as for trophoblastic cells via MAPK activation [18] in the sense that it inhibits apoptosis and promotes proliferation [19]. In vitro studies have demonstrated that among the different hormones secreted by the placenta, leptin and others hormones like human chorionic gonadotropin (hCG) play an important role and they are involved in an autocrine/paracrine loop regulating placental function and the fetal growth. In this regard, we have recently demonstrated that hCG up-regulates leptin expression most likely by using the MAPK signal transduction pathway [20]. However, we have also demonstrated that leptin stimulates PIK3 pathway in JEG-3 cells as well as in trophoblastic cells obtained from placenta of healthy donors [18]. 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 [21–23].

In this context, the signaling mechanisms that mediate the regulation of protein synthesis involve the activation of ribosomal p70 S6 kinases [24]. Besides, protein synthesis is known to be regulated by insulin and other hormones like leptin by phosphorylation of

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different translation factors that are involved in the initiation and elongation stages of translation [25,26]. Thus, the initiation factor EIF4E binds to the cap structure at the 50-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) [27]. 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 [28]. The activity of this complex is also regulated by phosphorylation of EIF4E [27,29]. Consistent with this observation, our group has demonstrated EIF4E and EIF4EBP1 phosphorylation in adipocytes [30], hepatocytes, in rat hepatoma cell line HTC [31] as well as in JEG-3 cells and explants obtained from placenta [25]. In human placenta, leptin effect on protein synthesis 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, as assessed by using pharmacological inhibitors. Interestingly, in vitro studies have demonstrated that leptin is capable of stimulating placental system of amino acid transport in a time-specific manner [32] and that this is dependent on activation of the JAK-STAT signaling pathway [33]. 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 [34–36]. In order to further investigate the pathway by which leptin stimulates protein synthesis, we studied the phosphorylation state of different proteins of the initiation stage of translation and [^{3}H]leucine incorporation in JEG-3 cells using the kinase inactive mutant of the major signaling pathways of the receptor leptin, MAPK and PI3K. In this study, we show that leptin stimulates protein synthesis by activating the translation machinery via both PI3K and MAPK pathways.

2. Materials and methods

2.1. Reagents

Human recombinant leptin was from R&D Systems. Antibodies against phosphorylated EIF4E (Ser209), phosphorylated EIF4EBP1 (PHAS-I) in Thr37/Thr46, and phosphorylated p70 S6K (Thr 421/Ser 424) were from Cell Signaling. Monoclonal antibodies against EIF4E, EIF4EBP1, PKB, and actin were from Santa Cruz Biotechnology. [^{3}H]leucine (162 Ci/mmol) was purchased from Amersham Pharmacia. The pcDNA1 plasmids encoding the kinase inactive mutant of MAPK (designated MAPKkd) and the kinase inactive mutant of PI3K pathway (designed PKBkd) [37] was kindly supplied by Dr. J. Silvio Gutkind (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD).

2.2. Cell culture and treatments

The human choriocarcinoma cell line JEG-3 (generously provided by Susana Genti-Raimondi, Universidad Nacional de Córdoba, Argentina) was grown in DMEM-F12 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS), 100 μU/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine (Invitrogen), and 1 mM sodium pyruvate at 37 °C in 5% CO_2. JEG-3 cells were transfected with the plasmids MAPKkd and PKBkd and were treated with or without leptin 10 nM for 10 min, 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).

2.3. Protein synthesis

Cells were grown in 12-well plates (5×10^5 cells/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. JEG-3 cells were transfected with the plasmids MAPKkd and PKBkd and were treated with or without leptin 10 nM for 4 h. The empty plasmid was used as control. 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 (1 M). 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. Transfection was performed by triplicate in each of at least three independent experiments.

2.4. 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).

2.5. 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.

3. Results

The choriocarcinoma cell line JEG-3 was used as a model for human trophoblastic cells. These cells maintain many characteristics of human trophoblast cells and have been widely used to study placental cellular signaling [38,39].

