Up-Regulation of Placental Leptin by Human Chorionic Gonadotropin

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Leptin, the 16,000 molecular weight protein product of the obese gene, was originally considered as an adipocyte-derived signaling molecule for the central control of metabolism. However, leptin has been suggested to be involved in other functions during pregnancy, particularly in placenta, in which it was found to be expressed. In the present work, we have found that recombinant human chorionic gonadotropin (hCG) added to BeWo choriocarcinoma cell line showed a stimulatory effect on endogenous leptin expression, when analyzed by Western blot. This effect was time and dose dependent. Maximal effect was achieved at hCG 100 IU/ml. Moreover, hCG treatment enhanced leptin promoter activity up to 12.9 times, evaluated by transient transfection with a plasmid construction containing different promoter regions and the reporter gene luciferase. This effect was dose dependent and evidenced with all the promoter regions analyzed, regardless of length. Similar results were obtained with placental explants, thus indicating physiological relevance. Because hCG signal transduction usually involves cAMP signaling, this pathway was analyzed. Contrarily, we found that dibutyryl cAMP counteracted hCG effect on leptin expression. Furthermore, cotransfection with the catalytic subunit of PKA and/or the transcription factor cAMP response element binding protein repressed leptin expression. Thereafter we determined that hCG effect could be partially blocked by pharmacologic inhibition of MAPK pathway with 50 μM PD98059 but not by the inhibition of the phosphatidylinositol 3-kinase pathway with 0.1 μM wortmannin. Moreover, hCG treatment promoted MAPK kinase and ERK1/ERK2 phosphorylation in placental cells. Finally, cotransfection with a dominant-negative mutant of MAPK blocked the hCG-mediated activation of leptin expression. In conclusion, we provide some evidence suggesting that hCG induces leptin expression in trophoblastic cells probably involving the MAPK signal transduction pathway. **(*Endocrinology* 150: 304–313, 2009)**

The process of embryo implantation and trophoblast invasion is currently considered as the most limiting factor for the establishment of pregnancy. Molecular interactions at the embryo-maternal interface during the time of adhesion and subsequent invasion are crucial to the process of embryonic implantation (1). This process takes place during the first weeks of pregnancy when the well-differentiated primary cells of the placenta, known as trophoblast cells, grow in an invasive fashion. There is evidence suggesting that cytokines produced by the maternal endometrium and the developing embryo play an important role in this signaling process. Although numerous cytokine-receptor pairs are expressed by the maternal endometrium and the embryo during implantation, their temporal pattern of expression is still incompletely understood (2). Several cytokines and growth factors are known to influence trophoblast migration, proliferation and invasion (3). In particular, leptin has been proposed to play a relevant role in implantation by virtue of its stimulatory effect on matrix metalloproteinase expression in cy-

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Abbreviations: $(Bu)_2$cAMP, Dibutyryl cAMP; C/EBP, CCAAT/enhancer-binding protein; CREB, cAMP response element binding protein; FCS, fetal calf serum; hCG, human chorionic gonadotropin; IBMX, 3-isobutyl-1-methylxanthine; MEK, MAPK kinase; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A.

totrophoblasts (4). On the other hand, 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 (5, 6).

Leptin hormone, the product of the *LEP* gene, is a 16,000 molecular weight nonglycosylated polypeptide of 146 amino acids discovered in 1994 by Zhang *et al.* (7). This cytokine-type hormone is able to exert multiple functions; the best characterized is the regulation of food intake and energy expenditure, especially under conditions of restricted energy availability. In this regard, leptin is produced by white adipose tissue and secreted in response to energy store. Thus, plasma leptin levels correlate with total adipose mass (8). Leptin is also secreted by placenta and gastric mucosa (9).

During the last years, pleiotropic effects of leptin have been identified, consisting in the modulation of several processes, such as thermogenesis, homeostasis, angiogenesis, hematopoiesis, osteogenesis, chondrogenesis, neuroendocrine, and immune functions as well as arterial pressure control (10). Compelling evidence also implicated leptin in reproductive functions such as the regulation of ovarian function, oocyte maturation, embryo development, and implantation (11–13). However, the complete regulation of leptin production is still poorly understood, and particularly it is unclear why the concentrations of this protein rise abruptly in early pregnancy (10).

Human chorionic gonadotropin (hCG) mediates its action through the LH/hCG receptor, and its major function is to maintain the progesterone production of corpus luteum during early pregnancy. hCG has probably many other functions, being one of the earliest embryonic signals; it is already expressed in eight-cell embryos and is secreted in high local concentrations by the blastocyst entering the uterine cavity. Therefore, it is probably one of the embryonic signals involved in the embryo-maternal dialogue regulating implantation (14). Both LH and hCG interact with the same receptor. It is a large cell surface glycoprotein with the characteristic structure of the G protein-coupled receptor superfamily (15, 16). Binding of hCG to its receptor generates signal transduction through the activation of the associated heterotrimeric G-proteins. In gonadal tissues, as it is in the classical response, after the binding of CG to the receptor there is an increase in cAMP upon activation of the adenylyl cyclase, and a consequent activation of protein kinase A (PKA) as well as an increase in the intracellular calcium through inositol triphosphate/phospholipase A2 pathway (17, 18). It was reported that hCG induces the expression of specific genes such as vascular endothelial growth factor, leukemia inhibitory factor, and metalloproteinase-9, all important in modulating both the immune system and cell survival at the maternal-fetal interface (16).

Syncytiotrophoblasts contain both leptin and functional LH/CG receptors (19, 20). Because hCG has long been associated with the initiation and maintenance of pregnancy, we hypothesized that it might be involved in the regulation of leptin expression in trophoblast cells. In this study we demonstrate that hCG indeed up-regulates leptin expression probably involving the MAPK signal transduction pathway.

Materials and Methods

Cell culture and treatments

The human choriocarcinoma cell line BeWo was grown in 45% DMEM and 45% HAM F-12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (Invitrogen), and 1 mM sodium pyruvate (Sigma Chemical Co., St. Louis, MO) at 37 C in 5% CO_2.

