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17Beta-Estradiol Enhances Leptin Expression in Human Placental Cells Through Genomic and Nongenomic Actions[1]

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ABSTRACT

The process of embryo implantation and trophoblast invasion is considered the most limiting factor in the establishment of pregnancy. Leptin was originally described as an adipocyte-derived signaling molecule for the central control of metabolism. However, it has been suggested that leptin is involved in other functions during pregnancy, particularly in the placenta, where it was found to be expressed. In the present work, we have found a stimulatory effect of 17beta-estradiol (E_2) on endogenous leptin expression, as analyzed by Western blot, in both the BeWo choriocarcinoma cell line and normal placental explants. This effect was time and dose dependent. Maximal effect was achieved at 10 nM in BeWo cells and 1 nM in placental explants. The E_2 effects involved the estrogen receptor, as the antagonist ICI 182 780 inhibited E_2-induced leptin expression. Moreover, E_2 treatment enhanced leptin promoter activity up to 4-fold, as evaluated by transient transfection with a plasmid construction containing the leptin promoter region and the reporter gene luciferase. This effect was dose dependent. Deletion analysis demonstrated that a minimal promoter region between −1951 and −1847 bp is both necessary and sufficient to achieve E_2 effects. Estradiol action involved estrogen receptor 1, previously known as estrogen receptor alpha, as cotransfection with a vector encoding estrogen receptor 1 potentiated the effects of E_2 on leptin expression. Moreover, E_2 action probably involves membrane receptors too, as treatment with an estradiol-bovine serum albumin complex partially enhanced leptin expression. The effects of E_2 could be blocked by pharmacologic inhibition of MAPK and the phosphoinositide-3-kinase (PI3K) pathways with 50 μM PD98059 and 0.1 μM Wortmannin, respectively. Moreover, cotransfection of dominant negative mutants of *MAP2K* or *MAPK* blocked E_2 induction of leptin promoter. On the other hand, E_2 treatment promoted MAPK1/MAPK3 and AKT phosphorylation in placental cells. In conclusion, we provide evidence suggesting that E_2 induces leptin expression in trophoblastic cells, probably through genomic and nongenomic actions via crosstalk between estrogen receptor 1 and MAPK and PI3K signal transduction pathways.

17beta-estradiol, gene expression, leptin, MAPK signal transduction pathway, placenta

INTRODUCTION

In pregnancy, materno-fetal dialogue is essential for implantation and requires spatial and time-coordinated molecular and cellular interactions. There is evidence suggesting that cytokines produced by the maternal endometrium and the developing embryo play an important role in this signaling process. Several cytokines and growth factors are known to influence trophoblast migration, proliferation, and invasion [1]. In particular, it has been suggested that leptin plays a relevant role in implantation by virtue of its stimulatory effect on matrix metalloproteinase expression in cytotrophoblasts [2]. On the other hand, deregulation of leptin metabolism and/or leptin function in the placenta has been implicated in the pathogenesis of various disorders during pregnancy, such as recurrent miscarriage, gestational diabetes, intrauterine growth retardation, and preeclampsia [3, 4]. Leptin hormone, the product of the *LEP* gene, is a 16 000-MW nonglycosylated polypeptide of 146 amino acids discovered in 1994 by Zhang et al. [5]. This cytokine-type hormone is able to perform multiple functions. The best characterized physiological role of leptin 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 storage. Thus, plasma leptin levels correlate with total adipose mass [6].

Over the last several years, the pleiotropic effects of leptin have been identified, consisting of modulation of several processes such as thermogenesis, homeostasis, angiogenesis, hematopoiesis, osteogenesis, chondrogenesis, neuroendocrine and immune functions, arterial pressure control, and different reproductive functions [7]. Leptin is secreted by placenta [8]; however, the complete regulation of leptin production in the placenta is still poorly understood, and it is particularly unclear why the concentrations of this protein rise abruptly in early pregnancy [7]. Clearly, a better understanding of the specific mechanisms regulating leptin biosynthesis is required to elucidate its physiological relevance during pregnancy.

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Steroids play an important role in the growth, differentiation, metabolism, reproduction, and morphogenesis of higher organisms and humans and are particularly required for the development and maintenance of reproductive tissues [9]. The most potent and dominant estrogen in humans is 17β-estradiol, but lower levels of the estrogens estrone and estriol are also present [10]. Circulating estrogens are primarily produced in the ovary in premenopausal women [11].

Estrogen concentration is significantly increased after the ninth week of gestation, reaching levels three to eight times higher than those of nongestating individuals. This increase results from a unique exchange between mother and fetus: the fetus uses pregnenolone produced by the placenta to produce adrenal dehydroepiandrosterone and dehydroepiandrosterone sulfate. These hormones are metabolized to androstenedione and testosterone at the level of the placenta. Finally, they are rapidly converted to estrone and estradiol, which are then released into the maternal circulation [12].

Human placenta has been shown to synthesize estrogens during pregnancy [13], in association with cytotrophoblast invasion [14, 15]. Moreover, there is evidence to suggest that placentally derived estrogen may play an autocrine role in trophoblast differentiation [16, 17].

Classical estrogen response involves the binding of estradiol to estrogen receptors ESR1 and ESR2 (formerly known as ERalpha and ERbeta), which consequently dimerize and act as ligand-activated transcription factors whose primary function is the modulation of gene expression (genomic or nuclear pathway). ESR-dependent activation of gene expression also involves DNA-bound ESR that subsequently interacts with other DNA-bound transcription factors as well as direct ESR-transcription factor (protein-protein) interactions, where ESR does not bind promoter DNA [18].

Recent evidence suggests that along with gene regulation, estradiol also mediates rapid cellular effects (nongenomic or extranuclear pathways). These ESRs are usually localized in the cell membrane and rapidly activate protein kinase pathways, alter membrane electrical properties, and modulate ion flux [19]. ESR1 and 2 proteins have also been localized in nuclei of cultured human syncytiotrophoblast cells using immunohistochemistry [17].

Some years ago, O'Neil et al. [20] demonstrated that estrogen could activate the *LEP* promoter in choriocarcinoma JEG-3 cells through ESR1 and suggested that regulation of leptin biosynthesis may depend on the existence of a functional ESR. Although these findings suggested the importance of estrogens in leptin biosynthesis, the mechanisms through which estrogen mediates leptin expression in human placenta remain undefined.

