Regulation of Placental Leptin Expression by Cyclic Adenosine 5′-Monophosphate Involves Cross Talk between Protein Kinase A and Mitogen-Activated Protein Kinase Signaling Pathways

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Leptin, a 16-kDa protein mainly produced by adipose tissue, has been involved in the control of energy balance through its hypothalamic receptor. However, pleiotropic effects of leptin have been identified in reproduction and pregnancy, particularly in placenta, where it was found to be expressed. In the current study, we examined the effect of cAMP in the regulation of leptin expression in trophoblastic cells. We found that dibutyryl cAMP [$(Bu)_2$cAMP], a cAMP analog, showed an inducing effect on endogenous leptin expression in BeWo and JEG-3 cell lines when analyzed by Western blot analysis and quantitative RT-PCR. Maximal effect was achieved at 100 μM. Leptin promoter activity was also stimulated, evaluated by transient transfection with a reporter plasmid construction. Similar results were obtained with human term placental explants, thus indicating physiological relevance. Because cAMP usually exerts its actions through activation of protein kinase A (PKA) signaling, this pathway was analyzed. We found that cAMP response element-binding protein (CREB) phosphorylation was significantly increased with $(Bu)_2$cAMP treatment. Furthermore, cotransfection with the catalytic subunit of PKA and/or the transcription factor CREB caused a significant stimulation on leptin promoter activity. On the other hand, the cotransfection with a dominant negative mutant of the regulatory subunit of PKA inhibited leptin promoter activity. We determined that cAMP effect could be blocked by pharmacologic inhibition of PKA or adenylyl ciclase in BeWo cells and in human placental explants. Thereafter, we decided to investigate the involvement of the MAPK/ERK signaling pathway in the cAMP effect on leptin induction. We found that 50 μM PD98059, a MAPK kinase inhibitor, partially blocked leptin induction by cAMP, measured both by Western blot analysis and reporter transient transfection assay. Moreover, ERK 1/2 phosphorylation was significantly increased with $(Bu)_2$cAMP treatment, and this effect was dose dependent. Finally, we observed that 50 μM PD98059 inhibited cAMP-dependent phosphorylation of CREB in placental explants. In summary, we provide some evidence suggesting that cAMP induces leptin expression in placental cells and that this effect seems to be mediated by a cross talk between PKA and MAPK signaling pathways. **(*Endocrinology* 151: 3738–3751, 2010)**

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Abbreviations: $(Bu)_2$cAMP, Dibutyryl cAMP; CREB, cAMP response element-binding protein; C_T, threshold cycle; FCS, fetal calf serum; hCG, human chorionic gonadotropin; Luc, luciferase; MEK, MAPK kinase; PKA, protein kinase A; PKI, PKA regulatory Iα-subunit; qRT-PCR, quantitative real-time RT-PCR.

Embryo implantation represents the most critical step of the reproductive process. It consists of a unique biological phenomenon, by which the blastocyst becomes intimately connected to the maternal endometrial surface to form the placenta that will provide an interface between the growing fetus and the maternal circulation (1, 2). A large number of identified molecular mediators have been postulated to be involved in the early feto-maternal interaction, including hormones, adhesion molecules, cytokines, growth factors, lipids, and others (3). During pregnancy, the placenta produces a wide number of these molecules that play essential roles in the establishment and maintenance of pregnancy, adaptation of the maternal organism to pregnancy, fetal growth, and development of the mechanisms involved in parturition (4).

In this context, leptin has emerged as an important player in reproduction, and in particular, a relevant role of leptin in implantation has been proposed. Leptin, the product of the *LEP* gene, is a small nonglycosilated peptide of 146 amino acid residues (16 kDa), firstly found to be secreted by adipose tissue (5), with the function of modulation of satiety and energy balance (6). Actually, leptin can be considered as a multifunctional hormone that regulates not only body weight homeostasis but also thermogenesis, angiogenesis, hematopoiesis, osteogenesis, chondrogenesis, neuroendocrine, and immune functions, as well as arterial pressure control (7). Compelling evidence also implicated leptin in reproductive functions, such as the regulation of fertility, ovarian function, oocyte maturation, embryo development, and implantation (8–10). This current status of leptin is consistent with its production by various tissues and organs, such as the stomach, the skeletal muscles, the pituitary cells, and the placenta (11). The synthesis and secretion of leptin, as well as its functional receptors by trophoblastic cells, has been widely established (12, 13), suggesting that leptin may act through a paracrine or autocrine mechanism. In this way, previous studies have demonstrated the interactions between leptin and some placental hormones, implicating leptin as a modulator of placental endocrine function (14). Moreover, leptin increases matrix metalloproteinase expression in cytotrophoblasts (15), stimulates the process of proliferation and protein synthesis, and inhibits apoptosis (16–18) in human trophoblastic cells. Plasma leptin concentrations are significantly elevated in pregnant women as compared with those in age and body mass index-matched nonpregnant women (12, 19) and drops sharply after delivery (20). 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 (7, 21). At present, the complete regulation of leptin production is still poorly understood. We have previously reported that human chorionic gonadotropin (hCG), a key hormone in pregnancy, up-regulates placental leptin, and such effect is counteracted by cAMP (22).

Many of the functions attributed to the human placental trophoblasts, including production of various pregnancy hormones and transport of essential nutrients to the fetus, are under the regulation of second messengers, including cAMP (23). The cAMP is one of the oldest known signaling molecules. It has been shown that increases in intracellular cAMP result in decreased expression of *LEP* mRNA and leptin secretion in rat adipocytes (24), and that this inhibitory effect is protein kinase A (PKA) dependent (25). In contrast, it has been demonstrated that leptin secretion in BeWo cells and in term human placental tissue cultured in monolayer is augmented by forskolin, an activator of PKA (26, 27). However, several experiments have provided evidence that cAMP affects some cellular processes independently on PKA (28–30).

It is well documented that cAMP has profound effects upon human trophoblast function as demonstrated in numerous *in vitro* studies of normal and transformed trophoblast cells (31). Because cAMP plays a critical role in controlling placenta-specific gene expression and mediates the action of numerous placental hormones, we aimed to investigate the regulation of leptin expression by cAMP in human placenta and to unravel the signaling pathways involved.

Materials and Methods

Cell culture and treatments

The human choriocarcinoma cell lines BeWo and JEG-3 were grown in DMEM 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.

To test the effect of cAMP, the cAMP analog dibutyryl cAMP [$(Bu)_2$cAMP] (0.1 μM to 10 mM) (Sigma Chemical Co.) was used to facilitate cell entrance. In experiments designed to analyze the cAMP-dependent signal transduction pathway, the cell-permeable adenylyl cyclase inhibitor SQ22,536 (100 μM), the selective inhibitor of cAMP-dependent protein kinase (PKA) H89 (10 μM), and the MAPK kinase (MEK) inhibitor PD98059 (50 μM) (Sigma Chemical Co.) were used. Inhibitors were added 30 min before $(Bu)_2$cAMP treatment, except in experiments performed to determine protein phosphorylation, where the inhibitors were added 10 min before $(Bu)_2$cAMP treatment. 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- to 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 by the local ethical committee.