The effect of recombinant hCG (Sigma) was tested at different doses (25–500 IU/ml). Once hCG maximal effect on leptin expression was determined, in the following experiments 50 or 100 IU/ml hCG were used. In experiments designed to analyze the hCG-dependent signal transduction pathway, the cAMP analog dibutyryl cAMP [$(Bu)_2$cAMP; 0.1 μM to 1 mM] was used to facilitate cell entrance. The nonspecific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 0.1 mM), the specific phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (0.1 μM), and the MAPK kinase (MEK) inhibitor PD98059 (10 or 50 μM) (Sigma) were also used. All treatments were performed in DMEM-F12 media supplemented with 1% FCS unless indicated. Serum present in the media of incubation was reduced from 10 to 1% to lower nonspecific effects.

Placental explants collection and processing

Human placentas (n = 9) were obtained after cesarean section or vaginal delivery after normal term pregnancies 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 (10–15 mg wet weight) and thoroughly rinsed with cold Hanks' medium (pH 7.4) (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 $NaHCO_3$). None of the donor patients suffered from anomalous pregnancy. This study was approved by the patient's written consent and the local ethical committee.

Treatments of placental explants

Placental explants were randomly distributed in tubes containing 1 ml of Hanks' medium (n = 1 explant/tube, four replicates per treatment). Placental explants were maintained in a shaking water bath at 37 C during 5 min to equilibrate temperature and incubated for 10 min in the same medium supplemented or not with 10–500 IU/ml hCG and/or 50 μM PD98059. Explants were removed from the bath, centrifuged for 2 min at 2000 $\times$ *g* at 4 C and resuspended in 500 μl of lysis buffer (1$\times$ PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 10 mg/ml phenylmethanesulfonyl fluoride) during 30 min at 4 C on an orbital shaker and later centrifuged at 10,000 $\times$ *g* for 20 min. Supernatants were analyzed by Western blot.

Western blot analysis

Total cell lysates were prepared in lysis buffer. The lysates were centrifuged at 10,000 $\times$ *g* for 10 min to remove cellular debris. The protein concentration of the supernatant was determined by the Bradford staining method (21), with BSA as standard. Lysates were mixed with Laemmli's sample buffer containing 2% sodium dodecyl sulfate 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, Buckinghamshire, UK). Membranes were equilibrated in 1$\times$ PBS, and nonspecific binding sites were blocked by 5% nonfat milk in PBS at room temperature for 1 h. The membranes were then immunoblotted with polyclonal rabbit antihuman leptin Y20 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), with polyclonal rabbit antiphospho-ERK 1/2 (Thr202/Tyr204; 1:3000) or polyclonal rabbit anti-phospho-MEK (Ser217/Ser221) antibody (1:3000; Sigma). Loading controls were performed by immunoblotting the same mem-

branes with polyclonal rabbit anti-β-actin (1:5000; Sigma), with polyclonal rabbit anti-total-ERK 1/2 (1:3000) or polyclonal rabbit anti-total-MEK antibody (1:3000; Sigma). The antibodies were detected using horseradish peroxidase-linked goat antirabbit IgG (1:10,000; Santa Cruz) and visualized by the Amersham Pharmacia enhanced chemiluminescence signaling system and a Bioimaging analyzer (LAS-1000; Fujifilm, Tokyo, Japan). Quantification of protein bands was determined by densitometry using Image Gauge version 3.12 software (ScienceLab; Fujifilm).

Plasmids

The luciferase reporter constructs based on PGL-3 basic vector are shown schematically (see Fig. 3). They were all kindly provided by Dr. Oksana Gavrilova (Bethesda, MD) (22). To simplify the notation, we renamed them indicating the number of base pairs upstream transcription initiation as follows: pL2922 (p1774), pL1951 (p1775), pL1546 (p1776), pL948 (p1777), and pL218 (p1779). pRSV-βgal contains the β-galactosidase gene under the control of the Rous sarcoma virus promoter and was used to normalize the efficiency of individual transfections. pMtC-α is a 5.4-kb expression vector plasmid containing the cDNA for the α-isoform of the mouse cAMP-dependent protein kinase (PKA) catalytic subunit (23). Expression vector for the wild-type cAMP response element binding protein (CREB) named pCREB3 (24) was kindly provided by Dr. P. Sassone-Corsi (Santa Cruz, CA). The pcDNA1 vector encoding the kinase inactive mutant of ERK2 (designated MAPK-kd) (25) was kindly supplied by Dr. J. Silvio Gutkind (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD). In experiments using expression plasmids, the empty vectors were used as controls. To perform transient transfection assays, plasmids were purified using the Maxipreps Wizard kit (Promega Co., Madison, WI), and the concentration of DNA was estimated spectrophotometrically.

Transient transfection experiments

For transient transfection experiments, BeWo cells were plated at a density of 2.5×10^5 cells/ml onto six-well dishes containing 2 ml DMEM-F12 plus 10% FCS. Cells were incubated for 24 h. 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 *vs.* light emission was performed (data not shown). Typically 5 μg of the luciferase reporter and 5 μg of pRSV β-galactosidase internal control construct were transfected using 5 μl of LipofectAMINE (Life Technologies, Inc., Grand Island, NY). The medium was replaced after 5 h with DMEM-F12 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 luciferase and β-galactosidase activities

Luciferase activity in cell lysates was measured using the luciferase assay system (Promega). 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 luciferase assay buffer. Luciferase activity was measured with a junior luminometer (Hidex, Turku, Finland). β-Galactosidase activity was assayed using 1 mg *o*-nitrophenyl β-D-galactopyranoside (AmResco, Solon, OH) as the substrate in buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.07% β-mercaptoethanol) and incubated at 37 C until yellow staining was obtained. 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 luciferase activity per unit of β-galactosidase activity. Duplicate samples were analyzed for each data point.