3.1. MAPK and PKB inhibition by dominant negative expression inhibits the effect of leptin on protein synthesis in JEG-3 trophoblastic cells

Previous results had already shown that leptin stimulates the protein synthesis in JEG-3 cells and human villous explants by [^{3}H]leucine incorporation. This effect of leptin on protein synthesis was prevented by inhibitors both the MAPK and PIK3 pathways in JEG-3 cells and villous explants [25]. In fact, leptin is known to stimulate MAPK and PI3K pathways in different systems including placenta [40–43,18]. In order to further investigate the pathway by which leptin stimulates protein synthesis, we studied the leptin effect on protein synthesis by [^{3}H]leucine incorporation in JEG-3 cells overexpressing dominant negative forms of MAPK or PI3K. Serum-starved trophoblastic cells were transfected with the plasmids MAPKkd and PKBkd and then, they were treated with or

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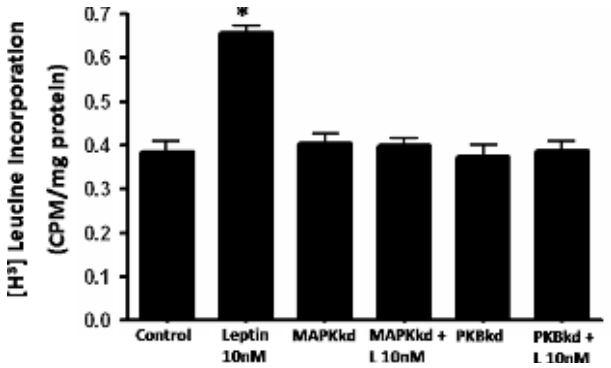

Fig. 1. Inhibition of leptin-mediated protein synthesis in the presence of dominant negative forms of MAPK and PKB in JEG-3 cells. Cells grown in DMEM-F12 medium were transfected with both plasmids and deprived of serum for 24 h prior to stimulation with leptin. Cells were treated with or without leptin (10 nM) for 4 h and then cells were processed as described in Section 2. The effect of leptin on protein synthesis was prevented by both PKBkd and MAPKkd. 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.

without leptin 10 nM for 4 h. The empty plasmid was used as control. As seen in Fig. 1, both the MAPK and PIK3 pathways are required to elicit the effect of leptin on protein synthesis in JEG-3 cells, although MAPK seems to be the major pathway whereby leptin exerts this effect.

3.2. MAPK and PKB inhibition by dominant negative expression inhibits leptin stimulation of S6 kinase, and phosphorylation of EIF4EBP1 and EIF4E in JEG-3 trophoblastic cells

Previous results showed that leptin stimulates the phosphorylation of S6K, EIF4EBP1 and EIF4E in trophoblastic cells and this effect was dose dependent [18,25]. Moreover we have also demonstrated, by using pharmacological inhibitors of MAPK and PIK3 pathways, that this effect may be mediated mainly by MAPK, but also in part by PIK3, at least in this cell line. To further understand the mechanism of action of leptin in trophoblastic cells we checked the relative importance of both pathways by using of plasmids encoding the kinase inactive mutant of MAPK and PKB, the central kinase of the PI3K pathway .We measured the activation of p70 S6K, EIF4EBP1 and EIF4E, by immunoblot using antibodies that specifically recognize the phosphorylated form of each protein.

Downstream of MAPK, as well as PI3K and 3-phosphoinositide-dependent protein kinase (PDK), p70 S6K is activated. p70 S6K is an important translational integration site [44]. Since p70 S6K activity is correlated with its phosphorylation state, specifically Thr421/Ser424 [45], we employed polyclonal antibodies that detect p70 S6K only when phosphorylated at Thr421/Ser424. As seen in Fig. 2, both the MAPK and PIK3 pathways prevented the effect of leptin on phosphorylation of p70 S6K. The effect of leptin on phosphorylation of p70 S6K was controlled by western blotting using anti-β-actin antibodies to exclude differences in protein content of samples.

