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Crosstalk between estradiol and NFκB signaling pathways on placental leptin expression

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Abstract

Pregnancy success requires a proper fetal maternal interaction at the establishment of implantation. Leptin has been described as a multitasking cytokine in pregnancy, particularly in the placenta, where it acts as an autocrine hormone. The expression of leptin in normal trophoblastic cells is regulated by different endogenous signals. We have previously reported that 17β-estradiol upregulates placental leptin expression through genomic and non-genomic mechanisms. To improve the knowledge of estrogen receptor mechanisms in regulating leptin gene expression, we examined transcription nuclear factor kappa B (NFκB) effect on estradiol leptin induction in human BeWo cell line and human term placental explants. We demonstrated that estradiol induction effect on leptin expression is blocked by the inhibition of NFκB signaling. We also found that the overexpression of p65 subunit, the active form of NFκB, induces leptin expression. Moreover, downregulation of estrogen receptor alpha (ERα), through a specific siRNA, abolished NFκB effect on leptin expression. We also demonstrated that ERα enhanced NFκB signaling pathway activation in trophoblastic cells. Estradiol treatment significantly increased p65 expression and phosphorylation of the inhibitory protein κB alpha (IκBα). A reporter plasmid containing NFκB elements was also induced in response to estradiol stimulation. Localization experiments revealed that estradiol treatment induced nuclear localization of overexpressed p65. Moreover, the overexpression of ERα produced a complete displacement of p65 protein to the nucleus. Finally, immunoprecipitation experiments showed the presence of a complex containing ERα and NFκB. All these evidences suggest a cooperative behavior between ERα and NFκB transcription factors to induce leptin transcription.

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Introduction

The success of pregnancy requires a time and dose dependent maternal–fetal crosstalk, which is crucial for implantation, placentation, remodeling of uterine vasculature, and development of maternal immune tolerance (Nair *et al.* 2017). The placenta works as an endocrine organ that produces growth factors, steroids, peptide hormones and cytokines that are important for the establishment and maintenance of pregnancy. Several cytokines and growth factors, such as leptin, are known to control trophoblast migration, proliferation and invasion (Dos Santos *et al.* 2015).

Leptin is a non-glycosylated peptide of 146 amino acid residues (16 kDa), found to be secreted by adipose tissue (Zhang *et al.* 1994), with the important function of regulating satiety and energy homeostasis (Farooqi & O'Rahilly 2014). Abundant evidence also linked leptin with reproductive functions, such as fertility, ovarian function regulation, embryo development and implantation (Henson & Castracane 2006, Perez-Perez *et al.* 2015). Trophoblast cells synthesize and secrete leptin and express its functional receptors (Masuzaki *et al.* 1997, Senaris *et al.* 1997), suggesting that leptin may play paracrine or/and autocrine functions. In this way, previous studies have demonstrated that leptin modulates the secretion of many placental hormones, implicating leptin as a regulator of placental endocrine function (Coya *et al.* 2006). Moreover, leptin stimulates cell survival in BeWo and JEG-3 human trophoblastic cell lines, enhancing cell proliferation and protein synthesis, and inhibits cell death by apoptosis (Magarinos *et al.* 2007, Perez-Perez *et al.* 2008, 2009, 2010).

Leptin metabolism and function alterations in the placenta are linked to different disorders during pregnancy, such as gestational diabetes, intrauterine

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growth restriction, recurrent miscarriage, and preeclampsia (Howell & Powell 2017, Briana & Malamitsi-Puchner 2020, Subiabre *et al.* 2020). Leptin levels are also elevated in obese pregnant women correlating with more pronounced insulin resistance and activation of mTOR signaling in the placenta, that might contribute to fetal overgrowth and adiposity in the offspring (Kelly *et al.* 2020).

Leptin expression in the placenta is strictly regulated and there are differences between the transcriptional regulation of human placental and adipose leptin (Coya *et al.* 2001). The human leptin gene has at −1.9 kb an enhancer that is activated by placental-specific transcription factors (Bi *et al.* 1997). We have previously published data confirming that estradiol, a key regulator during pregnancy, upregulates placental leptin expression (Gambino *et al.* 2010).

Steroids are key players in the development and maintenance of the placenta (Noyola-Martinez *et al.* 2019). The dominant estrogen in humans is 17β-estradiol (E_2) (Bjornstrom & Sjoberg 2005). During cytotrophoblast invasion, placenta growth and throughout pregnancy there is a tightly estrogen synthesis regulation (Everett & MacDonald 1979). Moreover, placenta derived estrogens play an autocrine regulation in trophoblast differentiation and gestational uterine vascular remodeling (Albrecht *et al.* 2006, Mandala 2020). Besides, estrogen receptors (ER) ERα and β proteins have been immunolocalized in nuclei of cultured term human placental syncytiotrophoblast cells (Billiar *et al.* 1997). Estradiol exerts its effect through genomic and nongenomic mechanisms. In the classical response, estradiol binds to its ER and generates the dimerization of the receptor, the translocation to the nucleus and the association with coactivators or corepressors that regulate transcription through the binding to its target genes (Nilsson *et al.* 2001). In previous works (Gambino *et al.* 2012*a*,*b*), we have reported that E_2 upregulates leptin expression in human placental cells by genomic and nongenomic mechanisms involving a crosstalk between ERα and MAPK or PI3K signal transduction pathways. ERα might be phosphorylated on multiple sites, and the activation of different protein kinases can lead to ligand independent ERα transcriptional promotion (Lannigan 2003). Gene expression regulation by ER complex might also involve the participation of other DNA-bound transcription factors such as AP-1, Sp1, NFκB and p300 (Safe & Kim 2008). Moreover we have previously described a cooperative behavior between ERα and Sp1 transcription factors to induce leptin transcription in placental cells (Schanton *et al.* 2018).

The nuclear factor kappa B (NFκB) is a transcription factor that regulates gene expression of targets associated to inflammation and immune response (Taniguchi & Karin 2018). The NFκB family consists of different DNA-binding proteins that form diverse homodimers and heterodimers, being the best characterized at present the p65 (Rel A)/p50 hetero-complex (Perkins 2012). Inactive NFkB is associated with IkB inhibitor proteins. Specific kinases, activated by extracellular signals, phosphorylate IkB and mark it for degradation, allowing the translocation to the nucleus and activation of NFkB complex (Ghosh & Karin 2002). In addition to its central role in immune response and inflammation, NFkB regulates the expression of genes that control cell viability (Monkkonen & Debnath 2018). In mammals, NFκB is considered a key player in the establishment of the implantation window and in the initiation of labor (King *et al.* 2001, Lappas & Rice 2007). In this context, NFκB expression is tightly regulated to sustain the cytokine profile necessary for the maternal tolerance to fetal antigens (McCracken *et al.* 2004).