Data analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as the mean ± SD. The statistical significance was assessed by ANOVA followed by different *post hoc* tests indicated in each figure and was calculated using the Instat computer program (GraphPad, San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

Recombinant hCG induces endogenous leptin expression in placental cells in a dose- and time-dependent manner

The choriocarcinoma cell line BeWo was used as a model for trophoblastic cells as previously reported (26, 27). Previous results showed that leptin and leptin receptor are expressed in this cell line, suggesting that leptin is probably exerting both a paracrine and an autocrine effect (19). In this work we investigated whether hCG was able to modulate leptin expression in not only BeWo cells but also human placental explants. Cells were seeded at 50–60% confluence in complete DMEM-F12 medium and 10% FCS. hCG treatment was performed in the same media supplemented with 1% FCS during 3 d. As seen in Fig. 1A, hCG enhanced leptin expression in BeWo cells as determined by Western blot. This effect was dose dependent, reaching a 3.5-fold increase that turned out to be statistically significant. Maximal effect was achieved at 100 IU/ml hCG. At a higher dose (500 IU/ml), leptin expression returned to control level. This phenomenon is probably due to transient loss of second-messenger production (desensitization) and loss of cell surface receptors (down-regulation) in response to pharmacological doses of the ligand (28). To achieve a more physiological system to study hCG effect, human placental explants from healthy donors were analyzed. The explants were incubated during 10 min in medium supplemented or not with different hCG concentrations (10–500 IU/ml). Figure 1B shows that hCG enhanced leptin expression in placental explants, measured by Western blot. This effect was dose dependent, reaching 5.5-fold increase that turned out to be statistically significant. Maximal effect was achieved at 100 IU/ml hCG. To further characterize hCG effect on leptin expression in trophoblastic cells, time-course experiments were carried out in BeWo cells stimulated with 50 or 100 IU/ml hCG. hCG treatment was performed in DMEM-F12 media supplemented with 1% FCS. As shown in Fig. 1C, the presence of the hormone in the culture media produced an increase in leptin expression measured by Western blot. Maximal effect was obtained at 72 h of treatment with 100 IU/ml hCG, reaching a 6.9-fold increase above control, even though at 24 and 48 h of treatment with 100 IU/ml hCG, a 5-fold leptin induction was already observed. These results reinforce the notion that hCG has a role in regulating leptin expression.

Leptin promoter activity is induced by recombinant hCG in a dose-dependent manner

Because hCG showed an inducing effect on leptin expression, we next studied whether this effect was exerted at the transcriptional level. Transient transfection assays were performed with a vector containing the regulatory region of leptin gene from −1951 bp fused to the luciferase reporter gene (pL1951). Luciferase expression under the control of leptin promoter was

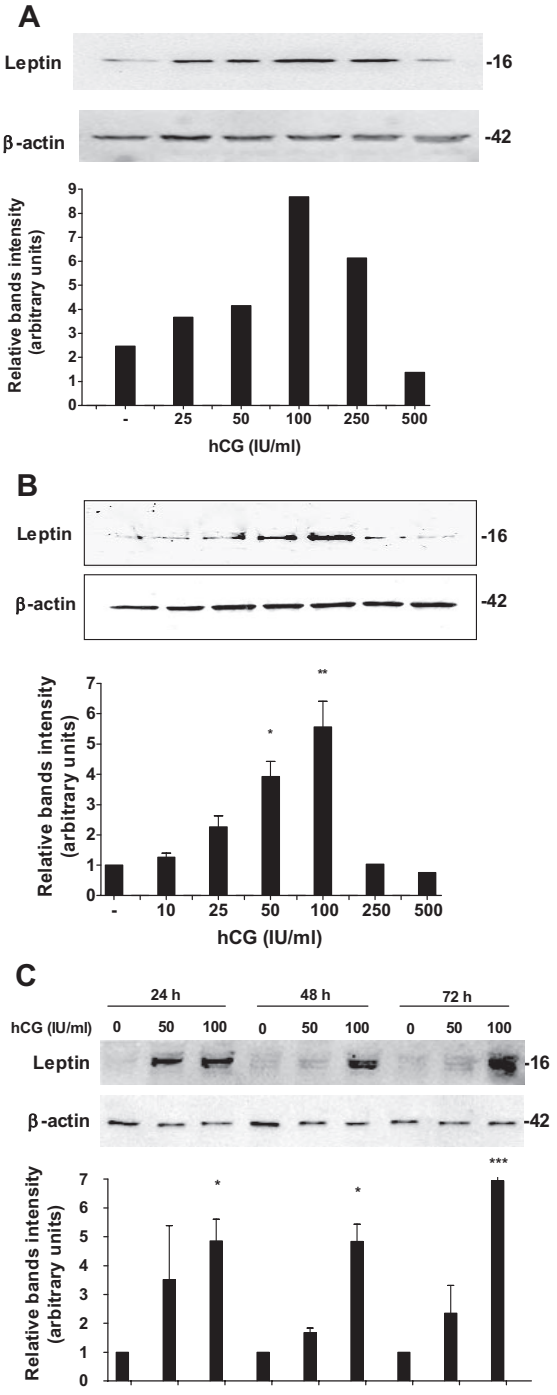


FIG. 1. hCG enhances leptin expression in placental cells. A, BeWo cells (1×10^6 cells) were plated in complete DMEM-F12 media supplemented with 1% FCS and incubated for 3 d with different doses of hCG (international units per milliliter). B, Placental explants were processed as previously described and treated with increasing hCG doses for 10 min. C, BeWo cells were incubated for 24 (lanes 1–3), 48 (lanes 4–6), or 72 h (lanes 7–9) and) treated with 50 IU/ml hCG (lanes 2, 5, and 8), 100 IU/ml hCG (lanes 3, 6, and 9) or not treated (lanes 1, 4, and 7). Cell extracts were prepared as indicated in *Materials and Methods*. Proteins were separated on SDS-PAGE gels and leptin expression was determined by Western blot. Molecular weights were estimated using standard protein markers. Loading controls were performed by immunoblotting the same membranes with anti-β-actin. Bands densitometry is shown in *lower panels*. Representative results from three replicates are shown. Statistical analyses were performed by ANOVA. *Asterisks* indicate significant differences from the control according to Bonferroni's multiple comparison *post hoc* test. *, $P < 0.05$; **, $P < 0.01$ *vs.* control. In all Western blots, molecular mass (kilodaltons) are indicated at the right of the blot.

proportional to the amount of plasmid transfected up to 15 μg (data not shown). As shown in Fig. 2A, treatment with hCG significantly increased Luc expression in cells transfected with pL1951. Maximal effect was achieved at 100 IU/ml hCG, where a 23-fold induction over control basal level was obtained. Promoter activity began to decrease at higher hCG doses. Ours results indicate that hCG enhances leptin promoter activity and regulates leptin gene expression at the transcriptional level.