The MAPK and PI3K pathways are known to activate protein synthesis by activation of translation and they converge in the phosphorylation of the proteins involved in the initiation of translation [46]. As shown in Fig. 3, the phosphorylation of both EIF4EBP1 (Fig. 3A) and EIF4E (Fig. 3B) was almost completely prevented by the dominant negative forms of MAPK and PKB. The amount of EIF4EBP1 and EIF4E in every sample was controlled using anti-EIF4EBP1 and anti-EIF4E antibodies.

4. Discussion

Pleiotropic effects of leptin have been identified during the last decade. Thus, leptin is involved in the modulation of several processes, such as angiogenesis, growth, and immunomodulation. [10]. Compelling evidence in recent years also suggested that leptin has a role in reproduction, particularly in the fetoplacental physiology [13–15]. In addition to white adipose tissue, the placenta is the second place of leptin synthesis in human pregnancy [9,47]. The presence of leptin receptor in trophoblast cells indicates that placental leptin may also have a role as an autocrine hormone [48,49]. 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 [13,16,35].

Leptin has been shown to induce proliferative activity in monocytes, lymphocytes, hematopoietic progenitors, and osteoblasts, among others [50–53]. Insulin also has growth-promoting effects in JAR placental cells [54,55], 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 gestational diabetes. Thus, to further understand the mechanism of action of leptin in trophoblastic cells, we decided to assess the effect and mechanism of action of leptin on protein synthesis. The stimulation of protein synthesis is known to be mediated by activation of p70 S6K and phosphorylation of the EIF4EBP1 [56]. Along these lines, we have recently found that leptin stimulates phosphorylation of p70 S6K in JEG-3 cell lines and in human trophoblast cells [18]. Moreover, we have also found that leptin stimulates phosphoryla-

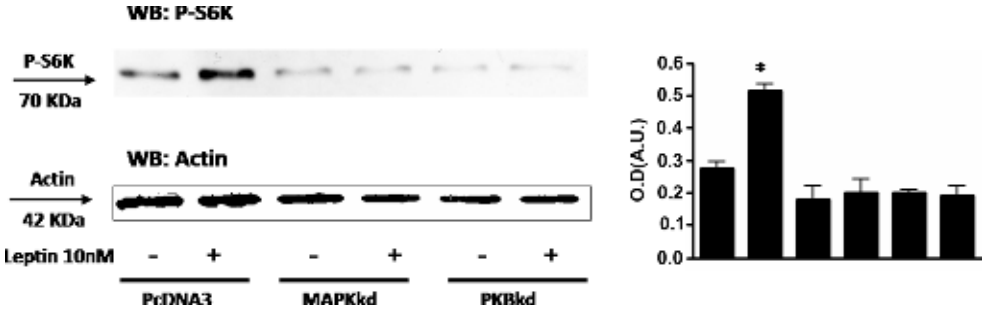

Fig. 2. Leptin stimulation of p70 S6K is abolished by the negative dominant forms of MAPK and PKB. Cells were seeded at 50–60% confluence and transfected with the plasmids MAPKkd and PKBkd. Serum-starved cells were incubated for 10 min with or without leptin. Cells were lysed, denatured, and subjected to SDS–PAGE. Proteins were transferred to nitrocellulose and analyzed by immunoblot with antibodies that recognize phosphorylated p70 S6K. The amount of protein in each sample was controlled by using anti-actin antibody. Each experiment shown is representative of three independent experiments. Densitograms with standard error are shown. $^{*}P < 0.01$ versus control.