Although crosstalk between NFκB and ER has been proposed, the mechanism by which NFκB pathway regulates ER function has not been fully resolved.

To improve the understanding of ER regulation on placental leptin expression, we examined, in this work, the effect of inflammatory NFκB signaling on leptin expression in human placental cells. Estradiol plays a critical role in the control of numerous placental hormones and seems to mediate leptin gene expression in placenta. We hypothesized that NFκB transcription factor participates in estradiol leptin expression regulation. Our study contributes with data demonstrating that NFκB enhances leptin expression in cooperation with estradiol/ERα signaling pathway in placenta.

Materials and methods

Ethics statement

Written informed consent was obtained from all subjects and all study procedures were approved by ethical review committee at the Alejandro Posadas National Hospital (Bioethics Committee 'Dr. Vicente Federico del Giudice').Cell culture and treatments

BeWo, a human choriocarcinoma cell line, was acquired from the American Type Culture Collection (ATCC). They were grown in 45% Dulbecco modified Eagle medium (DMEM) and 45% HAM F-12 (Invitrogen), 100 U/mL penicillin, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate (Sigma Chemical Company) and 2 nM glutamine (Invitrogen), at 37°C in 5% CO_2. Cell medium was replaced after 24 h of incubation, with DMEM-F12 0% or 1% FBS, later on, the cells were treated for 48 h for total protein immunoblotting or during shorter time laps for protein phosphorylation determination.

Optimal 17β-estradiol (E_2) (Sigma) concentrations were previously determined. In total, 10 or 100 nM E_2 was used. (Gambino *et al.* 2010). The specific Iκκ inhibitor sulfasalazine (Wahl *et al.* 1998) was used in different concentrations (10, 100 and 1000 nM) during 48 h.

BeWo cell line was also incubated with phorbol 12-myristate 13-acetate (PMA) (100 ng/mL). Afterwards, cells were washed with PBS and solubilized in lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1× PBS and 10 mg/mL phenylmethanesulfonyl fluoride (PMSF)). Western blot was performed with cell lysates. E_2 treatment was carried out in DMEM/F-12 media deprived of phenol red and supplemented with 1% charcoal stripped FBS unless indicated.

Placental explants collection and processing

Human placentas ($n=16$) at term were acquired from normal pregnant woman after delivery. Villous tissue free of calcification, hematoma or visible infarct, was sampled from different cotyledons a distance equidistant between the chorionic and basal plates. These parts of the cotyledons were cut into several cubic fragments (10–15 mg) and meticulously rinsed with cold DMEM-F12 0% FBS medium.

Treatment of placental explants

To represent a more physiological model, placental explants were incubated during 24 h with different treatments as indicated in each figure.

Placental explants were distributed in plates of 24 wells containing 1 mL of DMEM 0% FBS ($n=1$ explant/well, two replicates per treatment), maintained at 37°C during 30 min to equilibrate temperature, and incubated for 24 h in DMEM-F12 0% FBS in the presence or not of E_2. The explants were removed, centrifuged for 2 min at 2000 ***g*** at 4°C and suspended in 300 µL of lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1× PBS and 10 mg/mL phenylmethanesulfonyl fluoride (PMSF)) for 30 min at 4°C on an orbital shaker. Later, they were centrifuged at 10,000***g*** for 20 min. Western blot test was performed with the supernatants.

For qRT-PCR, after washing with PBS, the explants were frozen at −80°C and stored until extraction of total RNA.

Western blot

Complete 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 colorimetric method BCA Protein Assay Kit (Pierce, Thermo Scientific). Lysates were mixes with Laemmli's sample buffer having 2% SDS and 30 mM β-mercaptoethanol, boiled for 5 min, resolved by SDS-PAGE on a 12% gel, and electrophoretically transferred to nitrocellulose membrane (Hybond, Amersham Pharmacia). Membranes were equilibrated in 1× PBS and non-specific binding sites were blocked with 5% non-fat milk in PBS at room temperature for 1 h. Then they were immunoblotted with the specific polyclonal rabbit anti-human leptin Y20 (1:1000, Santa Cruz Biotechnology, Inc.), monoclonal mouse anti-p65 (1:1000, Santa Cruz Biotechnology, Inc.), or monoclonal mouse anti-pIκBα (1:1000 Santa Cruz Biotechnology, Inc.). Loading controls were done by immunoblotting the same membranes with monoclonal mouse anti-α-tubulin (1:1000, Santa Cruz Biotechnology, Inc.) or monoclonal mouse anti glyceraldehyde-3-phospate dehydrogenase (GAPDH) (1:1000, Santa Cruz Biotechnology, Inc.). The detection of the antibodies was made using horseradish peroxidase-linked goat anti-mouse IgG or anti-rabbit IgG (1:1000, Sigma and Co.), visualized by the Amersham Pharmacia ECL Chemiluminescence signaling system and a Bio-Imaging Analyzer G-box Chemi XT4 (Syngene). The quantification of protein bands was analyzed by densitometry using the software Image J version 1.45 (NIH).

Plasmids

Dr Oksana Gavrilova (Bi *et al.* 1997) generously provided the luciferase reporter construct, based on the PGL-3 basic vector. This vector contains a fragment of leptin promoter region upstream of the luciferase (Luc) reporter gene, which codify for the luciferase enzyme. The plasmid was renamed indicating the number of base pairs upstream of transcription initiation site: pL1951/42 (−1951 to +42 bp of leptin promoter). The expression plasmids HEGO and Rel A contain the cDNA of ERα and the subunit p65 of NFκB, respectively (provided generously by Dr Adali Pecci, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina). The pNFκB vector is a reporter plasmid that contains NFκB binding elements upstream a minimal promoter and the Luc reporter gene.

The efficiency of individual transfections was normalized using the pRSV-βgal vector containing the β-galactosidase gene under the control of the Rous sarcoma virus (RSV) promoter. The empty vector was used as control in transfection experiments with expression plasmids. Plasmids were purified using the Midiprep Wizard kit (Promega), and DNA concentration was estimated spectrophotometrically.

Transient transfection experiments

BeWo cells were plated in to six-well dishes containing 1 mL of DMEM-F12 10% FBS, at a density of 2.5×10^5 cell/mL, during 24 h. Normally, 5 µg of the pRSV-βgal, 5 µg of the luciferase reporter and 5 µg of each expression plasmid were transfected using 7,5 µL of PEI 2 mg/mL (Polyethylenimine, Polysciences, Inc). After 4 h the medium was replaced with DMEM-F12 1% FBS with the addition of the different treatments as statement in the corresponding legend. Each experiment was made in duplicate and it was performed at least three independent times.