In the present study, we analyzed the effect of E_2 on leptin expression in human placenta using two experimental models: BeWo choriocarcinoma cells and human placental explants. We demonstrated that E_2 indeed upregulates leptin expression in placental cells, and that this effect probably involves both genomic and nongenomic actions via crosstalk between ESR1 and MAPK and PI3K signal transduction pathways.

MATERIALS AND METHODS

Cell Culture and Treatments

The human choriocarcinoma cell line BeWo was grown in 45% Dulbecco modified Eagle medium (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 Company, St. Louis, MO) at 37°C in 5% CO_2.

The effect of 17β-estradiol (E_2; Sigma) was tested at different doses (0.01–10 μM). Once E_2 maximal effect on leptin expression was determined, 10 or 100 nM E_2 was used in subsequent experiments. The antiestrogen ICI 182 780 (AstraZeneca, London, U.K.) was used to assay the specificity of estrogen effects. Membrane estrogen actions were demonstrated by incubating with estradiol covalently linked to bovine serum albumin (BSA) (E-BSA; β-Estradiol 6-(O-carboxymethyl)oxime:BSA; Sigma). E-BSA was filtered prior to use in order to eliminate free E_2 according to a previously described method [21]. Briefly, 900 μl of E-BSA (400 μM in estradiol dissolved in 50 mM Tris-HCl [pH 8.5]) was added to a centrifugal filter unit with a MW cutoff of 3000 (Millipore, Billerica, MA) and then subjected to centrifugation at 13 000 × *g* for 30 min. The retained fraction was washed three times with buffer and recovered. Volume was adjusted to 900 μl. The efficacy of filtration to remove free E_2 was verified by Stevis et al. [21].

The specific PIK3 (PI3K) inhibitor Wortmannin (0.1 μM) and the MAP2K (MEK) inhibitor PD98059 (50 μM; Sigma) were also used. Treatments were performed in DMEM-F12 media without phenol red and supplemented with 1% charcoal-stripped FCS unless otherwise stated. Serum present in incubation media was reduced from 10% to 1% in order to lower nonspecific effects.

Placental Explants Collection and Processing

Human placentas (n = 9) were obtained after Cesarean section or vaginal delivery following normal term pregnancies and were 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 the cotyledons were cut into multiple cubic segments (10–15 mg wet weight) and thoroughly rinsed with cold DMEM-F12 medium. None of the donor patients suffered from anomalous pregnancy. Samples were obtained under informed consent, and the study was approved by the Hospital Universitario Virgen Macarena (Seville, Spain) ethics committee.

Treatments of Placental Explants

Placental explants were randomly distributed in tubes containing 1 ml of DMEM-F12 medium without FCS (one explant per tube, four replicates per treatment). Placental explants were maintained in a shaking water bath at 37°C for 5 min to equilize temperature and incubated for 4 h in the same medium supplemented with or without 0.01–1 μM E_2. Explants were removed from the bath, centrifuged for 2 min at 2000 × *g* at 4°C, resuspended in 500 μl of lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 10 mg/ml phenylmethanesulfonyl fluoride) for 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.

Western Blot Analysis

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 Bradford [22] using BSA as standard. Lysates were mixed with Laemmli sample buffer containing 2% SDS and 30 mM β-mercaptoethanol, boiled for 5 min, separated by SDS-PAGE on a 12% gel, and electrophoretically transferred to a nitrocellulose membrane (Hybond; Amersham Pharmacia, Buckinghamshire, U.K.). Membranes were equilibrated in 1× PBS, and nonspecific binding sites were blocked by 5% nonfat milk in PBS at room temperature for 1 h. Membranes were then immunoblotted with polyclonal rabbit anti-human leptin Y20 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) with polyclonal rabbit anti-phospho-mitogen-activated protein kinases 1 and 3 (MAPK1/3; Thr202/Tyr204; 1:3000) or with polyclonal rabbit anti-phospho-AKT (Ser 472/473) antibody (1:3000; BD Biosciences Pharmingen, NJ). Loading controls were performed by immunoblotting the same membranes with polyclonal rabbit anti-ACTB (1:10 000; Sigma), polyclonal rabbit anti-total-mitogen-activated protein kinases 1 and 3 (MAPK 1/3; 1:3000; Sigma), or polyclonal rabbit anti-total-AKT antibody (1:3000; Sigma). Bound antibodies were detected using horseradish peroxidase-linked goat anti-rabbit IgG (1:10 000; Santa Cruz) and visualized by the Amersham Pharmacia ECL Chemiluminescence signaling system and a Bio-Imaging Analyzer Fujifilm LAS-1000 (Fuji Photo Film Company, Tokyo, Japan). Quantification of protein bands was performed by densitometry using Scion Image software (Scion Corporation, Washington, DC).

Plasmids

The luciferase reporter constructs based on the PGL-3 basic vector and PGL-3 promoter vector are shown schematically in Figure 2, C and D. They were all kindly provided by Dr. Oksana Gavrilova (NIDDK, National Institutes of Health, Bethesda, MD) [23]. To simplify the notation we renamed some of