To identify the sufficient and necessary region responsible for such induction, transient transfection assays with plasmids containing serial deletions of leptin promoter were performed. We observed that hCG enhanced leptin expression in all the fragments tested (Fig. 2B). Regions spanning from −2922 to −1951 bp were induced almost 3 times. The construct containing the promoter region from −948 to −1546 bp probably contains inhibitory element/s because the promoter activity in the presence of hCG dropped abruptly. This activity is restored when using constructions that contain promoter regions up to −948 bp. In summary, these results show that hCG induces leptin promoter activity probably through different elements in its promoter and that a minimal −218 bp region is sufficient to evidence such induction.

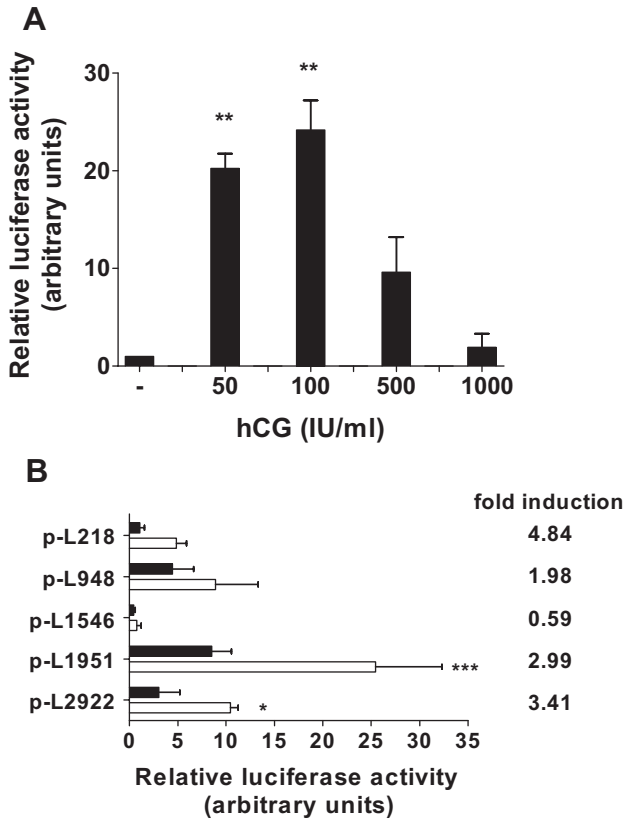


FIG. 2. hCG enhances leptin promoter activity in BeWo cells. A, Cells were transiently transfected with pL1951 plasmid construction and treated with hCG as indicated. B, Cells were transiently transfected with plasmids containing different leptin promoter deletions and treated (*white bars*) or not (*black bars*) with 100 IU/ml hCG. In all cases cells were incubated for 72 h in media of DMEM-F12 and 1% FCS. Luciferase activity was measured in cellular extracts and normalized to β-galactosidase activity. Activity obtained with empty vector (PGL-3 basic vector) was set as a control. Results are expressed as mean ± SEM for two independent experiments performed in triplicates. *, $P < 0.05$; **, $P < 0.01$ *vs.* control.

hCG induction of leptin expression is counteracted by the activation of cAMP-dependent pathway

It is generally accepted that hCG elevates the intracellular cAMP level and induces the activation of the PKA signal transduction pathway (29). Therefore, we evaluated whether the up-regulation of leptin gene by hCG in placental cells was mediated by this pathway. After treatment with hCG, $(Bu)_2$cAMP, or both, leptin expression was assessed by Western blot (Fig. 3A). In accordance with data shown in previous figures, hCG significantly stimulated leptin expression by 2.5-fold with 100 IU/ml (lane 2) and 2-fold with 50 IU/ml (lane 3). But as observed in the same figure, 0.1 or 1 μM $(Bu)_2$cAMP treatment had no effect on leptin expression (lanes 4 and 5). Moreover, when cells were treated with both hCG plus $(Bu)_2$cAMP, a complete loss of hormone induction was observed (lanes 6–9).

We next decided to investigate cAMP effect on hCG leptin induction at the transcriptional level. BeWo cells were transiently transfected with pL1951 luciferase reporter construct and treated with hCG, $(Bu)_2$cAMP, or IBMX, a nonspecific phosphodiesterase inhibitor. Results are shown in Fig. 3B. As expected, hCG significantly stimulated leptin promoter activity. The cotreatment with hormone and the cyclic nucleotide suppressed hCG induction of leptin expression by 3.8-, 5.1-, and 30-fold reduction when using 0.1 μM, 1 μM, and 1 mM $(Bu)_2$cAMP, respectively. Moreover, treatment with IBMX in combination with hCG also caused the suppression of hCG effect, leading to a 17-fold reduction in leptin promoter activity. These results reinforced the notion that the cyclic nucleotide seemed to suppress hCG effect on leptin expression. However, we observed that in transfection assays, the treatment with 0.1 or 1 μM $(Bu)_2$cAMP alone enhanced leptin promoter activity. These results require additional experiments to better understand this discrepancy.

To discriminate whether the blockage effect exerted by cAMP on leptin induction by hCG was directly dependent on the cyclic nucleotide or whether it was due to the activation of PKA, we performed cotransfection experiments with expression plasmids for the catalytic subunit of PKA or the transcription factor CREB. Results are shown in Fig. 3C. The overexpression of PKA or CREB proteins caused a significant 6- and 3-fold reduction, respectively, on leptin promoter activity, even in the absence of hCG stimulation. All these results strongly suggest that high cAMP level inhibits leptin induction by hCG and that, at least with these approaches, the activation of PKA does not explain hCG effect on leptin expression. Further studies are required to unravel the molecular mechanisms involved in the observed inhibition.