Assay for luciferase and β-galactosidase activities

Luciferase Assay System (Promega) was used to measure luciferase (Luc) activity in cell lysates. Seventy-two hours after transfection, cells were washed with PBS and harvested using 50 µL of lysis buffer provided by the manufacturer. GloMaxMulti + Microplate Multimode Reader Luminometer (Promega) was used to measure luciferase activity. β-Galactosidase activity was assayed using 1 mg of *o*-nitrophenyl β-D-galactopyranoside (AmResco) as the substrate in buffer Z (40 mM NaH_2PO_4, 60 nM Ns_2HPO_4,

10 nM KCl, 0.07% β-mercaptoethanol, 1 mM $MgSO_4$). Samples were incubated at 37°C until yellow staining. Enzymatic reaction was stopped with 75 µL of sodium carbonate. Color development was determined by absorption at 420 nm. The variation in transfection efficiency was corrected with this value. Luc results were calculated as the percentage of Luc activity per unit of β-galactosidase activity. For each data duplicates were performed.

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

Quantity of leptin mRNA was determined by qRT-PCR. TRISURE reagent was used to extract total RNA according to the manufacture instructions (Bioline Co). The concentration and purity of the RNA were estimated spectrophotometrically at 260 and 280 nm. For the synthesis of cDNA, 5 µg of total RNA was reverse transcribed at 55°C during 1 h using the fist Strand cDNA synthesis Kit (Roche). The following primers based on the sequences of the National Center for Biotechnology Information GenBank were used for quantitative real-time PCR: Leptin: forward 5′-GAACCCTGTGCGGATTCTT-3′; reverse 5′-CCAGGTCGTTGGATATTTGG-3′; and Cyclophilin: forward, 5′-CTTCCCCGATGAGAACTTCA-3′; reverse, 5′-TCTTGGTGCTCTCCACCTTC-3′. qRT-PCR Master Mix Reagent kit was obtained from Roche (Fast Start universal SYBR Green), and PCRs were performed on a Chromo 4 DNA Engine (BioRad).

The reaction contains 10 µM of forward and reverse primers, 3 µL of cDNA, and the final reaction volume was 25 µL. The reaction 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 describes: denaturation 15 s at 95°C and 1 min annealing and extension 1 min at 59°C. The threshold cycle (CT) from each well was calculated by the Opticon Monitor 3 Program. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative quantification (Livak & Schmittgen 2001). The evaluation of $2^{-\Delta\Delta CT}$ for treated samples indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophilin), and relative to the untreated control. To confirm the specificity of amplification a melting curve analysis was performed. To ensure the absence of sample contamination, reaction mixtures without reverse transcriptase or RNA were run in parallel.

Immunofluorescence assays

Bewo cells were grown on coverslips in DMEM-F12 10% FBS. The next day they were transiently transfected with Rel A and/or HEGO expression plasmids and were treated with 100 nM estradiol at the times indicated in the figures. Then they were washed three times with PBS, fixed 20 min with 3% *p*-formaldehyde and permeabilized with 0.1% Triton X-100 as described (Quinta *et al.* 2010). After the fixation, the cells were washed three times with PBS, and blocked for 30 min with AFI solution (BSA 0.5%, Tween 20 0.2%, sodium azide 0.1%, Tris 50 nM pH 8). The primary antibodies were diluted in AFI solution and were added to the cells ON at 4°C: rabbit polyclonal anti-ERα (1:50, Santa Cruz Biotechnology) or mouse monoclonal anti-Rel A (1:100 Santa Cruz Biotechnology). This incubation was followed by three washes with PBS for 10 min and then the cells were incubated with the secondary antibodies for 1 h: Alexa 488 nm anti rabbit IgG (1:100, Thermo Fisher) or Alexa 563 nm anti mouse IgG (1:100, Thermo Fisher). After further washings with PBS and counterstained with DAPI, samples were mounting with prolong Gold antifading solution (Thermo Scientific). The images were taken using an inverted fluorescence microscope (Nikon) and analyzed using the software Image J version 1.45 (NIH).

Immunoprecipitation assays

After E_2 treatment, cells were washed two times with PBS, scrapped, and homogenized in HEM buffer (1 nM EDTA, 10 mM Hepes buffer at pH 7.4 and 20 mM Na_2MoO_4). After a centrifugation for 10 min at 4°C at 12,000 ***g***, ERα and p65 were immunoprecipitated from 300 µL of cytosol (4 mg/mL protein), with 2 µL of anti-ERα or anti-Sp1 antibody and 30 µL of 50% (g/v) protein A-Sepharose (Sigma) under rotation for 3 h at 4°C. Pellets were washed five times with 1 mL of HEM buffer, and proteins were detected by Western blotting (0.05%, v/v dilution for primary antibodies and 0.02%, v/v dilution for horseradish peroxidase-labeled secondary antibody anti-rabbit). Antibodies were visualized by the Amersham Pharmacia ECL Chemiluminescence signaling system and a Bio-Imaging Analyzer G-box Chemi XT4 (Syngene).

Statistical analysis

To assure reproducible results experiments were repeated separately at least three times. In Western blot analysis arbitrary units were calculated from normalized bands intensity, and in reporter assays from normalized Luc activity. Controls values were set as 1. ANOVA followed by different post hoc tests, was used to assess statistical significance as indicated in each figure. A P-value <0.05 was considered statistically significant.

Results

NFκB inhibition blocked E_2 effect on leptin expression

It was extensively reported that ERα gene expression regulation could be mediated by the crosstalk with different transcription factors (Safe & Kim 2008). In this regard, we aimed to study NFκB signaling pathway on E_2 induced leptin expression in placental cells. Sulfasalazine, a potent and specific inhibitor of both transcription and nuclear translocation of the inflammatory transcription factor NFκB (Wahl *et al.* 1998), was used. Treatment with sulfasalazine 10, 100 and 1000 nM completely blocked E_2 induction of leptin expression determined by qRT-PCR in BeWo cells (Fig. 1A). Similar results were obtained when sulfasalazine effect was assessed on human term placental explants, a more physiological model (Fig. 1B). All these results were validated by Western blot analysis both in BeWo cells and placental explants (Fig. 1C and D). Moreover, in placental explants, sulfasalazine treatment decreased endogenous leptin, suggesting