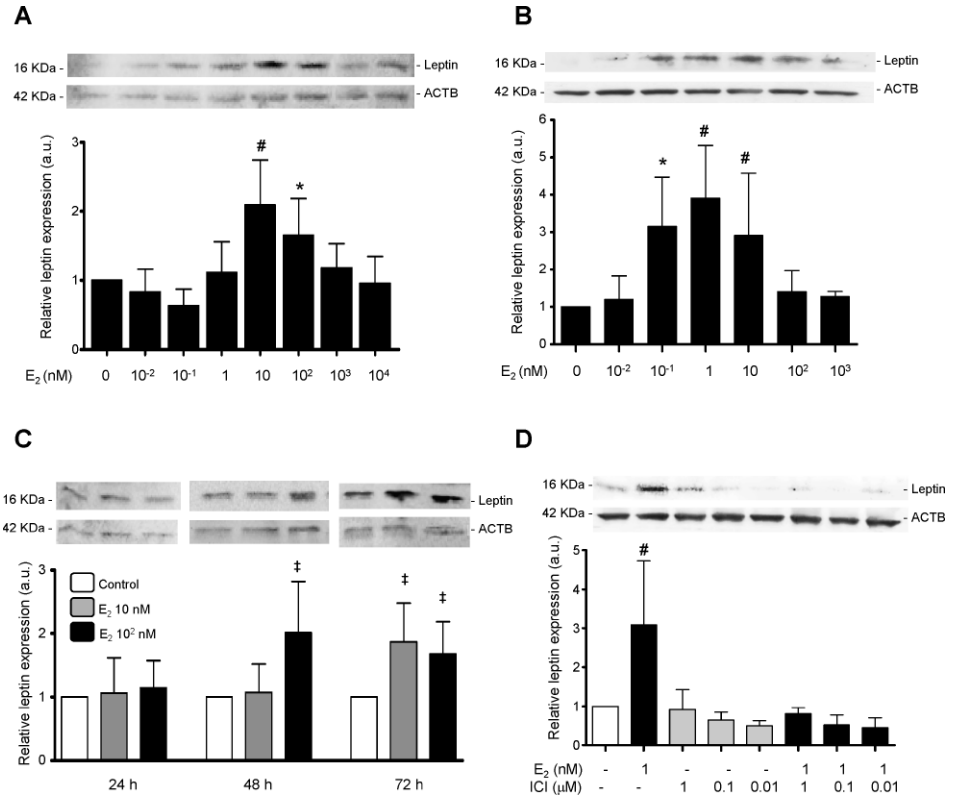

FIG. 1. E_2 enhances leptin expression in placental cells. **A**) BeWo cells were incubated for 72 h with different E_2 doses, as indicated, in DMEM-F12 media supplemented with 1% charcoal-stripped FCS. **B**) Placental explants were processed as described in *Materials and Methods* and treated with different E_2 doses for 4 h as indicated. **C**) BeWo cells were incubated for 24 (lanes 1–3), 48 (lanes 4–6), or 72 h (lanes 7–9) and treated with or without 10 and 100 nM E_2. **D**) Placental explants were incubated with or without different doses of the antiestrogen ICI 182 780 for 4 h, in the presence or absence of 100 nM E_2. In all cases, 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. Loading controls were performed by immunoblotting the same membranes with anti-ACTB. Band densitometry is shown in lower panels. Representative results from three replicates are shown. Statistical analysis was performed by ANOVA. Significant differences from control were determined by Dunnett multiple comparison post hoc test. $*P < 0.05$; $\#P < 0.01$; $\ddagger P < 0.001$ vs. control. a.u., arbitrary units.

them, indicating the number of base pairs upstream of transcription initiation as follows: pL1951 (p1775), pL1546 (p1776), pL948 (p1777), pL805 (p1778), and pL218 (p1779). An empty vector was used as control in reporter experiments. Plasmids derived from the PGL-3 vector contained fragments of leptin promoter regions upstream of the SV40 promoter as indicated in Figure 2D: (a) p1611 (empty construct), (b) p1842 (−1951 bp to −1546 bp), and (c) p1860 (−1951 bp to −1847 bp). The plasmid HEGO is an expression vector containing the cDNA of estrogen receptor 1 (*ESR1*), and the plasmid ERE-*Luc* is an estrogen-responsive element (ERE) promoter-driven luciferase reporter plasmid. These plasmids were kindly provided by Dr. Adalí Pecci (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina) [24]. The pcDNA1 vectors encoding the kinase-inactive mutants of *MAPK1* and *MAP2K1* (designated MAPK-kd and MAP2K-kd, respectively) were kindly supplied by Dr. J. Silvio Gutkind (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD) [25].

To normalize the efficiency of individual transfections, pRSV-βgal containing the β-galactosidase gene under the control of the Rous sarcoma virus (RSV) was used. In experiments using expression plasmids, the empty vectors were used as controls. To perform transient transfection assays, plasmids were purified using the Maxipreps 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 per milliliter 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. In order 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, 5 μg of pRSV-βgal internal control construct, and/or 5 μg of each expression plasmid were transfected using 5 μl of LipofectAMINE (Life Technologies, Inc., Grand Island, NY). The medium was replaced after 5 h with DMEM-F12 1% charcoal-stripped FCS as indicated in each figure, plus the addition of the different effectors. Transfection analysis was performed in duplicate in 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 the 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 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. 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 mean ± SD in arbitrary units (a.u.). Arbitrary units were calculated as normalized band intensity in Western blot analysis or normalized luciferase activity in reporter assays, where control values were taken as 1. Statistical analysis was performed using the Graph Pad Prism computer program (GraphPad 4.0; GraphPad Software, San Diego, CA). Statistical significance was assessed by ANOVA, followed by different post hoc tests, as indicated in each figure. $P < 0.05$ was considered statistically significant.

RESULTS

17β-Estradiol Enhances Endogenous Leptin Expression in Placental Cells

In this study, we investigated E_2 modulation of leptin expression in the choriocarcinoma cell line BeWo and in human placental explants. BeWo cells were previously used as a model for trophoblastic cells and express leptin and leptin receptor [26–28]. Cells were seeded at 50%–60% confluence in complete DMEM-F12 medium with 10% FCS. In all experiments, prior to E_2 treatment, media was replaced by DMEM-F12 without phenol red and 1% charcoal-stripped FCS to reduce basal estrogen effects. No differences were evident when complete DMEM-F12 was used (data not shown). As seen in Figure 1A, E_2 enhanced leptin expression in BeWo