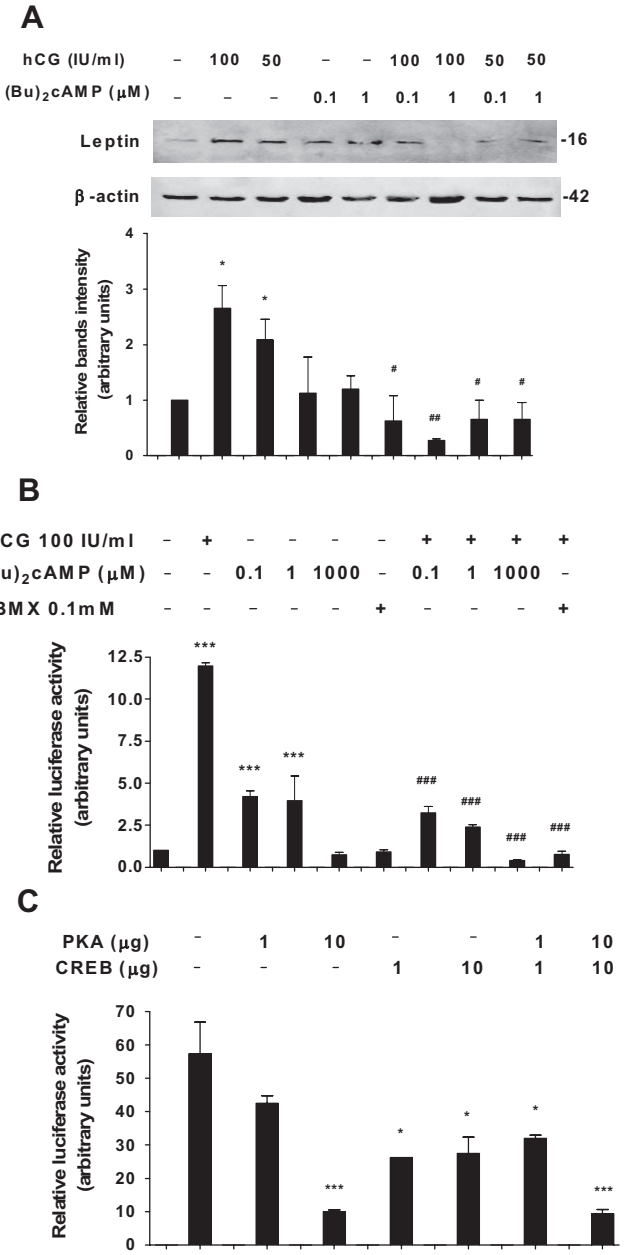


FIG. 3. cAMP counteracts hCG effects on leptin expression in BeWo cells. A, Cells were incubated for 3 d with different doses of hCG (international units per milliliter) and $(Bu)_2$cAMP as indicated. Extracts from cells were prepared as previously described and loaded in a 12% SDS-PAGE. Leptin expression was determined by Western blot. Loading controls were performed by immunoblotting the same membranes with anti-β-actin. Band densitometry is shown in the *lower panel*. Results shown are from a representative experiment. B, Cells were transiently transfected with pL1951 plasmid construction and treated with hCG, $(Bu)_2$cAMP, and IBMX, alone or combined as indicated. C, BeWo cells were transiently cotransfected with pL1951 and plasmids expressing CREB (1 μg/ml) or PKA (2.3 μg/ml), as indicated. Cells were incubated for 72 h in media of DMEM-F12 and 1% FCS. Luciferase activity was measured in cellular extracts and normalized to β-galactosidase activity. Activity obtained with empty vector (PGL-3 basic vector) was set as a control. Results expressed as mean ± SEM for three independent experiments. *, $P < 0.05$; ***, $P < 0.001$ *vs.* control; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ *vs.* hCG treatment.

The MAPK pathway is involved in the up-regulation of leptin by hCG in trophoblastic cells

If the PKA-dependent pathway was not involved in hCG induction of leptin expression in placental cells, our next question was to determine whether this hormone could stimulate other signal transduction pathways. It was previously reported the ability of the CG/LH receptor to signal via the MAPK/ERK pathway in primate endometrium (30) and porcine granulosa cells (31, 32). We then examined whether signaling pathways, such as MAPK or PI3K, could play a role in the hCG-mediated increased of leptin levels. BeWo cells were preincubated for 30 min in the presence or absence of different pharmacological inhibitors act-

ing on those signaling pathways and then incubated for a further 72 h with 100 IU/ml hCG in the continued presence or absence of these inhibitors. Pretreatment with inhibitors of PI3K (wortmannin; 0.1 μM) had no effect on the hCG-mediated up-regulation of leptin expression as measured by Western blot analysis (Fig. 4A). When the MEK inhibitor was tested by pretreatment with 50 μM PD98059, an inhibitor that blocks MEK's ability to activate ERKs, it completely blocked the effect of 100 IU/ml hCG, showing that ERK activation, but not PI3K activation, was necessary for hCG to cause the increase in leptin level in BeWo cells. To confirm the role of ERK activation on hCG-up-regulated leptin gene expression, similar experiments were performed in cells from placental explants. They were pretreated for 10 min with 50 μM PD98059 and then incubated in the presence of 50 or 100 IU/ml hCG, and leptin expression was assessed by Western blot analysis. As shown in Fig. 4B, hCG up-regulated leptin level and this effect could be inhibited by PD98059 pretreatment. We observed that treatment with 50 μM PD98059 also down-regulated leptin level in the absence of hCG, probably due to endogenous basal activation of MAPK in placenta explants.

To further investigate the MAPK/ERK involvement in the hCG signaling of leptin stimulation, we performed transient transfection experiments using the pL1951 construction. Cells were preincubated during 30 min with 10 or 50 μM PD98059 or 0.1 μM wortmannin and incubated later during 72 h with 100 IU/ml hCG. The hormone stimulated leptin promoter activity and this effect was fully blocked with 50 μM PD98059 but not 0.1 μM wortmannin (Fig. 4C). Further evidence implicating the MAPK pathway in hCG signaling on the activation of leptin was obtained from experiments in which BeWo cells were transiently cotransfected with a plasmid encoding a kinase mutant of ERK2 (MAPK-kd) and pL1951 reporter construction. As shown in Fig. 4D, cotransfection of MAPK-kd blocked the hCG-mediated induction of leptin promoter activity but did not modify its basal activity. Taken together all these data suggest that hCG induction of leptin gene in placental cells is dependent on the stimulation of the MAPK/ERK pathway.