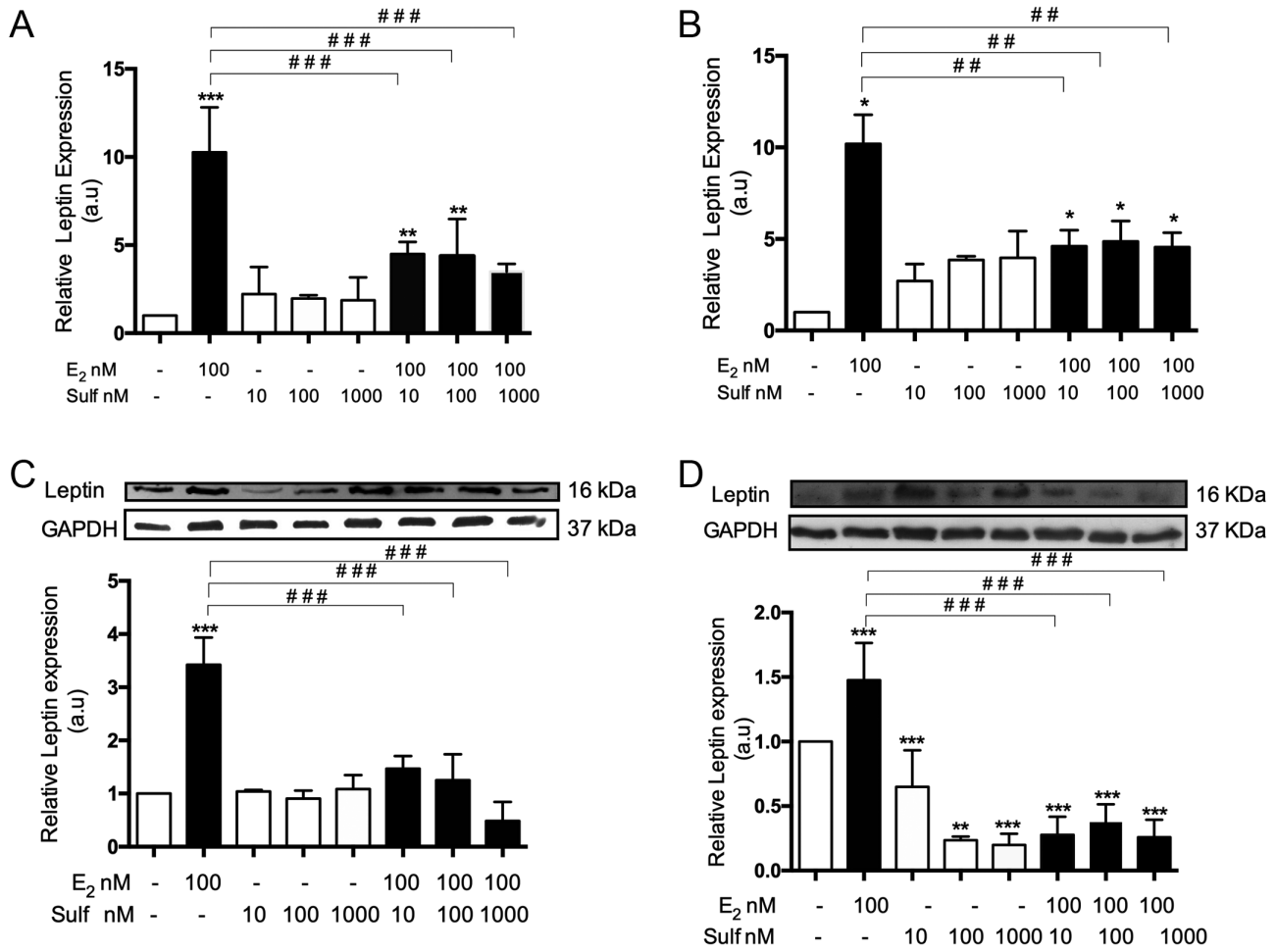

Figure 1 Inhibition of NFκB suppressed E_2 effect on leptin expression BeWo cells (A) and (C) and term placental explants (B) and (D) were pretreated as indicated with sulfasalazine during 30 min, afterwards E_2 was added. After 48 h (A) or 24 h (B), total RNA was extracted and Leptin mRNA expression was quantified by qRT-PCR. Cyclophilin was used as internal standard. Cell extracts (C) and placental explants extracts (D) were prepared as indicated in 'Materials and methods' section and proteins were separated on 12% SDS-PAGE gel. Leptin expression was determined by Western blot. Loading control was performed by immunoblotting the same membranes with anti-GAPDH. Standard protein markers were used to estimate the molecular weights. Molecular weight (kDa) is indicated at the right of each blot. Bands densitometry is shown in lower panels. Results are expressed as mean ± S.D. for ten independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control and $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs E_2 treatment. Statistical analyses were performed by ANOVA followed by Bonferroni post hoc test.

that NFκB pathway could be involved in basal leptin expression regulation (Fig. 1D).

Crosstalk between p65 and ERα on leptin expression

To analyze a putative cooperation between ERα and p65 transcription factors on placental leptin transcription, different approaches were performed. First, transient transfection experiments with different amounts of p65 (Rel A) expression vector and 100 nM E_2 treatment, were performed in BeWo cells. Reporter transcription was evaluated using the pL1951/42 plasmid construction that contains leptin promoter region from −1951 to +42 bp directing luciferase (Luc) expression. As it is seen in Fig. 2A 100 nM E_2 significantly increased leptin expression as already published (Gambino *et al.* 2010). The overexpression of the human transcription factor p65, with 0.5 µg plasmid, significantly enhanced basal leptin expression in BeWo cells. On the other hand, p65 overexpression did not alter E_2 effect on leptin promoter transcription.

Second, to further study the effect between estradiol, ERα and p65 transcription factors on leptin expression, similar transient transfection experiments were performed using p65 (Rel A) and ERα (HEGO) expression vectors. The transfection of 0.5 µg of Rel A plasmid significantly increased leptin expression as described earlier. The cotransfection with 10 ng of ERα expression vector significantly increased leptin expression but did not differ from the increase obtained with Rel A alone (Fig. 2B). It is worthy to notice that very small quantities of HEGO plasmid were used to not saturate the system.