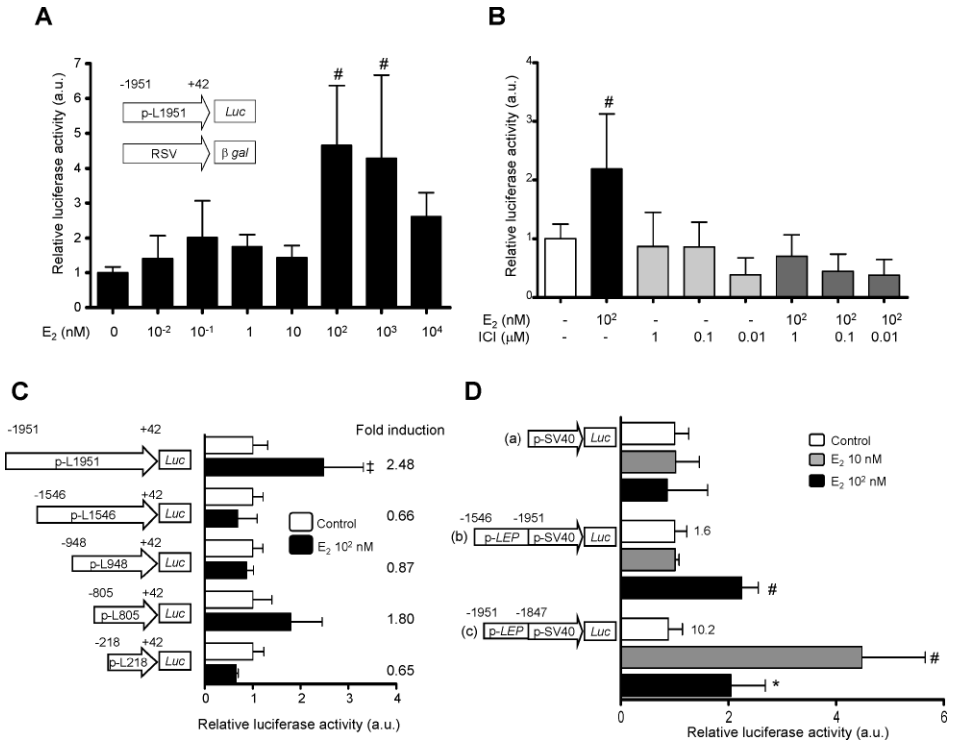

FIG. 2. E_2 enhances leptin promoter activity in BeWo cells. **A**) Cells were transiently transfected with pL1951 plasmid construction and treated with E_2 as indicated. **B**) Cells were transfected with pL1951 and incubated with different doses of ICI 182 780, in the presence or absence of 100 nM E_2. **C**) Cells were transiently transfected with plasmids containing different leptin promoter deletions and treated with (black bars) or without (white bars) 100 nM E_2. **D**) Cells were transfected with plasmids derived from pGL3 promoter containing fragments of leptin promoter upstream of SV40 promoter and incubated with 10 nM E_2 (gray bars), 100 nM E_2 (black bars), or not treated (white bars). Numbers at right show basal promoter activity of each construct relative to the empty vector. In all cases, after transfection, cells were incubated for 48 h in DMEM-F12 media supplemented with 1% charcoal-stripped FCS. Luciferase activity was measured in cellular extracts and normalized to β-galactosidase activity. Activity obtained in the absence of E_2 and ICI 182 780 was set as control. Results are expressed as mean ± SD for three independent experiments performed in duplicate. Statistical analysis was performed by ANOVA. Significant differences from control were determined by Dunnett multiple comparison post hoc test. *$P < 0.05$; #$P < 0.01$; ‡$P < 0.001$ vs. control. a.u., arbitrary units.

cells, as determined by Western blot. This effect was dose dependent, reaching a 2.1-fold increase that turned out to be statistically significant. Maximal effect was achieved at 10 nM E_2. At higher doses, leptin expression returned to control levels.

In order to study the effects of E_2 in a more physiological system, human placental explants from healthy donors were analyzed. Explants were incubated for 4 h in medium with or without different E_2 concentrations (0.01–1000 nM). Figure 1B shows that E_2 enhanced leptin expression in placental explants, as evidenced by Western blot. This effect was dose dependent, reaching a 3.9-fold increase that turned out to be statistically significant. Maximal effect was achieved at 1 nM E_2.

To further characterize the effects of E_2 on leptin expression in trophoblastic cells, time-course experiments were carried out in BeWo cells stimulated with 10 or 100 nM E_2 in DMEM-F12 without phenol red and supplemented with 1% charcoal-stripped FCS media. As shown in Figure 1C, the presence of the hormone in the culture media produced an increase in leptin expression, as measured by Western blot. Maximal effect was obtained at 48 h of treatment with 100 nM E_2, reaching a 2-fold increase above control. At 72 h of treatment, leptin induction could already be observed at 10 nM E_2.

ESR Antagonist ICI 182 780 Inhibits Leptin Expression

To analyze the role of the ESR in leptin expression in human placenta, placental explants were cultured for 4 h in DMEM-F12, either in the absence or presence of various concentrations of the ESR antagonist ICI 182 780 (0.01–1 μM). As shown in Figure 1D, leptin expression was decreased when explants were cultured in the presence of 0.01 μM ICI 182 780. Furthermore, E_2 enhancement of leptin expression was blocked using ICI 182 780, suggesting that the antiestrogen may exert its effect by antagonizing the action of E_2/ESR1 on leptin gene expression. These results reinforce the notion that E_2 has a role in regulating leptin expression in placental cells.

Leptin Promoter Activity Is Induced by E_2

Since E_2 was able to induce leptin expression, we decided to study whether this effect occurred at the transcriptional level. We performed transient transfection assays with plasmid pL1951, which contains the regulatory region of leptin gene, from −1951 bp to +42 bp, fused to the luciferase (*Luc*) reporter gene. In cells transfected with pL1951, treatment with E_2 for 48 h significantly increased LUC expression (Fig. 2A), and the maximal effect was achieved at 100 nM E_2. When transfected cells were incubated in the presence of the antiestrogen ICI 182 780, the effect of E_2 was antagonized (Fig. 2B). Taken together, these results suggest that E_2 enhances leptin promoter activity and regulates leptin gene expression at the transcriptional level.

Serial Deletion Analysis

In order to identify the minimal promoter region necessary for E_2 induction of leptin expression, BeWo cells were

transfected with plasmids containing serial deletions of leptin promoter. The stimulatory effect of E_2 on LUC activity was not observed when constructions containing regions below −1546 bp were used (Fig. 2C). These results suggest that the inducing effect of E_2 would involve the promoter region from −1951 to −1546 bp.

