

Transcriptional effect of hypoxia on placental leptin

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Abstract We observed recently that placental leptin is markedly increased in preeclampsia. Since this disorder is associated with vascular disorders, we have tested the hypothesis that hypoxia regulates leptin expression. We show that hypoxia increased leptin mRNA and secretion in trophoblast-derived BeWo cells. This effect was mediated through leptin promoter activation. 5' deletion analysis allowed us to delineate two regions containing 1.87 kb and 1.20 kb of the promoter which conferred respectively high and low responsiveness to hypoxia. These data indicate that leptin is up-regulated by hypoxia through a transcriptional mechanism likely to involve distinct hypoxia-responsive *cis*-acting sequences on the promoter. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Placenta; Leptin; Preeclampsia; Hypoxia; BeWo cell; Hypoxia-inducible factor-1

1. Introduction

Leptin, the protein product of the *ob* gene, regulates body weight homeostasis by controlling food intake through its hypothalamic receptors [1–3]. The leptin gene is expressed in a restricted number of tissues. In the adult, the major source of leptin is the adipose tissue and circulating levels are proportional to fat mass [4]. Another leptin-producing organ is the placenta [5]. During pregnancy, leptin concentration gradually increases in maternal blood, out of proportion with change in adipose tissue mass, and drops abruptly after delivery [5,6]. These observations suggest that leptin is released in the maternal circulation from the placenta. In support, recent data obtained in dually perfused human placenta indicate that 95% of placental leptin is released into the maternal circulation [7,8]. The rate of placental leptin production amounts 115 ng/min, representing 14% of the estimated whole-body leptin production [9]. This points out the placenta as an important site of leptin production in humans.

Placental leptin mRNA [10] and protein [11] are markedly increased in preeclampsia, a disorder associated with maternal hypertension, reduction in placental blood flow and placental hypoxia [12]. This raises the hypothesis that decreased oxygen tension is a positive regulatory factor of leptin gene expression. In the present study, we examined this proposal by as-

sessing the effect of hypoxia in human trophoblast-derived, leptin-expressing cells (BeWo). Our data show that leptin gene expression is up-regulated under hypoxic conditions and that this effect is mediated through activation of distinct *cis*-acting sequences of the human promoter.

2. Materials and methods

2.1. Cell cultures

JAr, JEG and BeWo cells were obtained from ATCC (Rockville, MD, USA) and cultured in DMEM (JAr and JEG) or RPMI 1640 medium (BeWo) supplemented with 10% fetal calf serum and antibiotics. Fetal calf serum and culture media were purchased from Gibco BRL (Gaithersburg, MD, USA). Hypoxic conditions were applied by placing the culture dishes in a sealed jar containing an oxygen chelator (Oxoid Ltd, Basingstoke, UK). According to the manufacturer, an atmosphere with 6% O₂ and 12% CO₂ is achieved in the jar within 1 h after sealing. Chemical hypoxia was produced by adding cobalt chloride (CoCl₂) or desferrioxamine (DFO) (Sigma, St Louis, MO, USA) to the culture medium. Both agents were dissolved in water and added to the medium to achieve a final concentration of 50 µM. After various time in culture as indicated, cells and culture medium were harvested and separately frozen until use. Leptin was determined in the medium by radioimmunoassay, as described by the manufacturer (Linco Research, St. Charles, MO, USA).

2.2. Immunocytochemistry

BeWo cells were seeded on 15-mm glass slides placed in 6-well plastic plates and grown to 60–70% confluence. Fixation was carried out for 45 min at room temperature in 4% PBS-paraformaldehyde. Cells were washed in PBS prior to addition of the leptin antibody (kind gift of Dr. S.D. Holmes, SmithKline-Beecham, Harlow, UK). The primary antibody was added for 30 min, followed by sequential 10-min incubations with solutions of biotinylated second antibody, streptavidin-peroxidase and the substrate AEC chromogen (3-amino-9-ethylcarbazole in *N,N*-dimethylformamide). Negative staining was performed by omitting the primary antibody. A counterstaining was performed with Mayer's hematoxylin. Sections were photographed using a Leitz Dialux 22 microscope (Leitz, Wetzlar, Germany).