Treatments of placental explants

Placental explants were randomly distributed in tubes containing 1 ml of DMEM-F12 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 4 h in the same medium supplemented or not with 0.1 μM to 1 mM $(Bu)_2$cAMP and/or 50 μM PD98059 or 10 μM H89. Inhibitors were added 10 min before $(Bu)_2$cAMP treatment. Explants were removed from the bath, centrifuged for 2 min at 2000 × *g* at 4 C, and resuspended in 500 μl of lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% 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 × *g* for 20 min. Supernatants were analyzed by Western blot analysis.

For real-time PCR, after thoroughly washing with phosphate buffer saline, the tissues were immediately frozen at −80 C and stored until extraction of total RNA.

Western blot analysis

Cells were seeded at 50–60% confluence in DMEM-F12 medium supplemented with 10% FCS. Each treatment was performed in the same media supplemented with 1% FCS during 3 d for leptin immunoblot or during 10 min for protein phosphorylation determinations. Total cell lysates were prepared in lysis buffer. The lysates were centrifuged at 10,000 × *g* for 10 min to remove cellular debris. The protein concentration of the supernatant was determined by the Bradford staining method (32), 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 Biotech, Piscataway, NJ) thereafter. Membranes were equilibrated in 1× 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, Inc., Santa Cruz, CA), with polyclonal rabbit antiphospho-ERK 1/2 (Thr202/Tyr204) (1:3000) or with polyclonal rabbit antiphospho-cAMP response element-binding protein (CREB) (Ser133) antibody (1:1000) (Sigma Chemical Co.). Loading controls were performed by immunoblotting the same membranes with polyclonal rabbit anti-β-actin (1:5000) (Sigma Chemical Co.), with polyclonal rabbit antitotal-ERK 1/2 (1:3000) or with polyclonal rabbit antitotal-CREB antibody (1:1000) (Sigma Chemical Co.). The antibodies were detected using horseradish peroxidase-linked goat antirabbit IgG (1:10,000) (Santa Cruz Biotechnology, Inc.) and visualized by the Amersham Pharmacia enhanced chemiluminescence signaling system and a Bio-Imaging Analyzer Fujifilm LAS-1000 (Fuji Photo Film Co., Ltd., Tokyo, Japan). Quantification of protein bands was determined by densitometry using Image Gauge version 3.12 software (ScienceLab, Fuji Photo Film Co., Ltd.).

Plasmids

The luciferase (Luc) reporter constructs are based on pGL-3 basic vector. They were all kindly provided by Oksana Gavrilova (33). pRSV-βgal contains the β-galactosidase gene under the control of the Rous sarcoma virus (RSV) 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 (34). pMt-REV is a 7.6-kb expression vector that contains a dominant negative mutant cDNA of the mouse PKA regulatory Iα-subunit (PKI) inserted between the mouse metallothionein-promoter and the polyadenylation signal sequence of the human GH gene (35). Expression vector for the wild-type CREB named pCREB3 (36) was kindly provided by P. Sassone-Corsi. 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 Corp., 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 of 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 Luc reporter and 5 μg of pRSV βgal internal control construct were transfected using 5 μl of LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD). 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 Luc and β-galactosidase activities

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

Quantitative real-time RT-PCR (qRT-PCR) assay

Abundance of leptin mRNA was determined by qRT-PCR. Total RNA was extracted from JEG-3 or placental explants using TRISURE reagent, according to the manufacture instructions (Bioline Co., Essex, UK). Concentration and purity of the isolated RNA were estimated spectrophotometrically at 260 and 280 nm. For cDNA synthesis, 5 μg of total RNA was reverse transcribed at 50 C during 1 h using the Transcriptor first Strand cDNA synthesis Kit (Roche, Indianapolis, IN). Quantitative real-time PCR was performed using the following primers based on the sequences of the National Center for Biotechnology Information GenBank database: leptin: forward, 5′GAACCCTGTGATTCTT 3′; reverse, 5′CCAGGTCGTTATTTGG 3′; and cyclophilin: forward, 5′CTTCCCCGATACTTCA 3′; reverse, 5′TCTTGGTGCTACCTC 3′. Quantitative RT-PCR Master Mix Reagent kit was obtained from Roche (Fast Start universal SYBR Green), and PCRs were performed on a Chromo 4 DNA Engine (Bio-Rad, Hercules, CA). A typical reaction contained 10 μM of forward and reverse primer, 3 μl of cDNA, and the final reaction volume was 25 μl. The reaction was initiated by preheating at 50 C for 2 min, followed by heating at 95 C for 10 min. Subsequently, 41 amplification cycles were carried out as follows: denaturation 15 sec at 95 C and 1 min annealing and extension at 58 C. The threshold cycle (C_T) from each well was determined by the Opticon Monitor 3 Program. Relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method. For the treated samples, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophylin), and relative to the untreated control.

Data analysis

For Western blots analysis, representative images of at least three independent experiments are shown along with quantification of immunoreactive bands. Quantitative RT-PCR experiments were repeated separately at least three times to ensure reproducible results. Transient transfection experiments were repeated at least three times and each treatment performed by duplicates. Results are expressed as the mean ± SD. The statistical significance was assessed by ANOVA followed by Bonferroni's multiple comparison *post hoc* test and was calculated using the GraphPad Instat computer program (GraphPad, San Diego, CA). A P value less than 0.05 was considered statistically significant.

Results

cAMP induces leptin expression in trophoblastic cells

The choriocarcinoma cell lines BeWo and JEG-3 were used as models for trophoblastic cells as previously reported (12, 37). Previous results showed that leptin and leptin receptor are expressed in these cell lines, suggesting that leptin is probably exerting both a paracrine and an autocrine effect (16). We have previously observed that hCG was able to induce leptin expression not only in BeWo and JEG-3 cells but also in human placental explants. Moreover, we have found that treatment with $(Bu)_2$cAMP causes a complete loss of hCG leptin induction (22). In this regard, we aimed to study the regulation of leptin expression by cAMP in human placenta. As seen in Fig. 1A, $(Bu)_2$cAMP (0.1–100 μM) enhanced leptin expression in BeWo cells as determined by Western blot analysis. This effect was dose dependent, reaching a 2.9-fold maximal increase at 100 μM. At a higher dose (1 mM), leptin expression returned to control level. We next examined whether cAMP could stimulate leptin at the transcriptional level. Figure 1B shows that $(Bu)_2$cAMP enhanced leptin mRNA expression in JEG-3 cells, measured by qRT-PCR. Maximal effect was achieved at 100 μM with a 22-fold increase. We next performed transient transfection assays with a vector containing the regulatory region of leptin gene from −1951 bp fused to the Luc reporter gene (pL1951). Luc expression under the control of leptin promoter was proportional to the amount of plasmid transfected up to 15 μg (data not shown). In transfected BeWo cells, 100 μM $(Bu)_2$cAMP induced a 4.2-fold increase in Luc activity. Taken together, these results demonstrate that cAMP increases not only leptin mRNA expression but also leptin synthesis in placental cells.