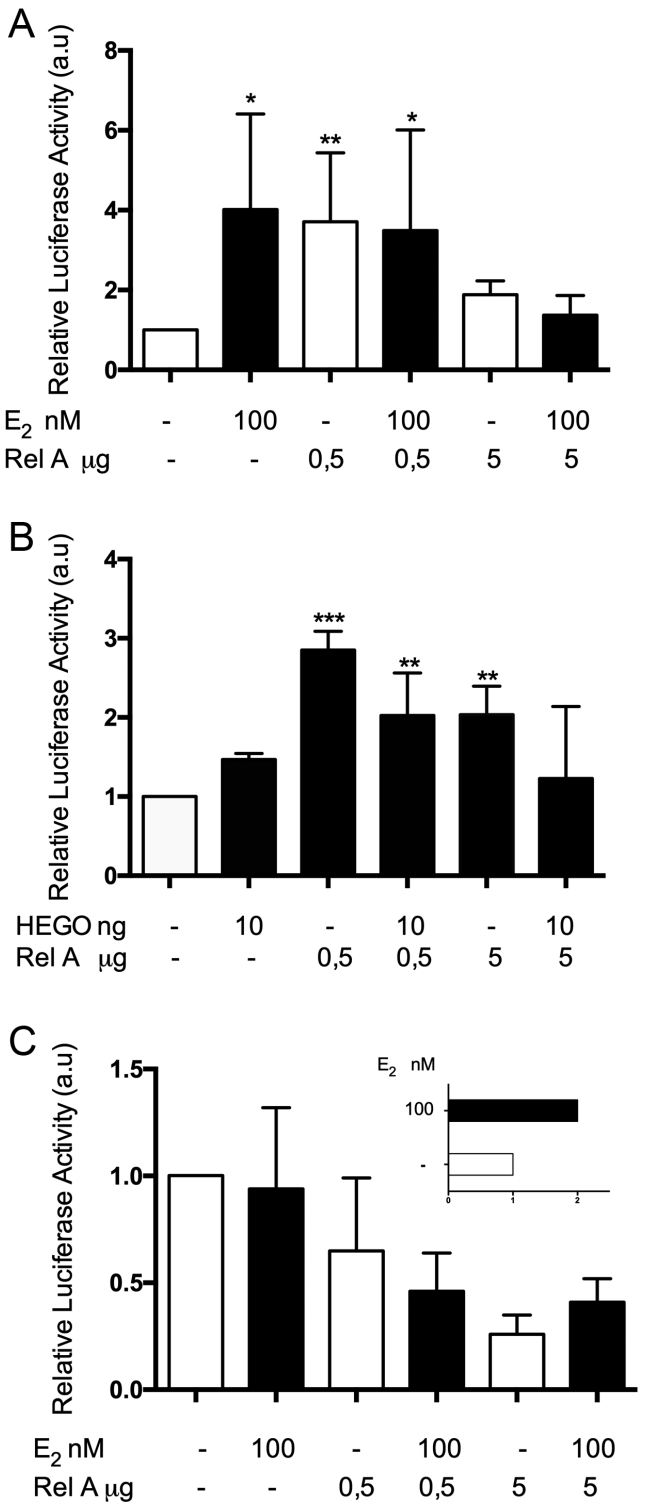

Figure 2 Effect of p65 overexpression on E_2 induced placental leptin expression. (A) BeWo cells were transiently transfected with pL1951 plasmid construction containing the promoter region of leptin gene from −1951 to +42 bp and different amounts of p65 expression plasmid. After transfection, BeWo cells were incubated for 48 h in DMEM-F12 media supplemented with 1% FBS and treated with E_2 as indicated. (B) BeWo cells were transiently transfected with pL1951 plasmid construction, different amounts of plasmid expressing human ERα (HEGO) and p65 expression plasmid. After transfection, cells were incubated for 48 h in DMEM-F12 media supplemented with 1% FBS. (C) BeWo-Sh2 cells were transiently transfected with pL1951/42 plasmid construction and Rel A expression plasmid and treated with different doses of E_2, and with 5 mg/mL of doxycycline, to induce the expression of the ERα interference RNA. Cells were incubated for 48 h in DMEM-F12 media supplemented with 1% FBS. As control, doxycycline was omitted, corroborating the inductive effect of E_2 on the pL1951/42 vector (inset figure). (A–C) Cell extracts were prepared as indicated in 'Materials and methods' section, Luc activity was measured in the cellular extracts and normalized to β-galactosidase activity. Results are expressed as mean ± S.D. for four independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control. Statistical analyses were performed by ANOVA followed by Bonferroni post hoc test.

In a third approach, BeWo cells with ERα downregulated expression were generated using siRNA strategy. Cells were stably transfected with an inducible expression plasmid encoding a precursor RNA that after processing maturates in a siRNA against ERα. This cell line called BeWo-Sh2 was cotransfected with the pL1951/42 reporter vector and Rel A expression plasmid. The transcription of ERα siRNA was induced with doxycycline and Luc activity was determined after 48 h. We observed that the downregulation of ERα abolished Rel A induction of leptin expression suggesting that ERα is necessary to evidence p65 inductive effect (Fig. 2C). In these cells E_2 induction of leptin expression is lost as expected. As a positive control, an experiment was performed in BeWo-Sh2 cells in which doxycycline was omitted corroborating the inductive effect of E_2 on the leptin reporter plasmid pL1951 (Fig. 2C inset). All these results suggest a regulatory effect of NFκB pathway activation on estradiol leptin expression regulation.

Estradiol increased NFκB signaling pathway activation in trophoblastic cells

The effect of E_2 on different steps of NFκB signaling pathway activation was analyzed. Reported data show that the expression of the Rel A protooncogene could be regulated in invasive cells (Dominska *et al.* 2017). Considering that first trimester extravillous cytotrophoblast are prone to proliferate and invade the decidua and the fact that estrogens promote growth, the aim of the present experiment was to evaluate changes in the expression of NFκB Rel A proto-oncogene p65 subunit. The addition of 10 and 100 nM E_2 significantly increased p65 expression in BeWo cells after 48 h of treatment. No changes in the expression of p65 were observed at 24 h of treatment (Fig. 3A). Similar experiments were performed in a more physiological model, using term human placental explants. In this model, the incubation with 100 nM E_2 during 24 h significantly increased p65 expression (Fig. 3B).

On the other hand, in the NFκB signaling pathway, extracellular factors interact with cell surface receptors and cause the release of active NFκB from phosphorylated