2.3. Northern blot analysis

Total RNA was extracted with 4.4 M guanidine thiocyanate [13] and analyzed by Northern blotting after electrophoresis in 1.2% formaldehyde agarose gel, transfer and fixation onto nitrocellulose filters (Hybond-N+, Amersham Pharma Biotech Inc, Piscataway, NJ, USA). Hybridization was performed with a human GLUT3 cDNA (kind gift of Dr. G. Bell, Chicago, IL, USA) and a ribosomal 18S probe for control of RNA loading. Blots were subjected to autoradiography and image analysis (Perfect-Image, Numeris, Nanterre, France).

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Complementary DNA was synthesized at 37°C for 50 min from 500 ng total RNA using random hexanucleotides primers (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA) and Moloney murine

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leukemia virus reverse transcriptase (Superscript II, Life Technologies SARL Cergy Pontoise, France). Briefly, 100 ng of reverse-transcribed RNA was amplified with 2.5 U *Taq* polymerase in 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM MgCl₂, 0.2 mM dNTP and 0.5 μM of gene-specific sense and antisense primers. The PCR products were visualized on 2% agarose gels by ethidium bromide staining and exposed to UV transilluminator for photography (Perfect-Image, Numeris, Nanterre, France). The primer pair derived from the human leptin cDNA sequence [1] was as follows: upstream primer 5'-CATTGGGGAACCCTGTGCGGATTC-3'; downstream primer 5'-TGGCAGCTCTTAGAGAAGGCCAGC-3'. Amplification consisted of one cycle at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 60°C for 1 min and 68°C for 2 min, with a final extension step at 68°C for 7 min. This amplified a fragment of 348 bp. The primer pair derived from the mouse β-actin cDNA sequence [14] was as follows: upstream primer 5'-GAGACCTTCAACACCCC-3'; downstream primer 5'-GTGGTGGTGAAGCTGTAGCC-3'. Amplification consisted of one cycle at 94°C for 2 min, followed by 30 cycles at 94°C for 40 s, 62°C for 40 s and 74°C for 1 min, with a final extension step at 74°C for 7 min. This amplified a fragment of 236 bp.

2.5. Transient transfections

Epididymal rat adipose cells were isolated and transfected by electroporation as described in [15]. For BeWo cells, Lipofectamine®-mediated transfection (Gibco BRL, Gaithersburg, MD, USA) was performed according to manufacturer's recommendations, when cells have reached approximately 50% confluence. After 5 h, the medium was changed and the cells were cultured for 24 h in normoxic or hypoxic conditions. Activity of the human leptin promoter was assessed using a series of luciferase reporter constructs (kind gift of J. Auwerx), containing serial 5' deletions extending from -2.38 (Δ2), -1.87 (Δ5), -1.20 (Δ14), -0.98 (Δ12), and -0.17 (Δ4) kb upstream of the transcription initiation site [16,17]. pGL3-Basic vector (Promega Corp. Madison, WI, USA) was the negative control. To normalize for transfection efficiency, a pRSV-β-galactosidase expression vector was co-transfected with each construct. Transfections were performed at least in triplicate with different plasmid preparations. In each experiment, individual data were calculated as mean of replicates and expressed as the ratio of luciferase over β-galactosidase activity. Luciferase assays were carried out as described [15].

2.6. Preparation of nuclear extract

Nuclear extracts were prepared essentially as previously described [18]. Briefly, confluent BeWo cells were broken in a dounce homogenizer in 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.05% Triton X-100, and protease inhibitor mixture containing 20 μM leupeptin, 20 μM pepstatin, 2 μM aprotinin, 50 μM AEBSF, 50 mM NaF, and 2 mM sodium metavanadate. Nuclei were pelleted by 10 min centrifugation at 600×g, at 4°C. The nuclear pellet was then resuspended in 100 μl of hypotonic buffer (25% glycerol, 20 mM HEPES, 1.5 mM MgCl₂, 0.2 mM EDTA, 1.2 M KCl and protease inhibitors). Nuclei were allowed to swell for 30 min at 4°C and a clear nuclear extract was obtained by centrifugation 30 000×g, 30 min at 4°C. Proteins were assayed by the Bio-Rad protein assay procedure (Richmond, CA, USA), with bovine serum albumin as standard.