To analyze the importance of this fragment in the regulation of leptin expression, different reporter vectors containing the leptin promoter region from −1951 to −1546 bp (p1842, Fig. 2D b) and -1951 bp to -1847 bp (p1860, Fig. 2D c) were used. These fragments were cloned in PGL-3 vector (Fig. 2D a) upstream from the SV40 promoter that directs reporter *Luc* expression. BeWo cells were transiently transfected with these constructions and incubated with 10 or 100 nM E_2 during 48 h. As shown in Figure 2D, E_2 increased LUC activity in cells transfected with plasmid (b) or with plasmid (c). Maximal E_2 effect was observed in cells transfected with construction (c) and treated with 10 nM E_2 (4-fold increase above control). Both constructions showed a significant increase in LUC activity when cells were treated with 100 nM E_2.

As shown in the inset in Figure 2D, the promoter fragment from −1951 to −1847 bp raised basal LUC activity significantly, suggesting that this region contains strong positive elements that regulate leptin expression in placenta.

E_2 Action Probably Involves Both Soluble and Membrane Receptors

In order to study the involvement of ESR1 in E_2 leptin induction, cotransfection experiments with different concentrations of an ESR1 expression vector (HEGO plasmid) and reporter plasmid pL1951 were performed. As shown in Figure 3A, the transcriptional activity of the leptin promoter was significantly enhanced when cotransfected with 50 ng of HEGO plasmid. The transfection with HEGO plasmid increased the luciferase activity of the ERE-*Luc* reporter construct in BeWo cells, both in the presence or absence of E_2, suggesting that HEGO actually increased ESR1 protein (Fig. 3B). The overexpression of ESR1 enhanced E_2 induction at almost all concentrations studied (Fig. 3A, inset). These results strongly suggest that ESR1 is involved in E_2 regulation of leptin expression and that this regulation is dependent on E_2 concentration.

Recent evidence suggests that, along with gene regulation, estrogen also mediates rapid cellular effects through membrane receptors. Membrane-initiated action can be mimicked with membrane-constrained E_2 conjugates such as, for example, E-BSA, which prevents E_2 from entering cells due to the large