hCG activates MAPK/ERK signaling transduction pathway in placental explants

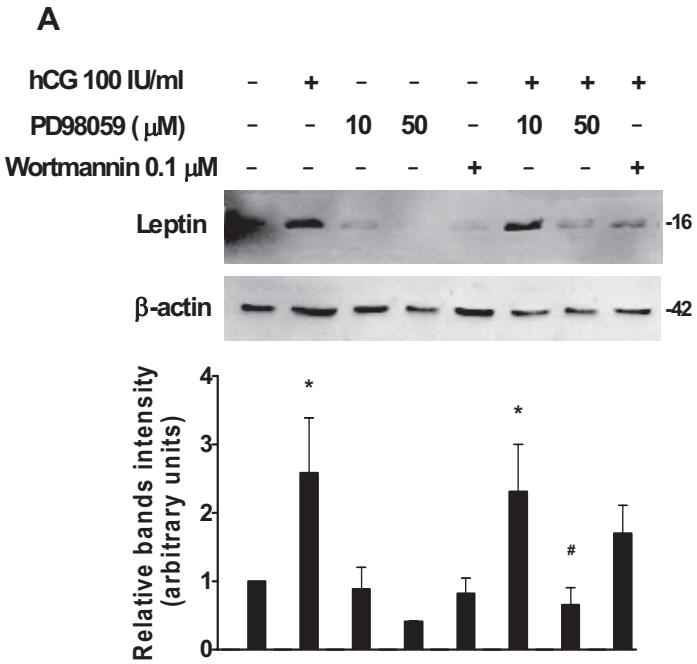
To confirm, in a more physiological model, whether hCG action on leptin expression could be mediated by MAPK/ERK signaling pathway in human placenta, we examined activation of ERK 1/2 and MEK in placental explants treated with different doses of hCG. Kinase phosphorylation was determined by Western blot using specific antibodies raised against the phosphorylated forms. As shown in Fig. 5 ERK 1/2 phosphorylation was significantly increased with hCG treatment. Maximal effect was achieved with 25, 50, and 100 IU/ml hCG. MEK phosphorylation was also enhanced with hCG incubation and maximal effect was achieved with 25, 50, and 100 IU/ml hCG (Fig. 5B). Furthermore, ERK1/2 phosphorylation stimulated by 100 IU/ml hCG was significantly blocked when explants were preincubated for 10 min with 50 μM PD98059. All together, these results demonstrate that hCG induces MEK and ERK1/2 phosphorylation in placental cells and that this signaling pathway might be involved in the regulation of leptin expression.

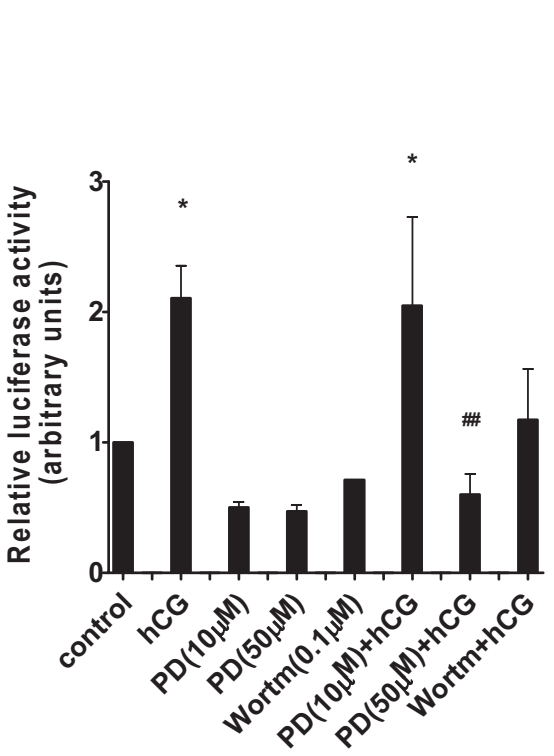
Discussion

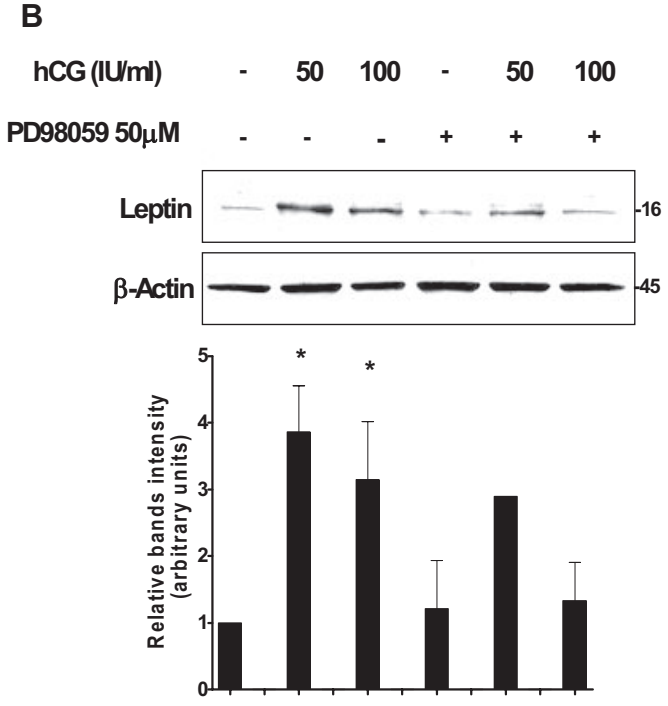
Previously published results suggested that leptin has a role in reproduction, particularly in the fetoplacental physiology. Circulating leptin levels are elevated during pregnancy, reaching a peak during the second trimester and at the end of pregnancy, whereas maternal plasma leptin levels decline to normal values 24 h after delivery (33). Consistent with this, leptin is produced by the human placenta and in normal pregnancy was found to be in the range 7.4–19 ng/ml (26). Even though, as trophoblastic cells produce leptin locally, the effective concentration of this hormone may be higher in the placenta. During the third trimester of pregnancy, leptin receptor levels also show a marked expression (34) The autocrine action of leptin may be important for trophoblast cell survival and also be relevant for pathophysiological conditions because mRNA leptin expression is known to be increased in preeclamptic placenta in which the fetus would be under chronic stress (35).

In this study, the regulation of leptin expression in BeWo human choriocarcinoma cells and human placental explants was investigated. BeWo cells express both leptin and its receptor (19) and maintain many characteristics of human trophoblast cells, and they have been widely used to study placental cellular signaling (36–38). Little is known about the regulation of leptin expression in the placenta. It has been reported that the regulation of leptin synthesis is mediated by steroid hormones (39, 40) and glucocorticoids (41). A placental-specific enhancer located 1.9 kb upstream of the human leptin gene was identified that works in choriocarcinoma lines but not adipose cells (22). It was also demonstrated that the human leptin gene is actively engaged by hypoxia through mechanisms that are common to other hypoxia-inducible genes (42). We proposed that some important pregnancy signals would be probably involved in the regulation of leptin expression. Placenta and, in particular, its cytotrophoblast component play a major endocrine function regarding fetus growth and development. hCG is secreted by villous trophoblasts and is likely to act on other receptor-bearing cells in the fetoplacental environment (16). When the effect of this hormone was analyzed on leptin expression in BeWo cells, a significant up-regulation was observed and this effect was dose and time-dependent. Nevertheless, we observed that at a higher dose of hCG (500 IU/ml hCG), leptin expression was down-regulated. These results were confirmed performing similar experiments in placenta explants, a more physiological model. It was previously demonstrated that in first trimester placental explants hCG release is pulsatile and that this episodic secretion is enhanced both by GnRH and leptin (43, 44). It was also demonstrated that leptin has a stimulatory effect on hCG secretion added to primary cultures of human term placental trophoblast cells (45) and in first-trimester trophoblast cells (46).