2.7. Western blotting analysis

Nuclear proteins from BeWo cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 7.5% resolving gel, and electroblotted onto nitrocellulose membrane (Hybond ECL, Amersham Pharma Biotech Inc, Piscataway, NJ, USA). Following blocking with low-fat milk, membranes were incubated for 1 h with an anti-hypoxia-inducible factor-1 α subunit (HIF-1α) antiserum [19] (kind gift of J. Pouyssegur). The blots were washed and exposed for 1 h to horseradish peroxidase-conjugated anti-rabbit IgG. The immune complex was detected by luminescent visualization (ECL, Amersham Pharma Biotech Inc, Piscataway, NJ, USA) on X-ray film (Kodak Biomax MR). As a control of nuclear extract integrity, the membrane was stripped and blotted with an anti-Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.8. Statistical analysis

Data (mean of replicates) are mean ± S.E.M. for the indicated number of experiments. Statistical analysis was performed using Student's *t*-test for unpaired data with significance defined as *P* < 0.05.

3. Results

A first series of time course experiments were conducted to assess the capacity of trophoblast-derived cell lines to produce leptin in culture. Among JAr, JEG and BeWo cell lines, only the latter released consistent amounts of leptin (Fig. 1A). In these cells, the rate of leptin secretion was stable around 0.2 ng/10⁶ cells/24 h over 5 days in culture. Moreover, leptin was readily detected by immunocytochemistry (Fig. 1B). Therefore, the BeWo cells were chosen as a physiological relevant model to assess placental leptin production.

To assess the effect of hypoxia, BeWo cells were exposed to either low oxygen (6%) or chemical hypoxia induced by CoCl₂ or DFO for various periods of time. Induction of the glucose transporter GLUT3 mRNA was first measured to assess the cell responsiveness to hypoxia, since this transporter has been

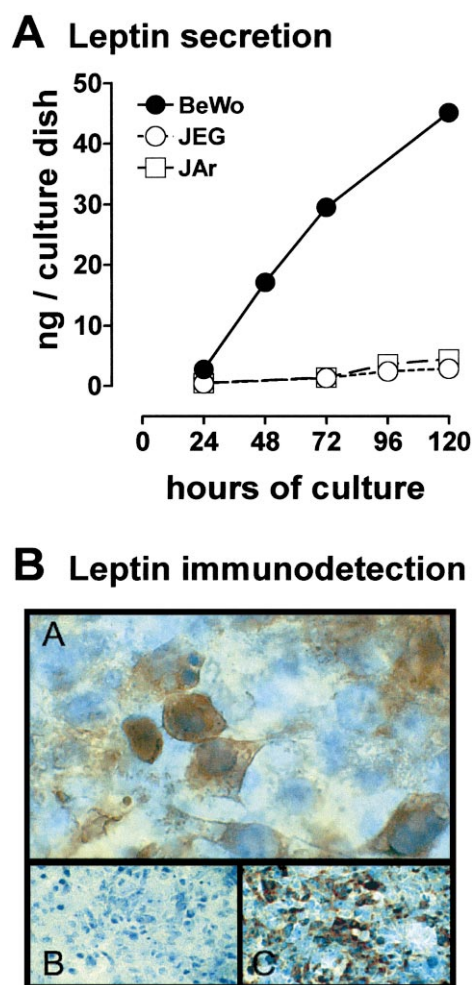


Fig. 1. Leptin production in trophoblast-derived cell lines. A: Leptin secretion in JEG, JAr and BeWo cells. Cells were cultured in normoxia for 120 h. The culture medium was sampled at the indicated period of time to measure leptin by radioimmunoassay (Linco Research, St. Charles, MO, USA). B: Immunodetection of leptin in BeWo cells. Immunostaining was carried out with a specific antibody for leptin (A, C), followed by biotinylated second antibody, streptavidin-peroxidase and the substrate AEC chromogen (3-amino-9-ethylcarbazole in *N,N*-dimethylformamide). Negative staining was performed by omitting the primary antibody (B). A counter-staining was performed with Mayer's hematoxylin. Magnifications are 595 (A), 425 (B) and 425× (C).