Leptin expression is enhanced by cAMP in placental explants

To study cAMP effect in a more physiological system, human placental explants from healthy donors were analyzed. The explants were incubated during 4 h in medium supplemented or not with different $(Bu)_2$cAMP concentrations (0.1–1000 μM). Figure 2A shows that cAMP enhanced leptin expression in placental explants, measured by Western blot analysis. This effect was dose dependent, reaching a 2.5-fold increase that turned out to be statistically significant. Maximal effect was achieved at 10 μM $(Bu)_2$cAMP. To further characterize cAMP effect on leptin expression in trophoblastic cells, qRT-PCR experiments were carried out in placental explants stimulated with $(Bu)_2$cAMP as previously described. As it can be observed in Fig. 2B, $(Bu)_2$cAMP increased leptin expression, and maximal effect was obtained at 100 μM, reaching a 56-fold increase above control. Moreover, at 1 mM $(Bu)_2$cAMP, a 11-fold leptin induction was already observed. These results reinforce the notion that cAMP has a role in regulating leptin expression.

PKA signaling is involved in cAMP effect on leptin expression

Because cAMP showed an inducing effect on leptin expression, we next investigated whether this second messenger could exert this action through the activation of the PKA signaling transduction pathway. We first determined whether cAMP was able to activate PKA signaling in human placenta. We examined CREB phosphorylation by

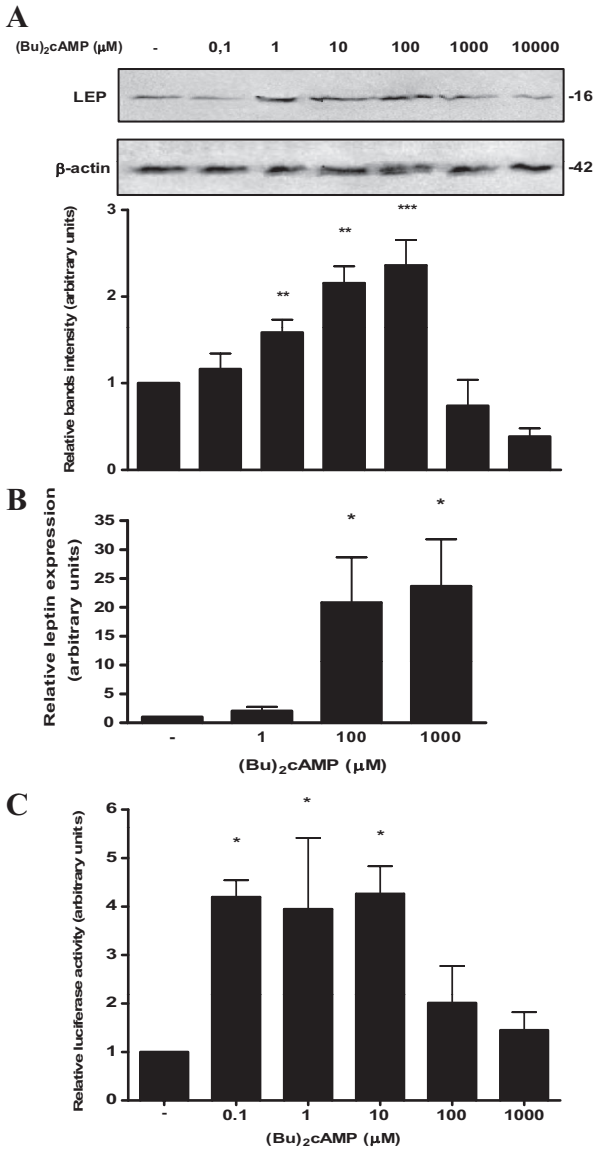

FIG. 1. cAMP enhances leptin expression in placental cells. A, BeWo cells (1×10^6 cells) were plated in complete DMEM-F12 media supplemented with 1% FBS and incubated during 3 d with different doses of $(Bu)_2$cAMP (μM). Cell extracts were prepared as indicated in *Materials and Methods*. Proteins were separated on SDS-PAGE gels, and leptin expression was determined by Western blot analysis. 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*. Molecular mass (kDa) is indicated at the *right of the blot*. A representative result from three replicates is shown. B, JEG-3 cells were processed as previously described for BeWo cells and treated with increasing $(Bu)_2$cAMP doses during 3 d. Total RNA was extracted as described in *Materials and Methods*. Leptin mRNA was quantified with real-time RT-PCR. Cyclophilin was used as internal standard. C, Cells were transiently transfected with pL1951 plasmid construction and treated with $(Bu)_2$cAMP as indicated during 72 h in DMEM-F12 media supplemented with 1% FBS. Luc 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 ± SD for two independent experiments performed in triplicates. Statistical analyses were performed by ANOVA. *Asterisks* indicate significant differences from the control according to Bonferroni's multiple comparison *post hoc* test. *, $P < 0.05$; **, $P < 0.01$. LEP, Leptin.

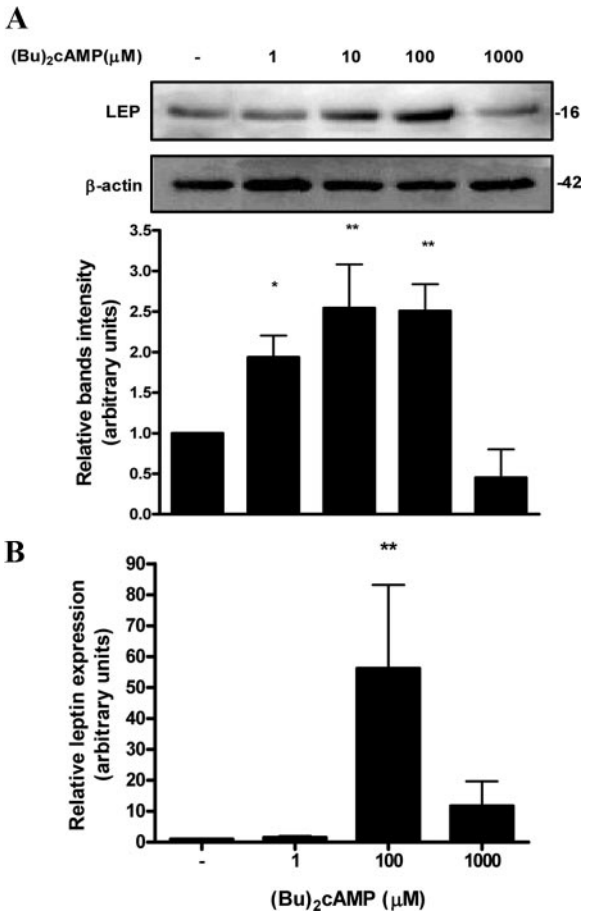

FIG. 2. cAMP induces leptin expression in human placental explants. A, Placental explants were processed as described in *Materials and Methods* and treated with increasing $(Bu)_2$cAMP doses during 4 h. Placental extracts were prepared and proteins were separated on SDS-PAGE gels. Leptin expression was determined by Western blot analysis. 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*. B, Placental explants were obtained as indicated in *Materials and Methods* and treated with $(Bu)_2$cAMP as indicated. Total RNA was extracted as previously described. Leptin mRNA was quantified with qRT-PCR. Cyclophilin was used as internal standard. Results shown are from a representative experiment and are expressed as means ± SD for three independent experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* control. LEP, Leptin.