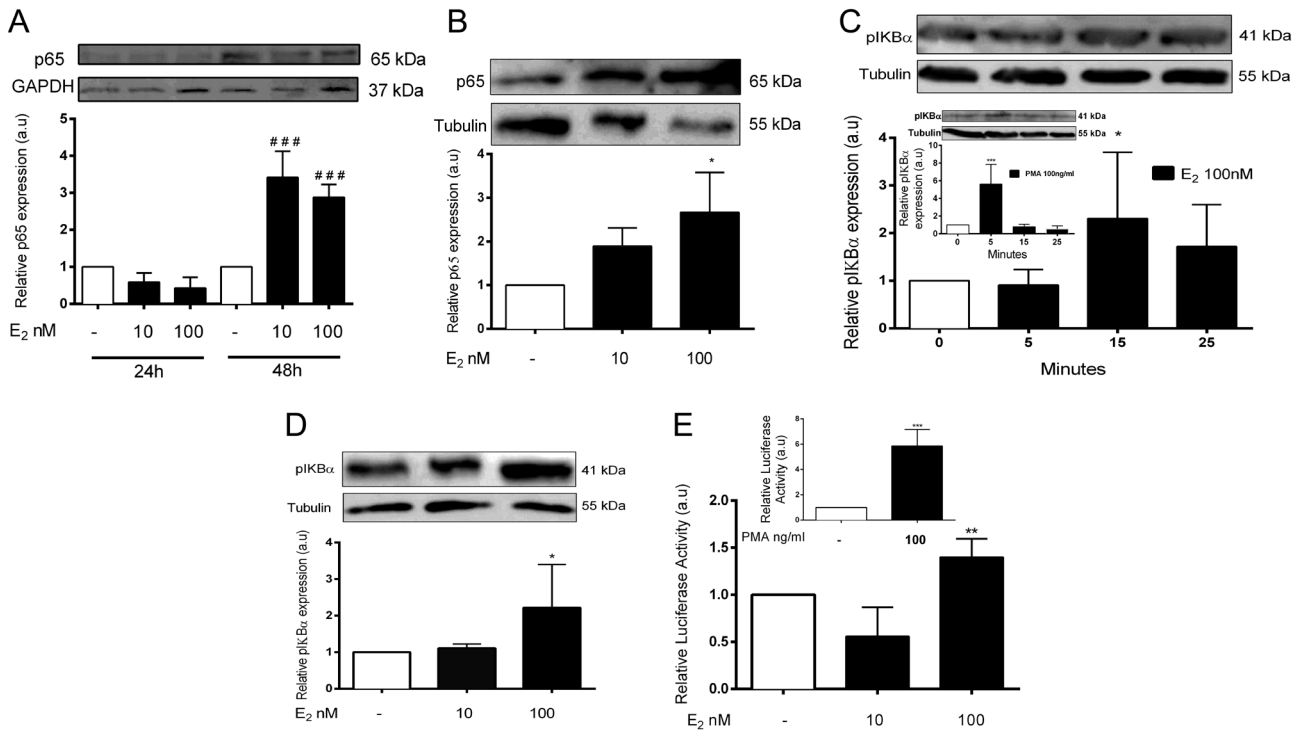

Figure 3 Estradiol increased NFκB signaling pathway activation in trophoblastic cells. BeWo cells (A) and placental explants (B) were treated with 10 and 100 nM E_2 as indicated, during 24 and 48 h (A) or 24 h (B). Proteins were separated on 12% SDS-PAGE gels and p65 expression was determined by Western blot. (C) BeWo cells were treated with 100 nM E_2 during 0, 5, 15, 25 min. As control BeWo cells were treated with 100 ng/mL phorbol 12-myrisate 13-acetate (PMA) in the same conditions (inset figure). Proteins were separated on 12% SDS-PAGE gels and IκBα phosphorylation (pIκBα) was determined by Western blot. (D) Placental explants were incubated with 10 and 100 nM E_2 during 24 h. Proteins were separated on 12% SDS-PAGE gels and IκBα phosphorylation (pIκBα) was determined by Western blot. (A–D) Protein extracts were prepared as previously described in 'Materials and methods' section. Loading control was performed by immunoblotting the same membranes with anti-GAPDH or anti-α-Tubulin. Standard protein markers were used to estimate the molecular weights. Molecular weight (kDa) is indicated at the right of each blot. Bands densitometry is shown in lower panels. (E) BeWo cells were transiently transfected with pNFκB reporter plasmid construction, and treated with 100 and 10 nM E_2 as indicated. As control BeWo cells were incubated with 100 ng/mL phorbol 12-myrisate 13-acetate (PMA) (inset figure). Cell extracts were prepared as indicated in 'Materials and methods' section and Luc activity was measured in the cellular extract and normalized to β-galactosidase activity. Results are expressed as mean ± S.D. of six independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs each control. Statistical analyses were performed by ANOVA followed by Bonferroni post hoc test.

cytoplasmic inhibitory protein κB (IκB). The next challenge was to determine the phosphorylation of IκBα in response to E_2 stimulation. Treatment with 100 nM E_2 during 15 min significantly increased pIκBα (Fig. 3C). Positive control of IkBα phosphorylation was performed by the incubation of BeWo cells with the phorbol ester phorbol 12-myristate 13-acetate (PMA), a well-known stimulator of NFκB signaling. The addition of 100 ng/mL of PMA produced a significant enhancement of pIκBα at 5 min incubation as expected (Fig. 3C inset). The phosphorylation of IκBα was also significantly increased after 24 h of treatment with 100 nM E_2 in term human placental explants (Fig. 3D).

Finally, to analyze the putative involvement of E_2 treatment on NFκB target gene activation, transient transfection experiments with an NFκB reporter construction were performed. The reporter plasmid contains multiple binding sites for NFκB dimers regulating Luc expression. Treatment with 100 nM E_2 significantly stimulated Luc expression (Fig. 3E). A positive control was included incubating BeWo cells with 100 ng/mL PMA. In this case a significant induction of Luc expression was observed as expected (Fig. 3E inset).

Localization and interaction of ERα and p65 in placental cells

To further study a possible crosstalk between the estradiol and NFκB signaling pathways, localization experiments were performed. In control cells, there was a homogeneous distribution of endogenous ERα or p65 proteins within the cytoplasm and nucleus (Fig. 4A). Estradiol treatment during 180 min induced endogenous ERα and p65 proteins to a perinuclear localization. Similar results were obtained when p65 protein was overexpressed. In addition when p65 protein was overexpressed and cells were treated with estradiol during 180 min nuclear p65 localization was induced. Moreover the overexpression of ERα produced