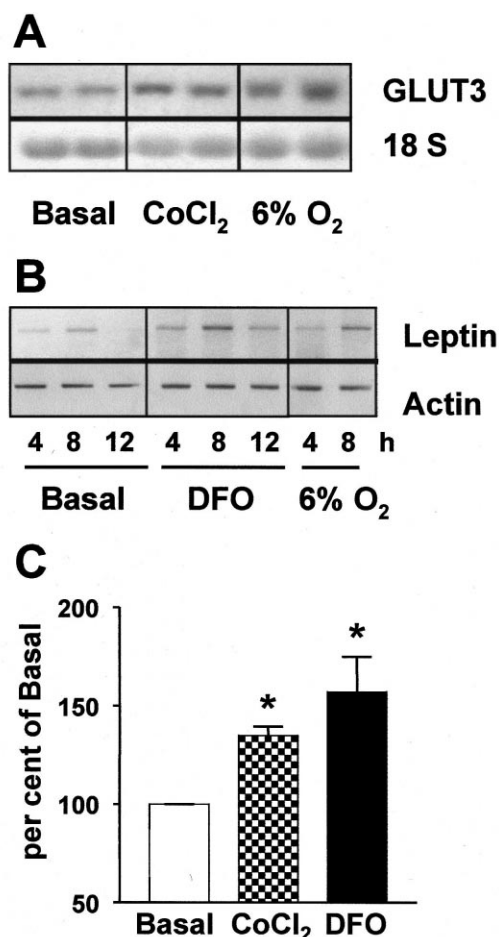


Fig. 2. Effect of hypoxia on GLUT3 and leptin in BeWo cells. The BeWo cells were cultured to confluence and exposed to 6% O₂ or chemical hypoxia induced by CoCl₂ (50 μ M) or DFO (50 μ M). At the end of the culture, total RNAs were extracted. A: Expression of GLUT3 mRNA and 18S rRNA was analyzed by Northern blotting. The blot shows signals arising from cells cultured for 24 h in normoxia (basal) or hypoxic conditions. B: Expression of leptin and actin mRNA was analyzed by RT-PCR after different periods of time as indicated. C: Leptin was measured in the culture medium by radioimmunoassay after 48 h. * P < 0.05 versus basal (n = 5).

previously shown to be induced under hypoxic stimuli in human trophoblast in culture [20]. In comparison with untreated (basal) cells, both low O₂ and chemical hypoxia-induced by CoCl₂ increased GLUT3 mRNA after 24 h (Fig. 2A). Similarly, leptin mRNA and leptin release were increased under exposure to hypoxic conditions (Fig. 2B,C). The effect of hypoxia on the amounts of leptin in the culture medium was detectable only after 48 h (data not shown), whereas leptin mRNA was increased after 4 to 8 h (Fig. 2B). This lag time between leptin mRNA induction and increased leptin release suggests a transcriptional effect of hypoxia.

To test this hypothesis, the effect of hypoxia was studied on the human leptin promoter. The activity of serial deletions extending from -2.38 to -0.17 kb upstream of the leptin gene transcription start site was assessed. The longest fragment, $\Delta 2$, included a region previously characterized as a placental enhancer (PLE) [21,22], whereas shorter fragments of the promoter were deleted of this domain. As shown in Fig. 3, absence of the PLE in $\Delta 5$ and $\Delta 4$ fragments led to a marked decrease in promoter activity in the BeWo cells. Nevertheless,

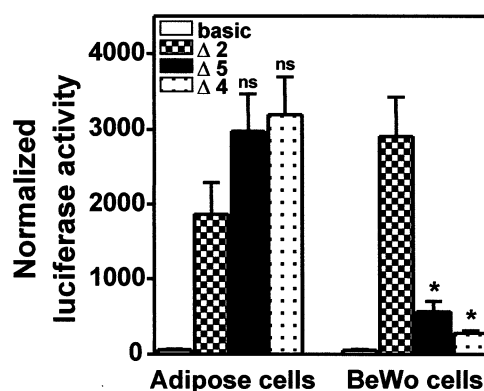


Fig. 3. Activity of the human leptin promoter in adipose and BeWo cells. Primary rat adipose cells were transfected by electroporation with 5 μ g of luciferase reporter constructs, either promoterless (basic) or containing various lengths of the human leptin promoter ($\Delta 2$: -2.38 kb and $\Delta 5$: -1.87 kb). BeWo cells were transfected by lipofection with 0.5 μ g of the same constructs. A pRSV- β -galactosidase expression vector was co-transfected with each construct. Luciferase activity was assessed after 24 h. Data represent the luciferase normalized to β -galactosidase activity and are the mean \pm S.E.M. for four (adipose) to seven (BeWo) independent experiments. * P < 0.05 versus $\Delta 2$; ns, non-significant versus $\Delta 2$.