Western blot analysis in BeWo cells (Fig. 3A) and placental explants (Fig. 3B) treated with different doses of $(Bu)_2$cAMP. As shown in Fig. 3, A and B, CREB phosphorylation was significantly increased with $(Bu)_2$cAMP treatment, and this effect was dose dependent. Maximal effect was achieved with 10, 100, and 1000 μM $(Bu)_2$cAMP. To further confirm the involvement of this pathway, we performed cotransfection experiments with pL1951 plasmid and increasing quantities (I to IV) of expression plasmids for the catalytic subunit of PKA or the transcription factor CREB. Results are showed in Fig. 3C. The overexpression of PKA or CREB proteins caused a significant stimulation on leptin promoter activity. PKA

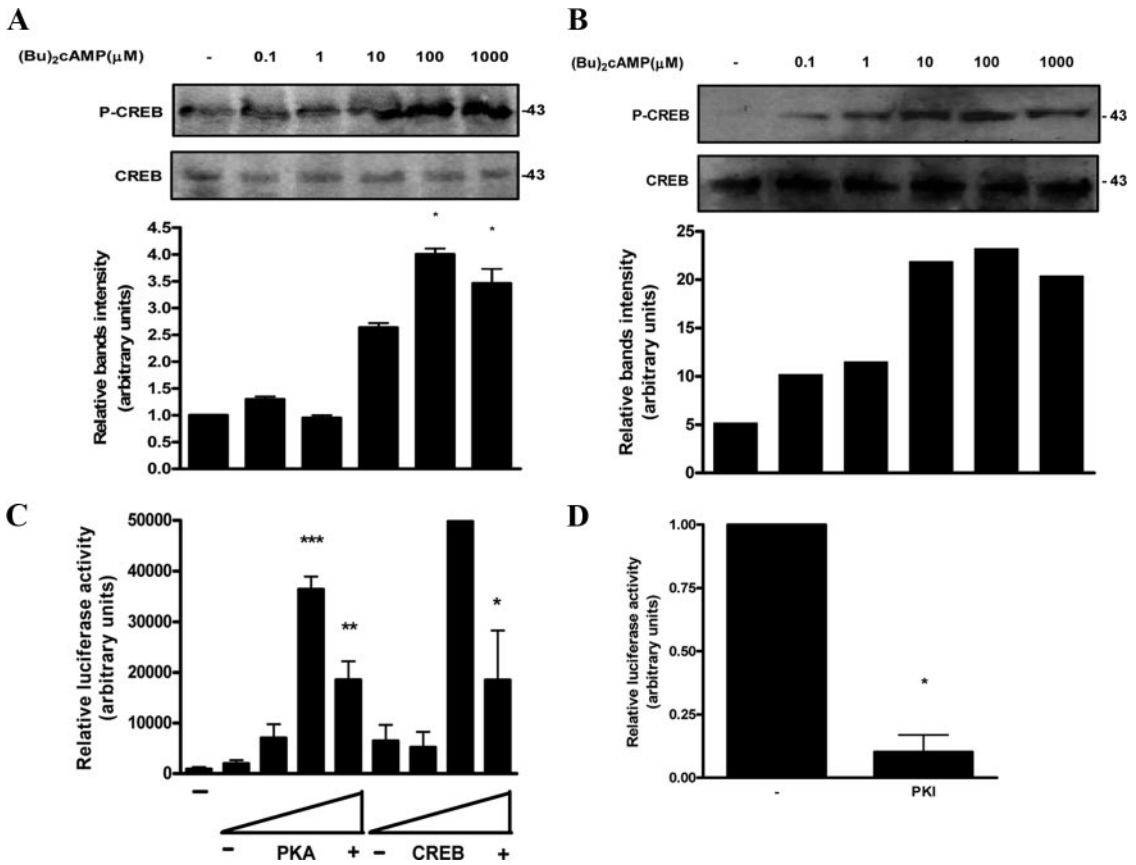

FIG. 3. PKA signaling participates in the induction of leptin by cAMP. A, BeWo cells (1×10^6 cells) were plated in complete DMEM-F12 media supplemented with 1% FBS and incubated for 15 min with $(Bu)_2$cAMP as indicated. B, Placental explants were processed as previously described and treated with $(Bu)_2$cAMP for 15 min. A and B, Extracts were prepared as indicated in *Materials and Methods*. Proteins were separated on SDS-PAGE gels. CREB phosphorylation was determined by Western blot analysis. Total CREB level in cell extracts was determined as loading control. 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 ± SD for three independent experiments. C and D, BeWo cells were transiently cotransfected with pL1951 and increasing concentrations of plasmids expressing the catalytic subunit of PKA (2.3 μg/ml) or CREB (1 μg/ml) (C) or with a dominant negative mutant of the regulatory subunit of PKA (PKI) (1 μg/ml) (D) as indicated. Cells were incubated during 72 h in DMEM-F12 1% FBS media. Luc 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 ± SD for three independent experiments performed in duplicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control.

and CREB caused a maximal 42.5- and 58-fold induction, respectively. These effects were dose dependent. In an opposite approach, we next performed a cotransfection with an expression plasmid for a dominant negative mutant of the PKI. As it is shown in Fig. 3D, PKI expression significantly inhibited leptin promoter activity with a 9.8-fold reduction. All together, these results demonstrate that cAMP induces CREB phosphorylation in human placenta and that PKA signaling pathway might be involved in the regulation of leptin expression by cAMP.

Inhibition of PKA-dependent pathway prevents leptin induction by cAMP

Our next aim was to confirm whether PKA-dependent pathway was involved in cAMP induction of leptin expression in placental cells. BeWo cells were treated with 1 μM $(Bu)_2$cAMP in the presence or absence of H89, as previously described. Leptin expression was measured by Western blot analysis (Fig. 4A). As expected, 1 μM $(Bu)_2$cAMP produced a 7-fold induction of leptin expression compared with control. This effect was inhibited by the pretreatment with 10 μM H89. To confirm the role of PKA activation on cAMP-up-regulated leptin expression, similar experiments were performed in cells from placental explants. As it is shown in Fig. 4B, cAMP up-regulated leptin level, and this effect was inhibited by H89 pretreatment. We next decided to investigate H89 effect on cAMP leptin induction at the transcriptional level. BeWo cells were transiently transfected with pL1951 Luc reporter construct and treated with $(Bu)_2$cAMP or/and 10 μM H89. Results are shown in Fig. 4C. As previously observed, cAMP significantly stimulated leptin promoter activity. The cotreatment with the cyclic nucleotide and PKA inhibitor suppressed cAMP induction of leptin expression by 12- and 4-fold reduction when using 1 μM $(Bu)_2$cAMP and 1 mM $(Bu)_2$cAMP, respectively. Moreover, Fig. 4D