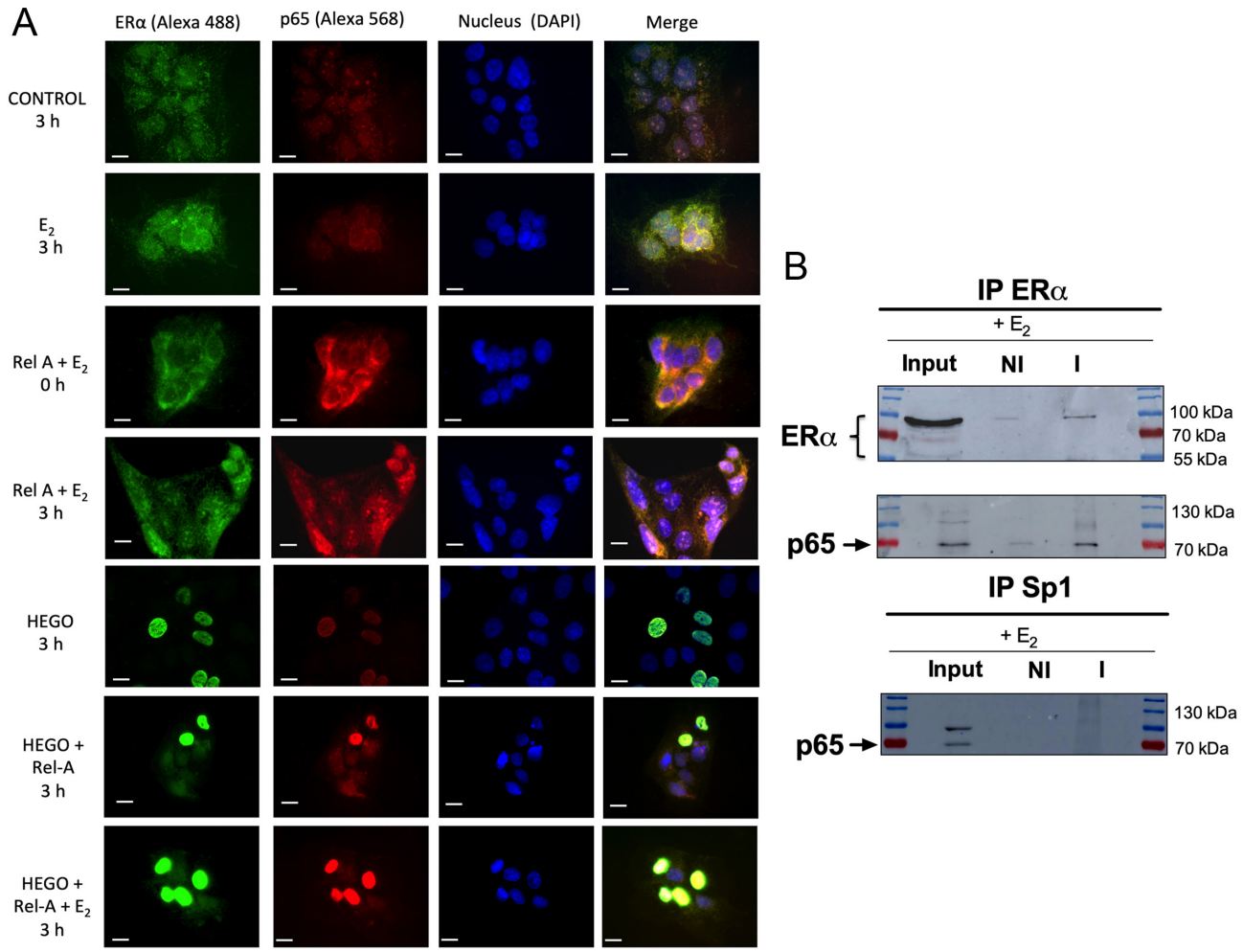

Figure 4 Localization and interaction of ERα and p65 in placental cells. (A) BeWo cells were seeded on coverslips in six-well plates and incubated in DMEM 1% FBS with estradiol 100 nM (E_2) or without E_2 (CONTROL) during 3 h. Also, BeWo cells were transiently transfected with human ERα (HEGO) and p65 (Rel A) expression plasmids, and treated or not with 100 nM E_2, as indicated, during 3 h. Cells were fixed and ERα expression (green) was detected using Alexa 488 conjugated secondary antibody and p65 expression (red) was detected with Alexa 568 conjugated secondary antibody. The nuclei were stained with DAPI (blue). Scale bar: 8 µm. Representative results from four replicates are shown. Representative micrographs from BeWo at 60X are shown. (B) BeWo cells were treated with 100 nM estradiol (E_2) during 24 h and whole cell extract (Input) was prepared as indicated in 'Materials and methods' section. Endogenous ERα or Sp1 were immunoprecipitated from whole cell extract with a specific IgG (I) or a non-immune IgG (NI), and p65 or ERα were revealed by Western blot as indicated. Representative results from two replicates are shown.

a complete displacement of both endogenous and overexpressed p65 protein to the nuclear localization. Estradiol treatment in the ERα and p65 overexpression condition did not modify nuclear localization of these two proteins. All these results prompted us to study a possible interaction between ERα and p65 proteins. To analyze this hypothesis immunoprecipitation experiments were performed. BeWo cells were treated with 100 nM E_2 and endogenous ERα was immunoprecipitated to study the putative interaction with p65 in physiological condition. Protein p65 was revealed in the immunoprecipitate by Western blot. The presence of p65 in the ERα immunoprecipitate indicates that both proteins are present together in a complex. Previous unpublished results from our lab have shown an association between ERα and Sp1 transcription factor. To unravel the possibility that these proteins participate in the same complex Sp1 immunoprecipitation experiments were performed. P65 is not present in the Sp1 immunoprecipitate (Fig. 4B). These results strongly suggest that ERα is probably participating in different specific complexes with p65 or Sp1 proteins.

Discussion

The regulation of leptin expression in placenta is modulated by steroid hormones, glucocorticoids and insulin (Coya *et al.* 2001, Perez-Perez *et al.* 2013). It was

also demonstrated that human leptin gene is enhanced by hypoxia through mechanisms common to other hypoxia-inducible genes (Ambrosini *et al.* 2002). Moreover, we have previously shown that leptin expression in placenta is upregulated by very important pregnancy signals such as cAMP, hCG and E_2 (Maymo *et al.* 2009, 2010, 2012, Gambino *et al.* 2010). In the present work, we have studied the mechanisms involved in the regulation of leptin expression by E_2 and NFκB signaling pathway in BeWo human choriocarcinoma cells. These cells express both leptin and its receptor (Magarinos *et al.* 2007). Term human placental explants were also used to validate results in a more physiological model.

NFκB is a multifunctional transcription factor and modulates the expression of genes that influence cell cycle progression, cell death regulation by apoptosis, inflammatory reactions, immune response and metastasis. In this way, both ER and NFκB can induce expression of growth and survival genes by a crosstalk at the transcriptional level.