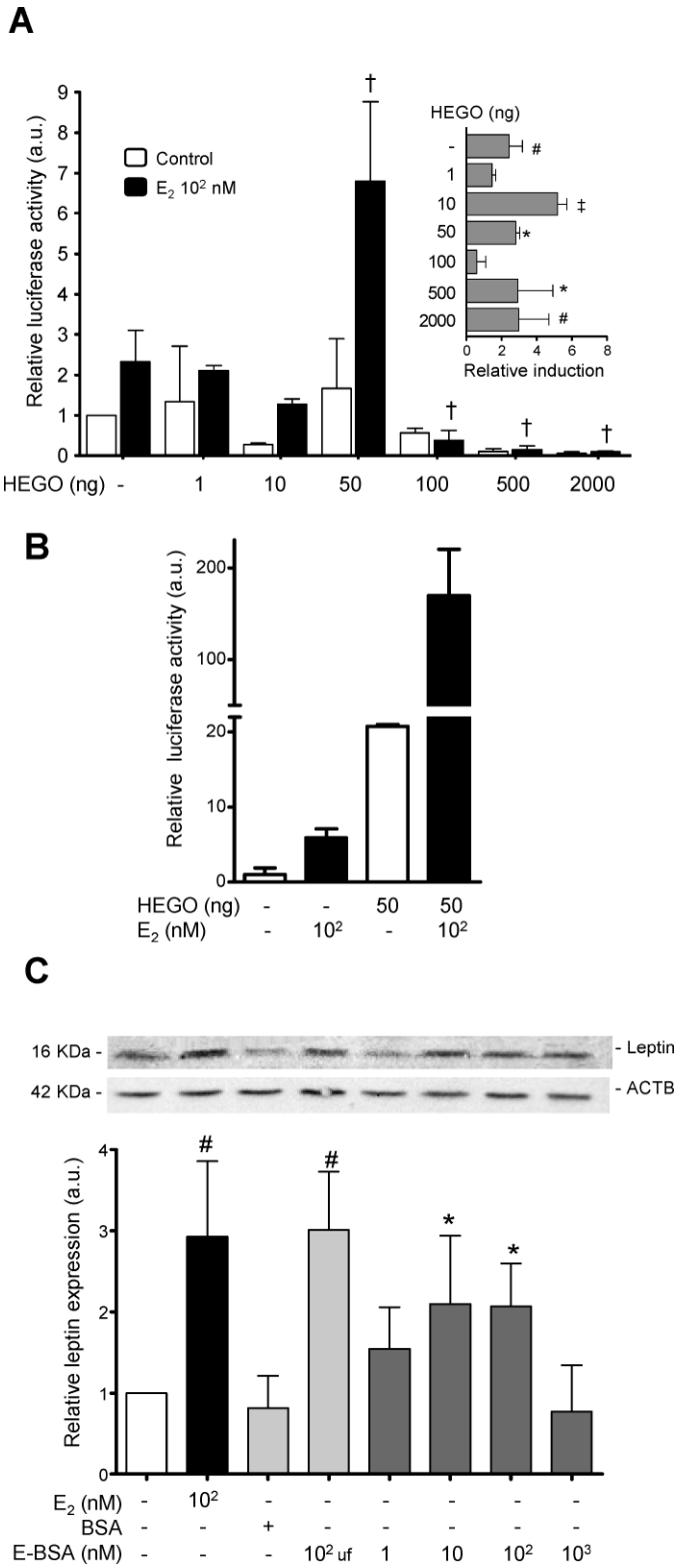

FIG. 3. E_2 action probably involves membrane receptors and *ESR1*. **A**) Cells were transfected with pL1951 and different amounts of plasmid expressing human *ESR1* (HEGO). After transfection, BeWo cells were incubated for 48 h in DMEM-F12 media supplemented with 1% charcoal-stripped FCS and 100 nM of E_2 (black bars) or not (white bars). Cell extracts were prepared as indicated in *Materials and Methods,* and luciferase activity was normalized to β-galactosidase activity. Activity obtained in the absence of E_2 and HEGO was set as control. In the inset, relative induction of luciferase activity observed in the presence of E_2 is shown for each amount of HEGO. Results are expressed as mean ± SD for two independent experiments performed in duplicate. Significant differences from control were determined by Bonferroni multiple comparison post hoc test. $*P < 0.05$; $\#P < 0.01$; $\ddagger P < 0.001$ vs. cells not treated with E_2 and $\dagger P < 0.01$ vs. cells treated with E_2 but not transfected with HEGO. **B**) Cells were transfected with an ERE promoter-driven luciferase reporter plasmid (ERE-*Luc*) and with or without HEGO plasmid. BeWo cells were incubated for 48 h in DMEM-F12 media supplemented with 1% charcoal-stripped FCS and treated with (black bars) or without (white bars) 100 nM E_2. Luciferase activity was measured in cellular extracts and normalized to β-galactosidase activity. Results are expressed as mean ± SD for two independent experiments performed in duplicates. **C**) BeWo cells were incubated for 48 h with E_2, BSA, and unfiltered (uf) or filtered E-BSA, as indicated. Leptin expression was detected by Western blot, and ACTB was used as loading control. Results are expressed as mean ± SD for four independent experiments. Significant differences from control were determined by Dunnett multiple comparison post hoc test. $*P < 0.05$; $\#P < 0.01$ vs. control. a.u., arbitrary units.

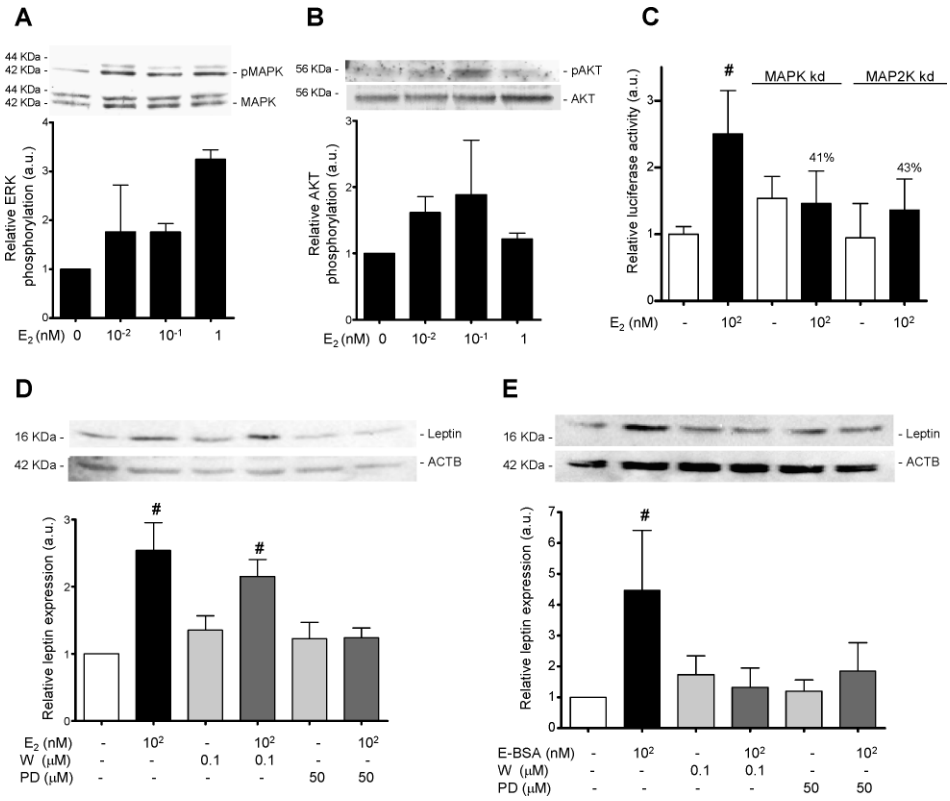

FIG. 4. MAPK1/3 and AKT are activated by E_2 and are involved in placental leptin expression. **A**, **B**) Placental explants were processed as previously described and treated with E_2 for 10 min. MAPK1/3 (**A**) or AKT (**B**) phosphorylation was determined by Western blot as indicated in *Materials and Methods*. Total MAPK or AKT protein levels in cell extracts were determined as loading controls. **C**) Cells were transfected with pL1951 and vectors encoding the dominant negative mutants for *MAPK1* and *MAP2K1* (MAPK-kd and MAP2K-kd, respectively). BeWo cells were incubated for 48 h with 100 nM of E_2 (black bars) or without (white bars). Cell extracts were prepared as indicated in *Materials and Methods*, and luciferase activity was normalized to β-galactosidase activity. Percentage of inhibition is shown above bars. #$P < 0.01$ vs. control. **D** and **E**) BeWo cells were pretreated with or without PD98059 and Wortmannin for 20 min and then were incubated for 48 h with E_2 (**D**), E-BSA (**E**), or without them. Leptin expression was determined by Western blot. In Western blot analysis, samples were loaded in a 12% SDS-PAGE. Band densitometry is shown in the lower panel. Results from a representative experiment are shown. They are expressed as mean ± SD of two independent experiments. Significant differences from control were determined by Dunnett multiple comparison post hoc test. #$P < 0.01$ vs. control. a.u., arbitrary units.

size of the conjugated molecule. To find out whether E_2 effect on leptin expression was dependent on membrane or soluble receptors, experiments with different concentrations of E-BSA were performed. E-BSA was filtered prior to use in order to eliminate free E_2. As shown in Figure 3C, treatment with 10 or 100 nM E-BSA significantly enhanced leptin expression as measured by Western blot analysis, suggesting the involvement of membrane receptors. Control treatment with unfiltered E-BSA elicited an increase in leptin expression similar to that obtained with E_2, indicating that the E-BSA unfiltered preparation contained free E_2.

MAPK1/3 and AKT Are Activated by E_2 and Are Involved in Placental Leptin Expression

Estrogen action mediated by membrane receptors rapidly activates several protein kinase pathways. To determine whether E_2 could stimulate MAPK and PI3K signaling pathways in trophoblastic cells, placental explants were incubated for 10 min in the presence of E_2, and MAPK1/3 and AKT phosphorylations were determined by Western blot. Figure 4, A and B, shows that 1 nM E_2 increased both MAPK1/3 and AKT phosphorylation. To analyze the role of these pathways in E_2-induced leptin expression, BeWo cells were pretreated for 30 min in the presence or absence of the pharmacological inhibitors PD98059 (50 μM) and Wortmannin (0.1 μM) to block MAPK and PI3K pathways, respectively. Subsequently, 100 nM E_2 was added and cells were further incubated for 48 h. Treatment with Wortmannin showed no significant effect on E_2-mediated upregulation of leptin expression, but treatment with PD98059 blocked the effect of E_2, suggesting that the MAPK pathway is involved in E_2 leptin induction (Fig. 4D).