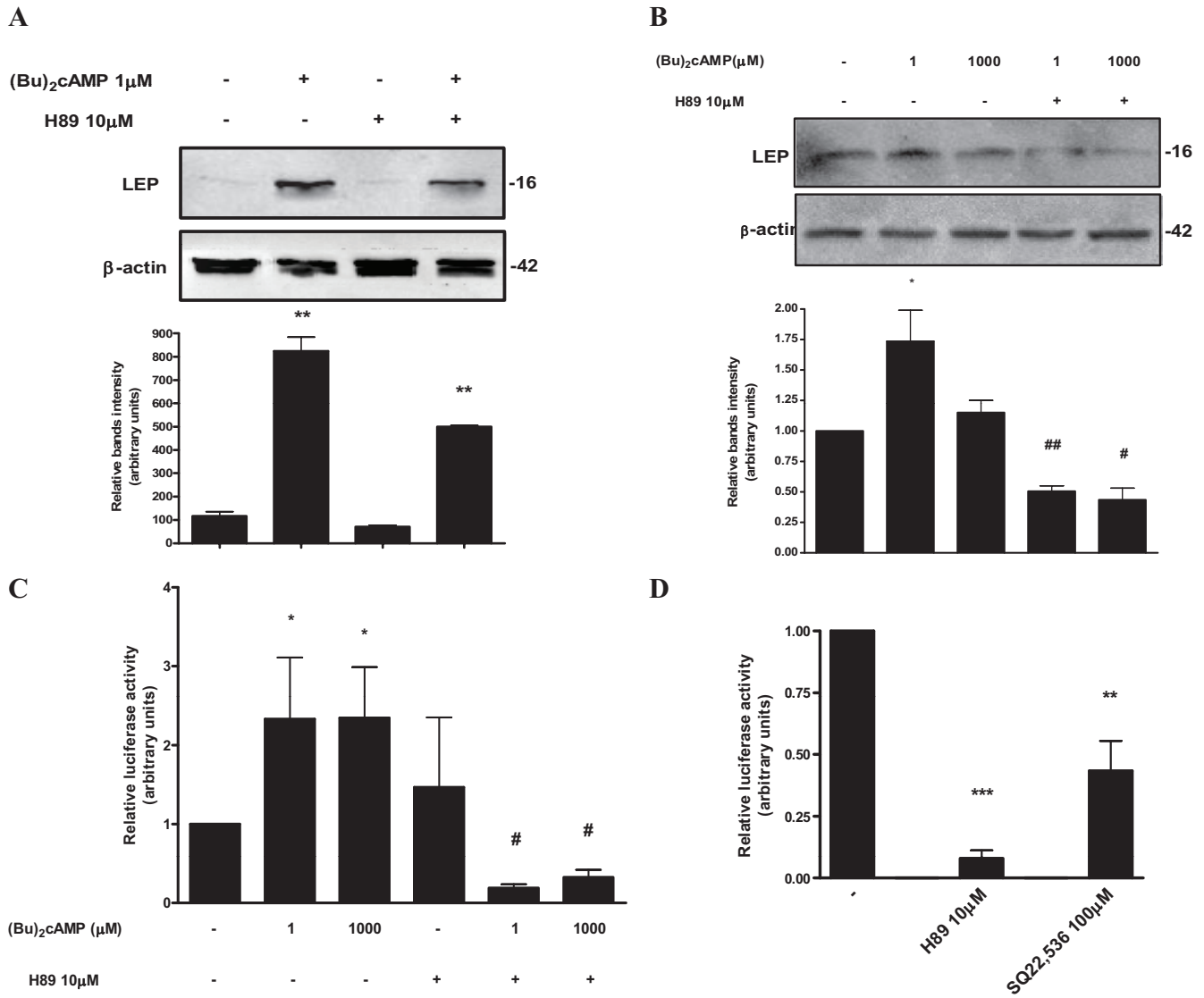

FIG. 4. PKA pathway inhibition blocks leptin induction by cAMP. A, BeWo cells (1×10^6 cells) were plated in complete DMEM-F12 media supplemented with 1% FBS and incubated for 3 d with 1 μM $(Bu)_2$cAMP and 10 μM H89 as indicated. B, Placental explants were processed as previously described and treated with 1–1000 μM $(Bu)_2$cAMP and 10 μM H89 for 4 h. A and B, Extracts were prepared as indicated in *Materials and Methods*. Proteins were separated on SDS-PAGE gels. Leptin expression was determined by Western blot analysis. 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 ± SD for three independent experiments. C and D, Cells were transiently transfected with pL1951 plasmid construction and treated with $(Bu)_2$cAMP, H89, and SQ22,536, alone or combined as indicated. Cells were incubated during 72 h in DMEM-F12 1% FBS media. Luc 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 ± SD for three independent experiments performed in duplicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control; #, $P < 0.05$ *vs.* $(Bu)_2$cAMP treatment. LEP, Leptin.

shows that when cells were treated with 100 μM SQ22,536, a pharmacological inhibitor of adenylyl cyclase, a significant 2.3-fold inhibition of the effect of cAMP on leptin expression was observed. These results reinforced the notion that PKA signaling pathway is involved in cAMP effect on leptin expression.

The MAPK pathway is involved in the up-regulation of leptin by cAMP in placental cells

It has been reported that cAMP stimulates (38–40) or inhibits (41, 42) the MAPK pathway in a variety of cell types. To study the MAPK involvement in the cAMP-dependent leptin stimulation, the effect of PD98059, a pharmacologic inhibitor that blocks MEK's ability to activate ERKs, was evaluated. BeWo cells were incubated with 1, 100, or 1000 μM $(Bu)_2$cAMP in the presence or absence of 50 μM PD98059. Leptin expression was measured by Western blot analysis. The MEK inhibitor blocked the effect of 1 and 100 μM $(Bu)_2$cAMP with 1.6- and 2.4-fold reduction, respectively (Fig. 5A). We next decided to evaluate ERK activation by cAMP in placental cells. BeWo cells were treated for 15 min with 0.1–1000 μM