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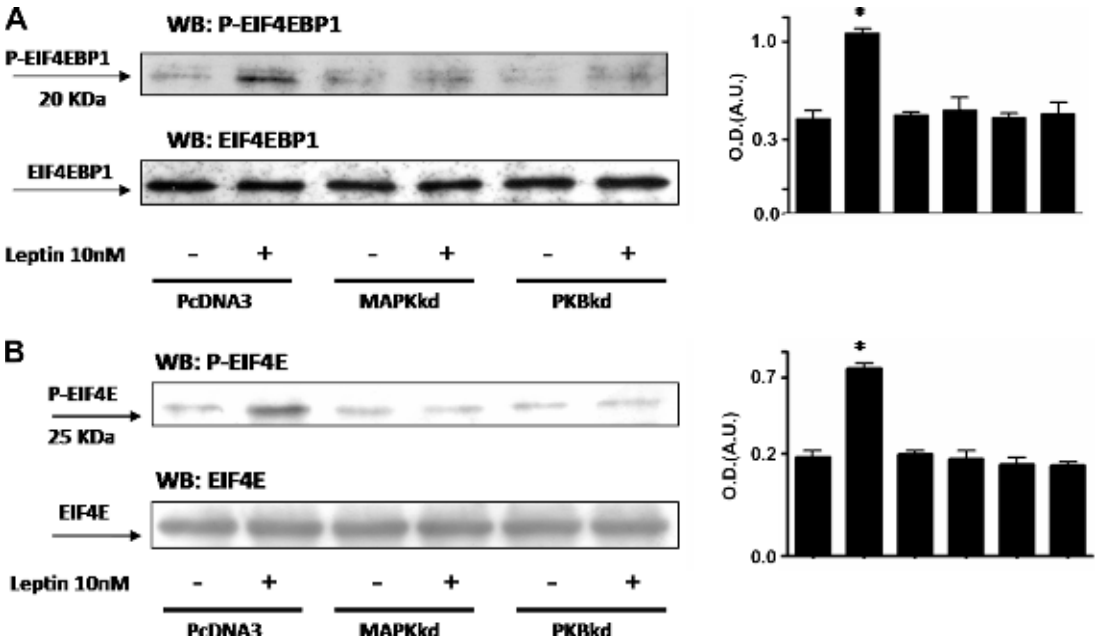

Fig. 3. Leptin stimulation of EIF4EBP1 and EIF4E phosphorylation in JEG-3 trophoblastic cells is prevented by the dominant negative forms of MAPK and PKB. Cells were treated as described in Fig. 2. Cells were lysed, denatured, and subjected to SDS–PAGE. Proteins were transferred to nitrocellulose and 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. Each experiment shown is representative of three independent experiments. Densitograms with standard error are shown. $^{*}P < 0.01$ versus control.

tion of EIF4EBP1 and EIF4E in both JEG-3 and trophoblastic cells in a dose-dependent manner [25]. In the same form, in the present study, we have found that this effect correlates with the protein synthesis rate.

In this context, it has also been extensively reported that MAPK is one of the known kinases that phosphorylates p70 S6K, EIF4EBP1, and EIF4E [57]. p70 S6K has also been shown to be activated by the PIK3-PDK pathway [58,59]. In fact, both PI3K and MAPK may regulate the activation of S6K [60]. The PIK3 pathway has been implicated in the regulation of many cellular processes, including resistance to apoptosis, cell motility, differentiation, and proliferation [61]. In this line, we have recently demonstrated the leptin activation of PIK3 and MAPK in human trophoblastic cells [18]. Thus, the activation of translation by leptin may be mediated by MAPK and PIK3 pathway. Moreover, we have also demonstrated 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 [25]. Now, these results have been confirmed using a more specific approach, such as the dominant negative forms of MAPK and PKB. Data obtained in the present work strongly suggest that both the MAPK and the PIK3 pathways are involved in the stimulation of protein synthesis by leptin. Thus, we have demonstrated in the present study that the stimulation of protein synthesis by leptin completely prevented by inhibition with the dominant negative mutant forms of MAPK and PI3K. 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 on protein synthesis, although, we can not discard the possibility that the activation of the initiation 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.

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 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 [62].

In conclusion, leptin effect on protein synthesis in JEG-3 human choriocarcinoma cells seems to be dependent on both MAPK and PIK3 activity, and both pathways might be necessary for leptin regulation of protein synthesis, not only regulating S6K but also 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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