Similar experiments using E-BSA instead of free E_2, together with 50 μM PD98059 or 0.1 μM Wortmannin, were performed to further analyze E_2 action through membrane receptors. BeWo cells were pretreated for 30 min in the presence or absence of the pharmacological inhibitors to block MAPK or PI3K pathways, and, subsequently, 100 nM E_2 was added and cells further incubated for 48 h. As shown in Figure 4E, the induction of leptin expression by E_2, measured by Western blot, was blocked when cells were preincubated with the pharmacological inhibitors for both MAPK and PI3K pathways, thus revealing the participation of both signal transduction pathways in membrane signaling. Taken together, these results suggest a close crosstalk between the different signaling pathways induced by E_2.

A different experimental approach was also used to evidence MAPK pathway involvement in E_2 induction of leptin expression. BeWo cells were transiently cotransfected with plasmids encoding a dominant negative mutant of the kinase *MAPK1* (MAPK-kd) or a dominant negative mutant of the kinase *MAP2K1* (MAP2K-kd) plus the pL1951 reporter

construction. As shown in Figure 4C, the expression of these mutants blocked E_2-mediated induction of leptin promoter activity, suggesting that the MAPK pathway is involved in leptin induction by E_2 in BeWo cells.

DISCUSSION

Leptin controls the functional integrity of the feto-placental unit, thereby maintaining pregnancy, and has a physiological effect on the development of the placenta. Deregulation of the autocrine/paracrine function of leptin at the feto-placento-maternal interface may be implicated in the pathogenesis of gestational diabetes, preeclampsia, and intrauterine fetal growth restriction [3, 4].

Placenta is a source of as well as a target for the action of leptin, as leptin receptor has been detected in placental trophoblast [29, 30]. During gestation, leptin concentration is greater than in nongravid women, and it increases with gestational age [31]. First trimester placental villi secrete 50-fold higher levels of leptin compared to term villi, and this secretion is potentiated by IL1A, 17β-estradiol, IL6, and CGB [31–34]. It has also been demonstrated that the human leptin gene is actively engaged by hypoxia through mechanisms that are common to other hypoxia-inducible genes [35].

Several studies have shown that leptin induces trophoblast cell proliferation by inhibiting apoptosis [26, 36], stimulates protein synthesis [37] and hormone and cytokine production in trophoblastic cells [38], and regulates fetal growth and development [39].

Human placental leptin is identical to that derived from adipose tissue in terms of size, charge, and immunoreactivity [40]. The leptin gene has a specific upstream enhancer, known as placental leptin enhancer region (PLE) [41], implying that leptin gene expression is regulated differently in placenta than in adipose tissue.

In this regard, it has been shown that E_2 is a regulator of leptin expression both in adipose tissue and in placenta. Estradiol administration enhances the expression of leptin mRNA transcripts and protein secretion by adipocytes, both in vitro and in vivo. Similarly, leptin expression in isolated rat adipocytes is inhibited by an ESR antagonist, and diminution of leptin expression in white adipose tissue in rats following ovariectomy is reverted with E_2 administration [42]. However, little is known about the regulation of leptin expression by E_2 in the placenta. Chardonnens et al. [32] observed that E_2 increased leptin production in cultured human cytotrophoblastic cells from first trimester placenta. Moreover, it was demonstrated that E_2 enhanced leptin promoter activity in JEG-3 cells, suggesting that E_2 regulates *LEP* gene expression via promoter activation [20]. Those investigations suggest that E_2 could be an important regulator of leptin expression in placental cells.

In this study, we investigated the regulation of E_2 on leptin expression in BeWo human choriocarcinoma cells and human placental explants. BeWo cells maintain many characteristics of human trophoblast cells and have been widely used to study placental cellular signaling [43–45]. Both experimental models express leptin and leptin receptor [26, 46], as well as ESR1 and ESR2 [47, 48].

When the effect of E_2 on leptin expression was analyzed in BeWo cells and human placental explants, a significant upregulation was observed. These effects were obtained using physiological E_2 concentrations and are in agreement with some in vivo studies in which a positive correlation between E_2 and serological levels of leptin in pregnant women was found [31, 49]. However, we observed that the highest doses of E_2 tested (1 and 10 μM) did not affect endogenous basal leptin expression. A similar biphasic pattern in E_2 response was previously obtained in cytotrophoblastic cells, but its underlying molecular mechanism is not completely understood [32]. It has been proposed that high doses of E_2 could downregulate the expression of ESRs, as observed in other reproductive tissues, but this hypothesis remains to be tested in placental cells [32].

In order to elucidate the specific effects of estrogen on leptin expression and to determine its molecular mechanism of action, we employed the luciferase reporter gene system to investigate leptin promoter activation directly. Because ERE consensus sequences have been identified in the leptin promoter region [50], we hypothesized that estrogen could activate the leptin promoter and that an ESR antagonist would inhibit this activation.

Using this experimental approach, we found that E_2 increased leptin expression acting at the transcriptional level. Treatment with ICI 182 780 completely blocked E_2 induction of leptin promoter activity and also inhibited basal leptin expression, suggesting a role of endogenous estradiol production in the autocrine control of leptin synthesis.

We also determined that a minimal region spanning −1951 to −1847 bp was sufficient to evidence such induction. In silico analysis of this region of the leptin promoter revealed potential consensus half-sites for ER element and also elements for different transcription factors such as SP1, with a core similarity of 1 (data not shown). Besides, this region includes a placental enhancer region, PLE, previously described [41].