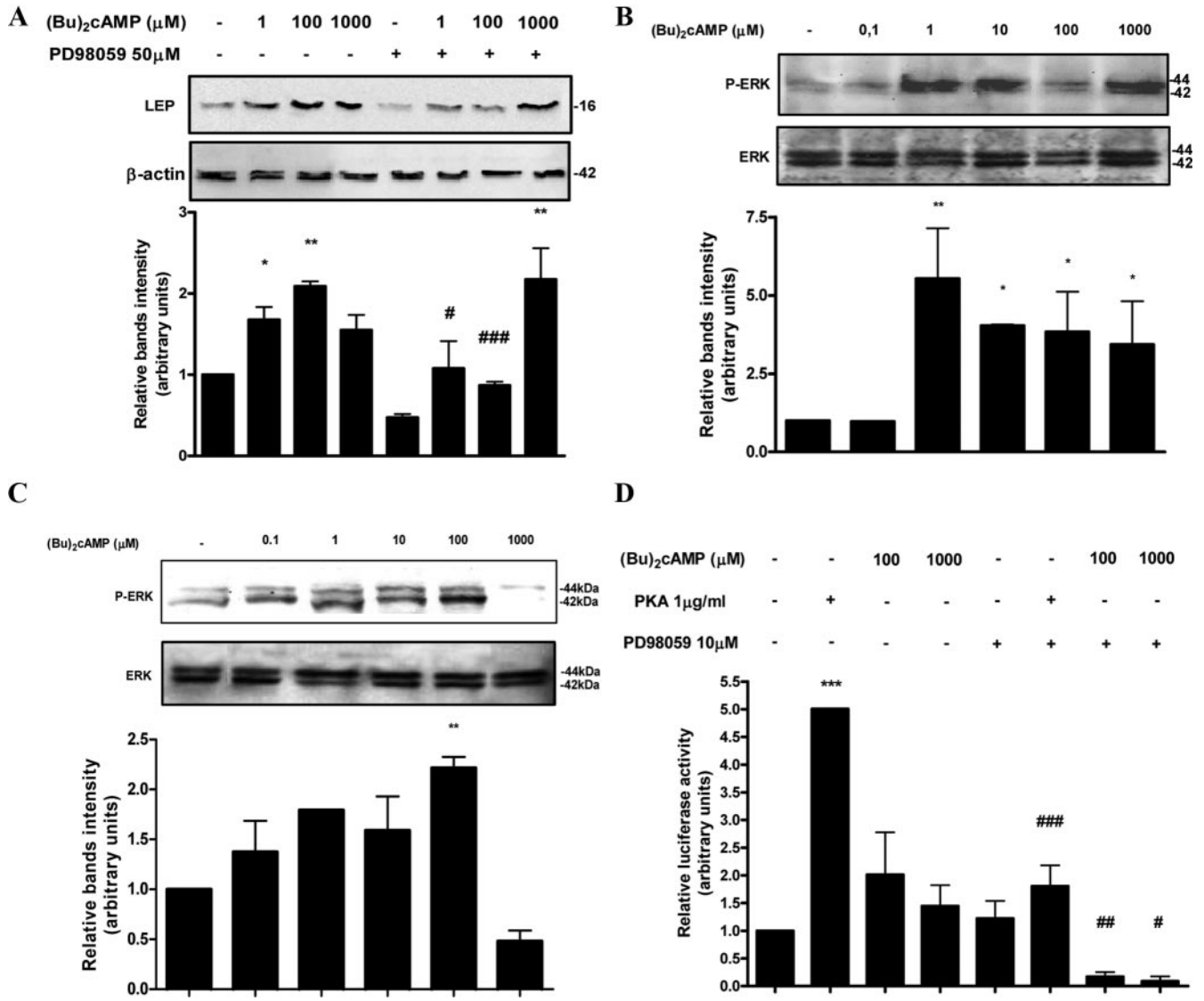

FIG. 5. cAMP activates MAPK pathway throughout leptin stimulation. A, Cells were incubated during 3 d with $(Bu)_2$cAMP and PD98059 as indicated. Extracts from cells were prepared as previously described and loaded in a 12% SDS-PAGE. Leptin expression was determined by Western blot analysis. Loading controls were performed by immunoblotting the same membranes with anti-β-actin. B, BeWo cells (1×10^6 cells) were plated in complete DMEM-F12 media supplemented with 1% FBS and incubated for 15 min with different doses of $(Bu)_2$cAMP (μM). C, Placental explants were processed as described in *Materials and Methods* and incubated with $(Bu)_2$cAMP as indicated. B and C, Extracts were loaded in a 12% SDS-PAGE, and ERK 1/2 phosphorylation was determined by Western blot analysis. Total ERK 1/2 protein level in extracts was determined as loading control. 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 means ± SD for three independent experiments. D, Cells were transiently transfected with pL1951 plasmid construction or cotransfected with plasmid encoding the catalytic subunit of PKA and treated with $(Bu)_2$cAMP and PD98059, alone or combined as indicated. Cells were incubated during 72 h in DMEM-F12 1% FBS media. Luc 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 ± SD for three independent experiments performed in duplicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ *vs.* $(Bu)_2$cAMP treatment. LEP, Leptin.

$(Bu)_2$cAMP, and ERK 1/2 phosphorylation was assessed by Western blot analysis. As it is shown in Fig. 5B, cAMP induced MAPK phosphorylation reaching a 2.3-fold maximal effect with 10 μM $(Bu)_2$cAMP. To confirm, in a more physiological model, whether cAMP could mediated MAPK signaling pathway activation in human placenta, we examined phosphorylation of ERK 1/2 in placental explants treated with different doses of $(Bu)_2$cAMP. As shown in Fig. 5C, ERK 1/2 phosphorylation was significantly increased with cAMP treatment. Maximal effect was achieved with 100 μM $(Bu)_2$cAMP. To further study the MAPK involvement in the cAMP effect on leptin expression, we performed transient transfection experiments using the reporter pL1951 construction, and cells were treated with 100 μM or 1 mM $(Bu)_2$cAMP and/or 10 μM PD98059. The cyclic nucleotide induced leptin promoter activity, and surprisingly, this effect was fully blocked with 10 μM PD98059 (Fig. 5D). Moreover, when

experiments of cotransfection with an expression vector encoding the catalytic subunit of the PKA were performed, we observed a significant stimulation of leptin promoter activity reaching a 5-fold induction over control. Treatment with 10 μM PD98059 blocked this PKA induction. Taken together, all these data suggest that cAMP induction of leptin gene in placental cells is mediated not only by the PKA signaling pathway activation but also by the MAPK/ERK pathway, and probably a cross talk between them is the responsible for the observed effects.

cAMP effect on leptin expression in placental explants involves cross talk between PKA and MAPK signaling transduction pathways

The PKA and MAPK pathways do not operate independently of each other, but multiple cross talk events between these pathways can occur. In this context, we decided to investigate the interrelationship between these two pathways in cAMP effect on leptin expression in placental cells. To establish whether inhibition of MAPK pathway could affect the activation of cAMP signaling in human placenta, we examined CREB phosphorylation by Western blot analysis in placental explants treated with 0,1 or 1 μM $(Bu)_2$cAMP and/or 50 μM PD98059. As expected, CREB phosphorylation was enhanced with cAMP incubation (Fig. 6A). Surprisingly this effect was blocked with 50 μM PD98059. With a similar approach, we next examined the phosphorylation of ERK 1/2 in BeWo cells preincubated with 10 μM H89 or 100 μM SQ22,536 and then treated with 1 or 1000 μM $(Bu)_2$cAMP. Kinase phosphorylation was determined by Western blot analysis. As shown in Fig. 6B, ERK 1/2 phosphorylation was significantly increased when PKA or adenylyl cyclase inhibitors were used. Moreover, when cells were treated with 1 or 1000 μM $(Bu)_2$cAMP plus H89, ERK 1/2 phosphorylation reached a 2,2- and 2,8-fold induction, respectively. These findings indicate that both PKA and MAPK signaling pathways are involved in cAMP up-regulation of leptin expression in placenta and that this effect probably occurs as a result of a cross talk between those signaling pathways. The molecular mechanisms underlying the current findings remain to be determined.

Discussion

The placenta is a unique, autonomous, and transient organ. It ensures maternal-fetal exchanges and is also involved in maternal tolerance of feto-paternal antigens (4). Human placental trophoblasts produce various hormones and cytokines that play essential roles in the establishment and maintenance of pregnancy and fetal growth (2, 43). The production of these substances is regulated by multiple factors and second messengers, including cAMP, which has a central function in this process (23). 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 (44). During the third trimester of pregnancy, leptin receptor levels show a marked expression (45). Many physiological roles have been suggested for leptin in human pregnancy (7, 21, 46–49). The placenta is a significant source of maternal circulating leptin, and the levels in the second and third trimesters are approximately 200% of those in the first trimester or in the nongravid situation (12, 19). However, because trophoblastic cells produce leptin locally, the effective concentration of this hormone may be higher in the placenta. The autocrine action of leptin may be important for trophoblast cell survival (16) and also be relevant for pathophysiological conditions, because 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 (21, 50). In the present study, the regulation of leptin expression in BeWo and JEG-3 human choriocarcinoma cells was investigated. BeWo and JEG-3 cells express both leptin and its receptor (16). They maintain many characteristics of human trophoblast cells and have been widely used to study placental cellular signaling (51–53). Normal trophoblastic explants from healthy donors were also studied to confirm the physiological relevance of the results.