the luciferase activity driven by these promoter sequences remained markedly higher (more than 25-fold) than the luciferase activity measured in cells transfected with the promoterless parent vector (basic). In contrast to the BeWo cells, deletion of the PLE did not significantly alter the luciferase activity measured in transfected adipose cells. These results confirm previous observations [22] and strengthen the physiological relevance of the cellular model used in the present study.

In comparison with basal conditions, the activity of the $\Delta 2$ promoter fragment increased by more than 4-fold under hypoxic conditions (Fig. 4). Deletion of the PLE did not preclude the effect of hypoxia. Rather, the stimulatory effect of hypoxia tended to be larger on $\Delta 5$ than on $\Delta 2$ promoter frag-

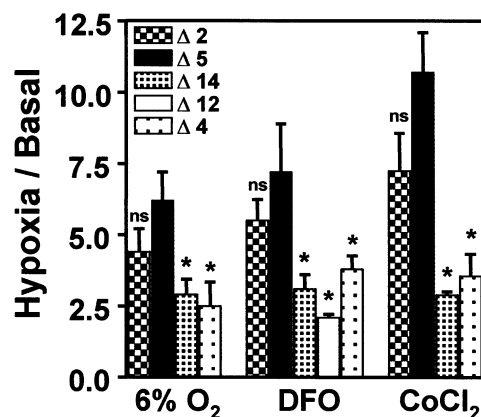


Fig. 4. Effect of hypoxia on human leptin promoter activity in BeWo cells. BeWo cells were transfected as in Fig. 3 and exposed to normoxia (basal) or various hypoxic conditions: 6% O₂, DFO (50 μ M) or CoCl₂ (50 μ M) for 24 h. The 5' end of leptin promoter deletions are: $\Delta 2$: -2.38 , $\Delta 5$: -1.87 , $\Delta 14$: -1.20 , $\Delta 12$: -0.98 , and $\Delta 4$: -0.17 kb. Data represent the ratio of hypoxic/basal expression for each construct and are the mean \pm S.E.M. of 5–7 independent transfections. * P < 0.05 versus $\Delta 5$; ns, non-significant versus $\Delta 5$.

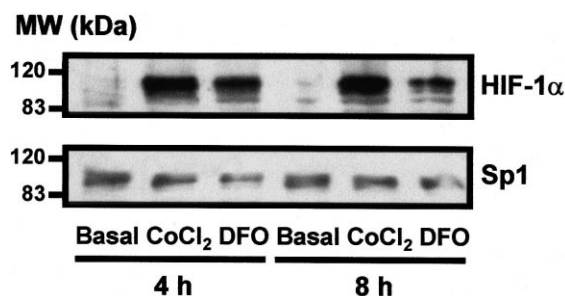


Fig. 5. Effect of hypoxia on HIF-1 α in BeWo cells. The BeWo cells were cultured for the indicated periods of time, in basal conditions or in the presence of CoCl₂ (50 μ M) or DFO (50 μ M). At the end of the culture, nuclear extracts (20 μ g) were analyzed by SDS-PAGE (7.5% gel) and immunoblotting using an anti-HIF-1 α antiserum. The membrane was stripped and blotted with an anti-Sp1 antibody.

ment activity. Depending of the stimulus, the luciferase activity driven by the Δ 5 deletion was enhanced by 7 to 12-fold. This demonstrates that the human leptin promoter is inducible by hypoxia and that this effect is mediated by the first 1.87 kb of the promoter, which do not contain the PLE. Shorter fragments (Δ 14, Δ 12 and Δ 4) were also inducible by hypoxia, but the ratio of hypoxic/normoxic expression of these constructs reached at most 3-fold.

HIF-1 α is induced in response to hypoxia through inhibition of ubiquitin-mediated proteasome degradation [23]. To determine whether this process occurred in the BeWo cells under the conditions used in this study, we prepared nuclear extracts to assess HIF1 α by western blotting analysis. As shown in Fig. 5, HIF1- α was markedly induced after 4 and 8 h of exposure to CoCl₂ or DFO. This effect was specific to HIF-1 α , as suggested by the constitutive expression of the transcription factor Sp1 in the same extracts.