Leptin action on hCG secretion apparently depends on the time of pregnancy, not affecting hCG secretion at term (47) but







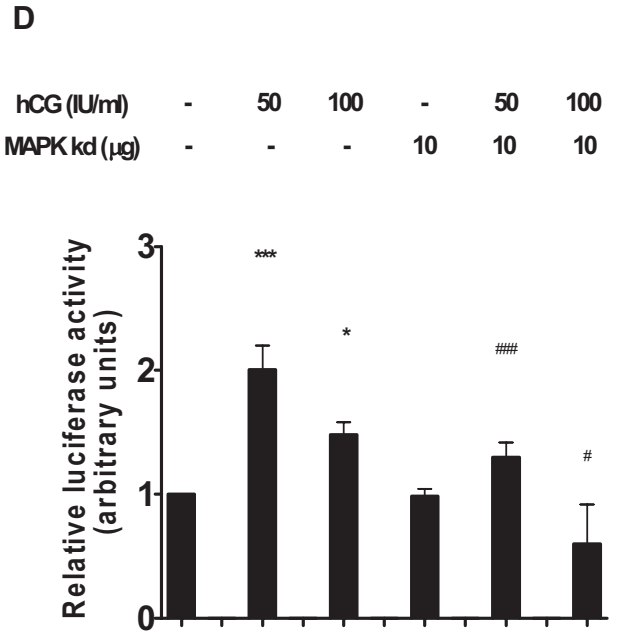


FIG. 4. hCG signal transduction pathways and leptin expression. A, BeWo cells (1 × 106 cells) were plated in complete DMEM-F12 media supplemented with 1% FCS and incubated for 3 d with hCG and PD98059 or wortmannin as indicated. B, Placental explants were processed as previously described and treated with PD98059 for 10 min followed by hCG treatment for another 10 min. In A and B, cell extracts were prepared as indicated in *Materials and Methods*. Proteins were separated on SDS-PAGE gels. Leptin expression was determined by Western blot. Loading controls were performed by immunoblotting the same membranes with anti-β-actin. Molecular weights were estimated using standard protein markers. Bands densitometry is shown in *lower panels*. Results shown are from a representative experiment and are expressed as means ± SEM for three independent experiments. *, $P < 0.05$ *vs.* control; #, $P < 0.05$ *vs.* hCG treatments. C, BeWo cells were transiently transfected with pL1951 plasmid construction and incubated in the presence or absence of hCG plus PD98059 (PD) or Wortmannin (Wortm). D, BeWo cells were transiently cotransfected with pL1951 and plasmid expressing MAPK-kd (1 μg/ml), and treated with hCG, as indicated. In C and D, luciferase activity was measured in cellular extracts and normalized to β-galactosidase activity. Activity obtained with empty vector (PGL-3 basic vector) was set as a control. Results are expressed as mean ± SEM for two independent experiments performed in duplicates. *, $P < 0.05$ *vs.* control; ***, $P < 0.001$ *vs.* control; #, $P < 0.05$ *vs.* hCG treatment; ##, $P < 0.01$ *vs.* hCG treatment, ###, $P < 0.001$ *vs.* hCG treatment.