Adipose leptin mRNA levels are increased by glucocorticoids and insulin (19–21) and decreased by β-adrenergic agonists (54). On the other hand, a rise of cAMP concentration decreases leptin secretion and a decrease exerts the opposite effect (55). Moreover, it has been reported that the regulation of leptin synthesis is mediated by steroid hormones (56, 57) and glucocorticoids (26). It was also demonstrated that the human leptin gene is actively engaged by hypoxia through mechanisms that are common to other hypoxia-inducible genes (58). Various regulatory elements have been identified within the leptin promoter (*e.g.* cAMP and glucocorticoid response elements and CCATT/enhancer and SP-1 binding sites), suggesting a direct regulation of leptin expression through different transcriptional pathways (5, 59–61). A placental specific enhancer located 1.9 kb upstream of the human leptin gene was identified (33). We have previously reported that hCG, a master hormone in pregnancy, up-regulates leptin expression in human placenta (22).

The regulation and physiology of leptin in placenta is largely unknown, and in particular, little is known about

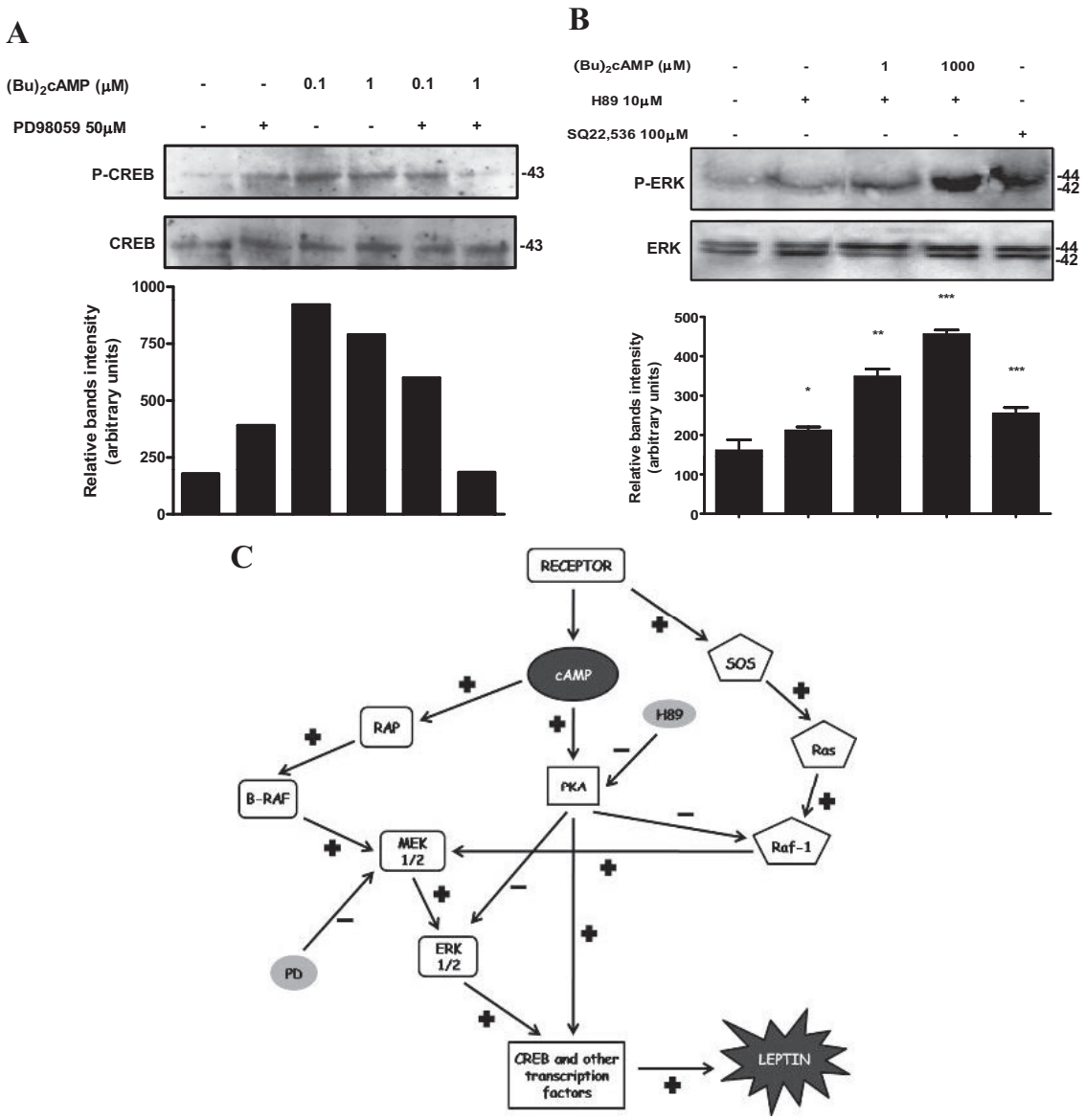

FIG. 6. A cross talk between PKA and MAPK signaling pathways is involved in cAMP effect on leptin expression in placenta. A, Human placental explants were processed as previously described and treated with $(Bu)_2$cAMP and/or PD98059 for 15 min. B, BeWo cells (1×10^6 cells) were plated in complete DMEM-F12 media supplemented with 1% FBS and incubated for 15 min with $(Bu)_2$cAMP, H89, or SQ22,536 as indicated. In all cases, cells or placental explants extracts were loaded in a 12% SDS-PAGE, and CREB (A) or ERK 1/2 (B) phosphorylation was determined by Western blot analysis as indicated in *Materials and Methods*. Total CREB or ERK 1/2 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 ± SD for two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control. C, Proposed model of the signaling pathways involved in cAMP stimulation based on current data and its relation with leptin expression. +, Stimulation; −, inhibition. RAP, Small Ras-like GTPase Rap1.

leptin regulation by cAMP. When the effect of cAMP on leptin expression in BeWo and JEG-3 cells was investigated, a significant up-regulation was observed. Moreover, we observed that cAMP was able to increase leptin promoter activity and leptin mRNA transcription. These results were confirmed performing similar experiments in placental explants at term, a more physiological model, where we found that cAMP enhanced leptin expression and leptin mRNA levels. Our results showed that low doses of cAMP enhanced leptin expression in a dose-dependent manner, both in cell line and placental explants, but at higher doses (>1 mM), the stimulation disappeared, resulting in an inverted U-shaped dose-response curve. It has been reported that activation of PKA was capable of regulating the expression of PKA subunits. A decrease in catalytic subunits and increase in regulatory subunit levels were observed in response to sustained activation of the PKA system by forskolin or cAMP analogs (62). This negative feedback regulatory mechanism helps to sustain cellular homeostasis under prolonged hormonal or neuro-

hormonal stimulation and prevents from overstimulation and might explain our results. The stimulatory effect of cAMP on leptin expression has also been reported in several nonadipose cells, such as C6 glioma cells, GH3 pituitary tumor cells (63, 64), MCF-7 breast cancer cells (65), gastric MKN-74 cells (11), and placental chorionic tissue (27). However, these results are in contrast to what has been demonstrated in adipose tissue. In explants cultures of mature adipocytes, PKA activation suppressed leptin secretion and leptin mRNA levels (24). Nevertheless, this discrepancy in the regulation of both leptin-producing tissues is in agreement with the fact that the human leptin gene has a placenta specific enhancer, and a placenta-specific nuclear binding protein is involved in leptin expression (33, 66). These evidences indicate that the regulation of leptin production in placental trophoblasts is likely different from that in adipocytes.