4. Discussion

It is established that the human placenta produces significant amounts of leptin [7,8], resulting in increased circulating levels in the maternal blood. The physiological role of this hormonal change has yet to be clarified, since it does not lead to a reduction in food consumption by the mother. We have recently demonstrated that the placenta expresses functional leptin receptors [24]. This suggests that leptin might also exert a local autocrine effect in this organ.

The regulation of leptin gene expression has been extensively studied in the adipose cells, but is less well characterized in cells of placental origin. Leptin mRNA and protein are markedly increased in placenta of insulin treated type I diabetic mothers, suggesting that insulin modulates expression of placental leptin [25]. Preeclampsia is another pathological situation associated with enhanced placental leptin [10,11]. Since preeclampsia is characterized by placental hypoxia, this raised the hypothesis that low oxygen could be a regulatory factor of leptin gene expression. The present study was designed to explore this proposal in a relevant cellular model. The trophoblast-derived BeWo cells were chosen since they both produce leptin in culture and are responsive to hypoxia, as demonstrated by increased GLUT3 gene expression.

The effect of hypoxia can be mimicked by various agents including transition metal ions, such as cobalt, and iron che-

lating agents, such as DFO. Although their mechanism of action is not fully understood, these factors are thought to alter the function of a putative heme protein participating to a O₂ sensor system [26]. These agents mimic the effect of hypoxia on the expression of a number of hypoxia-inducible genes, including erythropoietin, the GLUT1 isoform of glucose transporter and vascular endothelial growth factor (VEGF) [26–29].

A major finding of this study is that hypoxia induced either by low O₂, DFO or CoCl₂ markedly increases the activity of the human leptin gene promoter in placental cells. Deletion analysis allowed to map a domain encompassing the first 1.87 kb of the leptin promoter which conferred high responsiveness to hypoxic conditions. Exclusion of the PLE from this domain indicates that the *trans* factors involved are distinct from those, of yet unidentified nature, which bind to the PLE [21,22]. Transcription of hypoxia-inducible genes is regulated by hypoxia response elements (HREs) which contain binding sites for HIF-1. This transcription factor is composed of HIF-1 α and HIF-1 β (ARNT) subunits [30]. HIF-1 α is rapidly degraded by the ubiquitin–proteasome pathway in normoxia and this process is inhibited by hypoxia, allowing dimerization of HIF-1 α with HIF-1 β in the nucleus [23]. The HRE of the VEGF promoter has been shown to be composed of a HIF-1 binding site and a downstream ancillary sequence, forming an imperfect inverted repeat [31]. This consensus motif is conserved among several hypoxia-inducible genes. Analysis of the human leptin promoter revealed a similar structure (5'-TACGTG-10 nt-CACAGA-3'), which is included in the 1.87-kb promoter construct. Coupled with the marked induction of HIF-1 α by hypoxia, these observations argue for the implication of HIF-1 in leptin gene induction. Further studies are underway to confirm that this promoter region is mediating the hypoxic response.

The leptin promoter region extending from –1.20 kb to the transcription initiation site does not contain consensus HRE sequences. Nevertheless, it exhibited hypoxically inducible activity. These observations raise the possibility that hypoxia-dependent pathways target distinct *cis*-acting sequences on the leptin promoter, as previously described for GLUT1 gene [27].

Interestingly, the placental production of several cytokines, including TNF α , IL-1 α and IL-1 β , is increased under hypoxia in cultured villous explants from human placenta. This does not appear to be a general effect, since IL-6 production is not modified in the same model [29]. The authors suggest that inflammatory cytokines may contribute to the pathophysiology of preeclampsia. Whether this is also the case for leptin remains to be determined. Alternatively, since leptin is a potent angiogenic factor [32,33], enhanced placental leptin production could represent a compensatory response aimed at favoring neo-vascularization, in concert with other factors such as VEGF [34] and TGF β [35].

In conclusion, the present data show that the human leptin gene is transcriptionally up-regulated by hypoxia. This effect is mediated by activation of distinct domains of the promoter and is independent of the presence of the PLE.

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