Estrogen, acting via ESR1 or ESR2, modulates gene expression through multiple mechanisms. The traditional activation of an ERE involves ligand-bound receptor binding at a specific palindromic sequence of DNA within the promoters of estrogen-responsive genes [51]. However, genomic effects of estrogen could also be explained by an ERE-independent mechanism, because ESRs can regulate gene expression by modulating the function of other classes of transcription factors through protein-protein interactions in the nucleus. It was reported that ligand-bound ESR also mediates gene transcription from AP1 enhancer elements when in a complex with the AP1 transcription factors FOS and JUN [52–54]. In addition, several estrogen-responsive genes contain ERE half-sites, or binding sites for the orphan nuclear hormone receptor NR5A1, previously called SF-1 (SF-1 response elements [SFREs]) that serve as direct ESR binding sites [55]. ESR1, but not ESR2, is able to bind to SFREs [56]. It has also been demonstrated that genes containing GC-rich promoter sequences are regulated in a similar manner through the interaction of ESRs with the SP1 transcription factor [57].

Increasing numbers of genes are being found that are activated by E_2 through ESR-SP1 complexes, including the *LDLR* [low-density lipoprotein receptor] [58], *FOS* [59], and cyclin D1 [60] genes. The actions of ESRs at SP1 binding sites depend on the ligand, the cell type, and the receptor subtype [61]. On the other hand, it was reported that *Sp1/Sp3* compound heterozygous mice are not viable and manifest severe placental defects [62]. Previous reports have demonstrated the involvement of SP1 in the regulation of the leptin gene in adipocytes [63]. Moreover, there is a putative binding site for this transcription factor in the leptin promoter within the minimal region sufficient to demonstrate E_2 induction in placental cells. Further experiments will be needed to confirm that this transcriptional factor participates in E_2 upregulation of the leptin gene in placental cells and to study the mechanisms involved in this regulation.

It is known that E_2 exerts its actions through two members of the nuclear receptor superfamily, ESR1 and ESR2, and also through a recently discovered G protein-coupled membrane receptor, GPR30, now called GPER, G protein-coupled estrogen receptor 1 [64]. The presence of ESR in primate trophoblast suggests that, as in adipose tissue, the effect of estrogen on leptin expression is an ESR-mediated phenomenon [42]. It is likely that this effect is mediated by ESR1, because ESR1 protein and mRNA expression increase during syncytiotrophoblast differentiation, whereas ESR2 protein is undetectable and *ESR2* RNA levels are relatively low and decline with trophoblast differentiation [65].

Our results support the involvement of ESR1 in placental leptin expression based on the following evidence. In BeWo cells cotransfected with pL1951 and 50 ng of HEGO plasmid encoding the human ESR1, a significant increase of LUC activity was observed in the presence of E_2. Similar results were obtained by O'Neil et al. [20]in JEG-3 cells, where the presence of ESR1 and not the presence of ESR2 was enough to evidence a stimulatory effect of E_2 on leptin expression [20]. However, our results show that a high concentration of HEGO vector has an inhibitory effect on leptin promoter activity, both in control and E_2-treated cells. Such an effect was previously observed in HeLa and CV1 cells [66] and could be explained by the process of auto-inhibition, where free ESR1 molecules may compete with DNA-bound ESR1 for essential transcription factors, such as proteins of the basal transcription machinery [67]. Further experiments will be necessary to test this hypothesis in BeWo cells.

Previous reports have shown that estrogen can activate the *LEP* promoter in JEG-3 cells through ESR1 and that regulation of leptin biosynthesis may depend upon a functional ESR. Moreover, the *LEP* promoter region contains ERE half-sites, as well as AP1 sites [20]. These findings are consistent with our results in BeWo cells and placental explants, where E_2 stimulated leptin-luciferase, probably through ESR1, whereas ICI-182 780 blocked this stimulation. Further experiments will be needed to evaluate if AP1 transcriptional factor is involved in E_2 upregulation of the leptin gene in placental cells.

As previously stated, estrogens exert some of their effects through the action of ESRs on gene expression, but a number of other effects of estrogens are known as nongenomic actions and are believed to be mediated through membrane-associated ESRs. These actions are frequently associated with the activation of various protein kinase cascades [68]. However, nongenomic actions of estrogens may indirectly influence gene expression through the activation of signal transduction pathways that eventually act on target transcription factors. To test the hypothesis that E_2 regulation of leptin expression in placental cells could be mediated by membrane receptors, we performed experiments using the complex E-BSA, which prevents E_2 from entering the cell due to the large size of the conjugated molecule. Interestingly, in these experiments we determined that the effects of E_2 could be partially explained by the involvement of membrane receptors.

The presence of classical human ESR1 associated with membranes in endothelial cells was recently reported [69]. It is known that ESRs translocate to the plasma membrane, where the receptors are probably tethered to the cytoplasmic face of the bilayer and are contained within caveolae rafts [70]. We cannot rule out the possibility that ESR bound to the membrane may mediate the induction of leptin expression exerted by E-BSA.

The nongenomic actions of E_2 that have been reported include the activation of the MAPK signaling pathway, which has been extensively studied in several cell types, including breast cancer [71], endothelial [72], bone [73], and neuroblastoma cells [74]. It is known that ESR directly associates with PIK3R1, phosphoinositide-3-kinase, regulatory subunit 1 (alpha), previously known as the p85 alpha subunit of PIK3 (PI3K) [75], and that E_2 also activates this signaling pathway in endothelial [72], breast cancer [76], and liver cells [77].

We determined that both MAPK and PI3K signal transduction pathways can be activated in placental explants following E_2 treatment. Furthermore, the stimulatory effect of E_2 on leptin expression was blocked by a MAPK pharmacological inhibitor PD98059 or by the overexpression of dominant negative mutant forms of MAP2K or MAPK.

When analyzing the effect of blocking PI3K pathway by using a pharmacological inhibitor (Wortmannin), we obtained a complete block of leptin expression induction by E-BSA, suggesting that this transduction pathway is involved in nongenomic actions of E_2. However, a partial and not significant inhibition of free E_2 effects was observed with Wortmannin, due perhaps to activation of signaling pathways when E_2 is inside the cell that could overcome the inhibition of the PI3K/AKT pathway. All these results strongly suggest that E_2 was also acting nongenomically through activation of other transcription factors.

It has been reported that E_2 plays a role in the regulation of fetal growth, onset of parturition, and placental steroidogenesis [32]. In this study, we provide evidence that E_2 may induce leptin expression through direct and indirect pathways that include activating MAPK1/3 and AKT. This information, further implicating leptin with other hormones of trophoblastic origin, could associate this polypeptide with mechanisms involved in supporting pregnancy.

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