The synthesis and secretion of leptin in normal trophoblast cells are regulated by a variety of endogenous biochemical agents, which include growth factors, hormones, and cytokines (48). These agents initiate intracellular signaling events that result in the activation of several signal transduction pathways (31, 67), including the cAMP/PKA and MAPK pathways (68–71).

It is known that the PKA pathway plays a central role in biological signaling of various hormones in the placenta, such as epinephrine, prostanoids, and hCG (31).

Many peptides of the insulin superfamily also stimulate the PKA system in muscle tissues of vertebrates and invertebrates (72). Moreover, there is a large group of hormones and growth factors, such as glucagon, TSH, epidermal growth factor, platelet-derived growth factor, *etc.*, in which cAMP can have a role as a second messenger (73).

Insulin and glucocorticoids are good hormone candidates to regulate leptin expression and secretion in the placenta, because they are increased in maternal serum at the end of gestation and their receptors are expressed in the placenta (26, 74). Indeed, insulin and dexamethasone are important modulators of leptin gene expression, and secretion from freshly isolated rat adipocytes is acutely regulated by insulin (75). Dexamethasone-stimulated leptin secretion is mediated primarily by transcriptional mechanisms. By contrast, insulin's effects on leptin synthesis/secretion appear to be regulated in at least two posttranscriptional steps: 1) by mobilizing preexisting pools of leptin, and 2) by stimulating leptin protein synthesis. These studies also implicate a role for a phosphoinositide-3-kinase-dependent, a PD98059-sensitive and/or a rapamycin-sensitive signaling pathway in mediating leptin synthesis and secretion (76). Although there is available literature regarding the regulation of the adipose tissue leptin gene, the data regarding the regulation of placental leptin are meager by comparison. Insulin effects involve the recruitment of CCAAT/enhancer binding protein α through cAMP-dependent pathways (77). Binding of insulin to its receptor also activates the MAPK pathway (78, 79).

In the present work, we analyzed whether the induction effect of cAMP on leptin expression results from the activation of PKA or whether it is due to PKA-independent events induced by this nucleotide. The latter possibility cannot be excluded, because several experiments have provided evidence that cAMP affects some cellular processes independently of PKA (28–30). Among PKA intracellular substrates, it is the CREB (36, 80). CREB is a cAMP-regulated transcriptional regulatory protein that binds to consensus cAMP-responsive DNA elements within target genes. CREB is important for the expression of many cAMP-responsive genes in different cell types and in response to diverse signals (81). We confirmed that cAMP was able to induce CREB phosphorylation not only in choriocarcinoma cells but also in placental explants. Moreover, the overexpression of the catalytic subunit of PKA or the transcription factor CREB caused a significant increase in leptin promoter activity in a dose-dependent manner. The participation of PKA pathway in cAMP induction of leptin expression was further demonstrated when the overexpression of a dominant negative regulatory subunit of PKA (PKI), produced a decrease in leptin expression. We also examined the involvement of PKA-dependent pathway by using a PKA selective inhibitor, H89 (82). We observed that specific inhibition of PKA suppressed cAMP induction of leptin expression in BeWo cells and in placental explants. Furthermore, our results show that PKA pathway acts at a transcriptional level, because H89 was able to inhibit leptin promoter activity induced by cAMP. On the other hand, when cells were treated with SQ22,536, an adenylyl cyclase inhibitor, leptin promoter activity was reduced, demonstrating that a decreased in endogenous cAMP level also affects basal leptin expression. All these data strongly suggest that an increase in cAMP level stimulates leptin expression through the activation of the PKA signaling pathway.

MAPK pathway is essential for reproduction in general. It has been involved in oocyte maturation and in the control of trophoblast penetration and invasion (83). Moreover, it is well established that the ERK pathway is involved in placental development (84). We have also demonstrated previously that leptin promotes proliferation and prevents apoptosis in trophoblastics cells through MAPK pathway (17). These evidences prompted us to investigate whether the MAPK signal transduction pathway was involved in cAMP up-regulation of leptin gene in placental cells. The involvement of this signaling pathway was demonstrated using the pharmacologic MEK inhibi-

tor, PD98059, both by Western blot analysis and reporter experiments. We showed that MAPK signaling participates in the regulation of leptin expression by cAMP. In addition, PD98059 partially blocked leptin induction caused by the overexpression of the catalytic subunit of the PKA. In this context, it has been reported that MAPK cascade can modulate PKA activity by several mechanisms (85).

The cAMP/PKA pathway and the MAPK cascades modulate common processes in the cell and multiple levels of cross talk between these signaling pathways have been described (85–88). In some cell types, and under certain circumstances, activation of PKA results in activation of the ERK 1/2 pathway, whereas in other cell types and under other culture conditions, PKA blocks ERK 1/2 signaling (89). Although PKA-ERK 1/2 cross talk has been widely studied, the precise mechanisms involved in this process remain unclear. Possible cross talk between these pathways in trophoblastic cells was assessed to investigate the molecular mechanisms underlying cAMP effect on leptin up-regulation. In this way, we have demonstrated that cAMP treatment specifically activated ERK 1/2 phosphorylation in placental cells. Furthermore, the inhibition of MAPK pathway partially prevents CREB phosphorylation by cAMP. Phosphorylation of CREB at serine 133 was initially attributed to PKA. However, studies have established that several kinases can phosphorylate CREB at the same residue. ERK 1/2 cannot directly phosphorylate CREB, but it can activate by phosphorylation of members of the pp90rsk family of protein kinases (ribosomal s6 kinase 1–3), which in turn translocate to the nucleus and directly phosphorylate CREB (90). In addition, we showed that the inhibition of PKA resulted in a significant increase in ERK phosphorylation in placenta, suggesting that PKA activation might cause ERK inhibition and that cAMP activates ERK pathway in a PKA-independent manner. On the other hand, cAMP may activate MAPK through members of the Ras superfamily. In this mechanism, cAMP binds to guanine nucleotide exchange factor and activates Rap1A, which then increases MAPK phosphorylation (91, 92). These possible interrelationships are described in the model based in reported data (85, 93) shown in Fig. 6C.

In the present work we provide evidence that cAMP up-regulates leptin gene in human trophoblastic cells. More interestingly, we demonstrated that the cAMP regulatory effect on leptin expression probably involves both the PKA classic signaling pathway and the MAPK signal transduction pathway. A cross talk between these pathways would be responsible for the observed effects. However, further studies are needed to explain the molecular mechanisms underlying these current findings.

In summary, our results will lead to a better understanding of the regulatory mechanisms of leptin expression by human placental trophoblasts and further support the importance of leptin in the biology of reproduction.

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