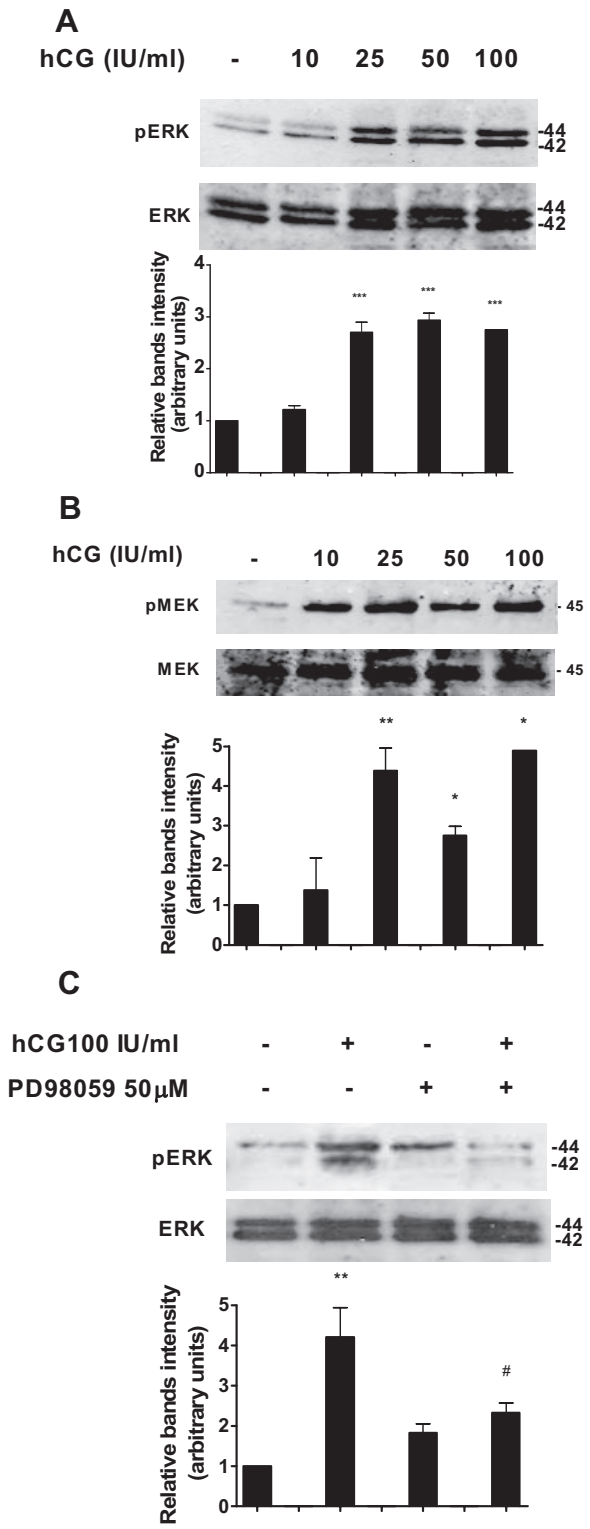


FIG. 5. hCG activates ERK 1/2 and MEK in placental explants. Placental explants were processed as previously described and treated with hCG for 10 min. In C, cells were pretreated with PD98059 for 10 min. In all cases explants samples were loaded in a 12% SDS-PAGE, and ERK1/2 (A and C) or MEK (B) phosphorylation was determined by Western blot as indicated in *Materials and Methods*. Total ERK or MEK protein levels in cell extracts were determined as loading controls. Molecular weights were estimated using standard protein markers. Bands densitometry is shown in *lower panel*. Results shown are from a representative experiment and are expressed as mean ± SEM for two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control; #, $P < 0.05$ *vs.* hCG treatment.

enhancing hCG secretion by first-trimester placental explants (44) and presents an interesting physiological situation to analyze. On the other hand, leptin secretion is significantly stimulated in cytotrophoblastic cells by treatment during 4 h with GnRH-II (44). In this scenario it is possible that in the physiological context changes in local hCG concentration might determine leptin expression. In this regard, it has been reported that leptin secretion in human cytotrophoblastic cells was significantly inhibited when incubated during 4 h with increasing concentrations of hCG (48). In these experiments the authors used concentrations from 5,000 to 30,000 IU/ml hCG. In agreement with these results, our findings showed a pronounced reduction in leptin expression with concentrations above 250 IU/ml hCG. We think that among the different hormones secreted by the placenta, GnRH, hCG, and leptin are involved in an autocrine/paracrine loop regulating placental function principally during the first trimester of pregnancy. Our results confirm the idea of a main role of cytokines on the fetal-placental-uterine dialog and open a very interesting possibility for further studies.

It has also been determined that leptin levels in sera from patients with different pregnancy pathologies such as recurrent miscarriage, gestational diabetes, intrauterine growth retardation, and preeclampsia were significantly higher, compared with normal controls (5). All these findings put leptin as a possible modulator of placental endocrine activity and, very specifically, as a possible connection between embryo and endometrium communication.

We found that hCG increased leptin expression acting at the transcriptional level and that a minimal region spanning up to −218 bp was sufficient to evidence such induction. *In silico* analysis of this DNA fragment revealed potential consensus elements for different transcription factors such as CCAAT/enhancer-binding protein (C/EBP) and specificity protein-1 with a core similarity of 1. Previous reports demonstrated the involvement of both C/EBP and specificity protein-1 in the regulation of leptin gene in adipocytes (49). Furthermore, C/EBP's transcriptional factors were reported to be regulated by hCG during follicular development (50). Other investigators also described an hCG up-regulation of C/EBPα expression in rat primary cultures of Leydig cells and human adipose cells (51, 52). Further experiments will be needed to confirm that this transcriptional factor is involved in hCG up-regulation of leptin gene in placental cells.

Binding of hCG to its receptor generates signal transduction through the activation of the associated heterotrimeric G proteins, and in the classical response, there is an increase in cAMP and a consequent activation of PKA (29). To analyze the molecular mechanisms underlying hCG effect on leptin up-regulation, we studied the effect of cAMP treatment on hCG-dependent up-regulation of leptin expression. We observed that $(Bu)_2$cAMP not only did not enhance hCG effect but also inhibited hCG-dependent leptin expression in placental cells. Moreover, the overexpression of the catalytic subunit of PKA or the transcription factor CREB completely down-regulated leptin expression. These results strongly suggest that high cAMP levels inhibit leptin induction by hCG and that the activation of PKA does not explain hCG effect on leptin expression. These findings are coincident with reported data from adipocytes (53) but are in dis-

agreement with the results shown by Zhao *et al.* (54). These authors demonstrated that expression of leptin and its secretion from the placenta are species specific and that 1 mM cAMP increased leptin expression in BeWo cells. Although we could not explain this discrepancy, further experiments are in progress in our laboratory to study the mechanism underlying cAMP action on leptin expression.

It was recently described that the down-regulation of the LH/CG receptor was obtained by treating rats with hCG or by the chronic elevation of cAMP production (55). It is possible that the down-regulation of the hCG receptor would explain the lack of cAMP stimulation on hCG effect when the level of the cyclic nucleotide was raised. Although the physiological role of hCG-promoted cAMP production is well documented, the LH/hCG receptor has also been shown to mediate activation of the MAPK (56, 57), Janus kinase-signaling, and PI3K pathways (58). It has also been suggested that LH-promoted MAPK stimulation may result in the desensitization of LH-stimulated steroidogenesis in granulosa cells (59).

For example, it is reported that in a human endometrial epithelial cell line (HES) and baboon epithelial endometrial cells, CG does not activate the adenylyl cyclase-cAMP-PKA pathway, but it can rapidly induce phosphorylation of ERK 1/2 in a PKA-independent manner (57). These evidences prompted us to investigate whether the MAPK and PI3K signal transduction pathways were involved in hCG up-regulation of leptin gene in placental cells. We demonstrated that hCG treatment specifically activated MEK and ERK1/2 phosphorylation in placental cells. The involvement of MAPK signaling pathway activation in leptin up-regulation was demonstrated by both pharmacological experiments using the MEK inhibitor PD98059 and the transfection of cells with a plasmid encoding a dominant-negative mutant of ERK2. Data obtained by using both methods are in good agreement with the notion that MAPK pathway plays a major role in transducing gonadotropin signaling toward leptin up-regulation. The PI3K pathway did not seem to be involved in leptin regulation by hCG.

In the present work, we provide evidence that hCG up-regulates leptin gene in human trophoblastic cells and that the hCG regulatory effect on leptin expression probably involves the MAPK signal transduction pathway. However, further studies are needed to explain the molecular mechanisms underlying these effects. In summary, our results further support the importance of leptin in the biology of reproduction.

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