To asses NFκB incidence on leptin expression inhibitory experiments with sulfasalazine were done. Sulfasalazine is a potent and specific inhibitor of both transcription and nuclear translocation of the inflammatory transcription factor NFκB. The in vitro doses of sulfasalazine we used were similar to other studies examining the effect of sulfasalazine on the vasculature (Kim *et al.* 2009) and in endothelial and cytotrophoblast cells (Brownfoot *et al.* 2019). The pharmacokinetics of sulfasalazine, during a normal therapy, in healthy individuals would show steady state serum concentrations of around 113 μM (Klotz 1985) when administered at 4 g per day. Therefore we examined the effect of sulfasalazine in vitro at doses straddling this threshold (Wahl *et al.* 1998). Treatment with sulfasalazine 10, 100 and 1000 nM, completely blocked estradiol action on leptin expression both in BeWo cells and placental explants. These results strongly suggest that NFκB transcription factor is modulating estradiol enhancement of leptin expression. To further characterize the effect of NFκB transcription factor, Rel A overexpression on leptin transcription was analyzed in BeWo cells. A significant upregulation was observed using reporter assays with the pL1951/42 construction. NFκB activation showed no effect on E_2 induction of leptin expression in transient transfection assays. At higher doses of Rel A plasmid, leptin expression returned to control levels regardless of E_2 presence. Similar results were obtained previously where leptin expression was diminished with high concentration of the inductors E_2 (Gambino *et al.* 2010), hCG (Maymo *et al.* 2009) or cAMP (Maymo *et al.* 2010). This phenomenon is probably due to a saturation of the transcription mechanisms.

In view of these findings the effect of ERα and p65 transcription factors on leptin expression was analyzed. The cotransfection of Rel A with ERα expression vectors significantly increased leptin expression but did not differ from the increase with Rel A alone. These results might suggest that the activation of the NFκB transcription factor is sufficient to increase leptin expression. To elucidate the involvement of ERα in NFκB induction of leptin expression, transient transfection experiments in cells with siRNA silenced ERα were performed. Our data show that the downregulation of ERα abolished Rel A induction of leptin expression. These results strongly suggest that ERα is necessary to evidence p65 inductive effect. All these evidences indicate a regulatory effect of NFκB pathway activation in cooperation with ER signaling on leptin expression. According to the 'classical genomic model' ligand-activated ERα binds to specific ERE to modulate transcriptional activity of E_2 target genes. Nevertheless, 35% of the categorized human E_2 responsive genes are transcribed via indirect ER-DNA association through protein–protein interactions with several trans factors such as Sp1, NFκB, or AP-1 (O'Connor *et al.* 2016). It was also described that ER family shares some common coactivators with NFkB, such as RAC3 (Klinge 2000). RAC3 is a member of the Steroid Receptor Coactivator (SRC) family that increases the transcriptional activity of several nuclear receptors and NFκB (Sheppard *et al.* 1999). The molecular mechanism by which coactivators induce chromatin remodeling and transcriptional activation involves a histone acetyltransferase (HAT) activity, present in RAC3 (Glass & Rosenfeld 2000) and the recruitment of other general coactivators such as CBP/p300 (Chen *et al.* 1999).

Crosstalk between the NFκB and ER pathways participates in the regulation of many cellular processes, for instance, both estrogen and active NFκB stimulate cell proliferation mediated by increased expression of the cell cycle regulatory protein cyclin D1. In this gene regulation, estrogenic signaling through ER modulates NFκB activation, in a manner that appears to be independent of gene transcription (Biswas *et al.* 2005). Therefore, the crossroads of these two pathways is important in cells that simultaneously carry both routes such as trophoblastic cells.

In view of this data, activation of the NFκB pathway by ER signaling was studied. Treatment with E_2 significantly increased p65 expression and phosphorylation of IκBα in BeWo cells and term human placental explants. These results support the hypothesis that ER signaling increases NFκB activation. To confirm this data a transient transfection assay was performed with a NFκB reporter construction. In these experiments, significant stimulation of Luc expression was observed after E_2 treatment. These results showed that NFκB signaling activation was evidenced at the transcriptional level.

In this work, our data support a mechanism activated by NFκB to induce leptin expression in coordination with ERα in placenta. It was seen that ERα could complex with different transcription factors to modulate transcription. For instance ERα and Sp1 interact in vivo to mediate E_2 induced activation of kisspeptin 1

gene in hypothalamic GT-1 cells (Li *et al.* 2007), and the integral membrane protein Claudin-5 (Burek *et al.* 2014), among other genes. Indeed, we have previously seen that ERα and Sp1 physically interact in BeWo trophoblastic cells (unpublished results). To analyse a possible regulatory interaction between ERα and NFκB, localization and immunoprecipitation experiments were performed. Similar localization pattern was observed after E_2 treatment of overexpressed ERα or p65 BeWo cells, suggesting a coordinated regulation. Moreover the presence of a complex between ERα and p65 was evidenced in trophoblastic cells. This complex differs with the one described previously between ERα and Sp1, suggesting the formation of different transcription factors associations modulating leptin expression.

A considerable body of experimental evidence suggests a strong association between NFκB and the development of pregnancy. NFκB is an important regulator of implantation, and plays a relevant role in the induction of parturition induction (Sakowicz 2018). On the other side, both leptin and NFκB were described as central nodes of action within a complex network aimed at preparing for pregnancy, particularly, they play an important role in Th1-Th2 immune modulation required for successful pregnancy (Faustmann *et al.* 2016). Moreover, an association between leptin and increased cardiovascular risk and increased levels of inflammatory factors have been reported in obesity (Wang & Nakayama 2010). In this pathology, leptin has pro-inflammatory action, involving pro-inflammatory cytokines expression, elevated ERK1/2 phosphorylation followed by increased NFκB activation and TNFα secretion (Lappas *et al.* 2005, Indra *et al.* 2013). On the other side, NFκB activation is detected in inflammatory diseases that are associated with increased amounts of inflammatory cytokines (Lawrence 2009). Some of these cytokine's genes are direct targets that are both expressed in response to NFκB activation and can feed-forward to activate NFκB by causing release of NFκB from IκB. This effect can amplify the immune response and prolong inflammation, either in a physiologic response or in a disease state. This positive feedback mechanism could give an explanation of NFκB effect on estradiol leptin expression. Undoubtedly, further studies are required to examine the interaction between the members of the NFκB transcription factor gene family and ERs in placental cells. On the other hand it has been postulated that maternal obesity and gestational diabetes may be associated with a state of chronic and low-grade inflammation termed 'metainflammation', where the placenta 'senses' and adapts to the maternal inflammatory environment (Pantham *et al.* 2015). In this manner, leptin could be acting as an inflammatory mediator.

In summary, in this study, we provide evidence that NFκB induces leptin expression in cooperation with E_2 action through ERα. The molecular mechanism of regulation of placental leptin expression by E_2 could be a multifactorial process, and may depend on the localization and concentration of ERs, the association with transcription factors such as NFκB and co-regulatory proteins and the activation of signal transducers.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M S performed experiments, analyzed data and wrote the paper. J M performed experiments and analyzed data. M F C performed experiments and analyzed data. A P P performed experiments and analyzed data. R C gave placental material and analyzed data. V S M discussed the results. A E discussed the results. C V conceived the study and wrote the paper.

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