Hypoxia-inducible Factor 1 Transactivates the Human Leptin Gene Promoter

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Increased placental leptin has been demonstrated in preeclampsia, a pregnancy disorder associated with placental hypoxia. This suggests that leptin gene expression is enhanced in response to oxygen deficiency in this organ. In support of this hypothesis, we have previously shown that hypoxia activates the leptin promoter in trophoblast-derived BeWo cells. Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric HIF-1α/HIF-1β complex that regulates the transcription of hypoxia-responsive genes. To test whether this factor is involved in hypoxia-induced leptin promoter activation, BeWo cells were transiently transfected with a HIF-1α expression vector. Exogenous HIF-1α markedly increased luciferase reporter activity driven by the leptin promoter when HIF-1β was co-expressed in the same cells. This effect was similar to that elicited by CoCl2, an agent known to stabilize endogenous HIF-1α. These data suggest that HIF-1α/HIF-1β dimers are involved in the effect of CoCl2 to activate the leptin promoter. To confirm the implication of HIF-1, the cells were transfected with a dominant negative form of HIF-1α producing transcriptionally inactive HIF-1β/HIF-1α dimers. This mutant HIF-1α protein abolished CoCl2 activation of the leptin promoter, providing direct evidence that the effect of CoCl2 is mediated by endogenous HIF-1α. Deletion analysis and site-specific mutagenesis demonstrated that a HIF-1 consensus binding site (HRE) spanning −120 to −116 bp relative to the start site was required for CoCl2 and exogenous HIF-1α induction of leptin promoter activity. Electrophoretic mobility shift assays performed with in vitro-translated HIF-1α and HIF-1β proteins demonstrated binding to this HRE and not to mutated sequences only when both subunits were used together. These data demonstrate that leptin is a new hypoxia-inducible gene, which is stimulated in a placental cell line through HIF-1 interaction with a consensus HRE site located at −116 in the proximal promoter.

Leptin, originally identified as a satiety factor secreted by adipose tissue, is also produced by the placenta in humans (1). Placental leptin mRNA (2) and protein (3) are markedly increased in preeclampsia, a disorder associated with maternal hypertension, reduction in placental blood flow, and placental hypoxia (4). These observations have led to the proposal that the leptin gene could be induced by hypoxia. In support of this hypothesis, we have previously shown that gene expression and leptin release were increased in trophoblast-derived BeWo cells in response to various conditions of natural or chemical hypoxia. Moreover, the human leptin promoter was activated by hypoxia in these cells (5).

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor of major importance in the cellular response to oxygen deficiency. HIF-1 comprises HIF-1α and HIF-1β subunits, which both belong to the basic-loop-helix-PAS protein family (for review, see Ref. 6). The HIF-1β subunit is constitutively expressed. By contrast, HIF-1α is maintained at a low level in normoxic cells through proteasomal degradation of the protein. The von Hippel-Lindau tumor suppressor protein is a component of the complex that targets HIF-1α for polyubiquitination and degradation (7). Two recent observations indicate that von Hippel-Lindau protein binds to HIF-1α when a proline residue at codon 564 is hydroxylated (8, 9). Hydroxylation of HIF-1α is controlled by a Fe3+-dependent hydroxylase activity that is inhibited by decreased oxygen. This mechanism accounts for HIF-1α stabilization in hypoxic cells, allowing nuclear translocation and dimerization with HIF-1β. Stabilization of HIF-1α is also induced by chelating or substituting Fe3+ with desferrioxamine and cobalt chloride (CoCl2), respectively. This provides a molecular mechanism accounting for the ability of these agents to mimic the effect of hypoxia in experimental cell systems.

The present study was designed to test whether HIF-1 is involved in hypoxia-induced activation of the human leptin gene promoter in the placental BeWo cells. To investigate this, the transcriptional activity of HIF-1 was manipulated by overexpressing the wild-type or dominant negative form of HIF-1α. Our data provide evidence that induction of leptin promoter activity by hypoxia is mediated by HIF-1, through a HIF-1α consensus binding site, located at −116 in the proximal promoter. This study adds the leptin gene to the list of hypoxia-inducible genes regulated by this transcription factor.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human choriocarcinoma cell line BeWo was obtained through American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum and antibiotics in a humidified atmosphere with 5% CO2 at 37 °C.

**Plasmids and Constructs**—The 5′-deleted constructs containing various lengths of the human leptin promoter sequences upstream of the luciferase reporter gene have been described previously (5). Promoter fragments are designated according to their length in bp (pX(X bp)), relative to the transcription start site described in Ref. 10. Expression

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Transactivation of the Human Leptin Gene Promoter by HIF-1

Fig. 1. Effect of overexpression of HIF-1α on leptin promoter activity. BeWo cells were transiently transfected with 500 ng of a luciferase reporter plasmid containing 1.872 kb of human leptin promoter (+1872) and 60 ng of a β-galactosidase expression vector. When indicated (+ HIF-1β), the cells were co-transfected with 1 μg of pcDNA3-HA-HIF-1β. Some cells were co-transfected with 1 μg of pcDNA3-HA-HIF-1α (gray and black bars). Whatever the experimental condition, the total amount of transfected DNA was kept at 2 μg by addition of pcDNA3 empty vector. Five h after transfection, the medium was changed, and the cells were cultured in serum-free medium in the absence (open and gray bars) or presence (hatched and black bars) of 100 μM CoCl2 for 24 h. The cells were then lysed, and luciferase activity and β-galactosidase activity were measured in cell lysate. Transfections were performed at least in triplicate, and triplicate values were averaged to give the result of one experiment. Relative luciferase activity is the ratio of luciferase over β-galactosidase activity. Data are the mean ± S.E. of four independent experiments.

vectors encoding wild-type HIF-1α (pcDNA3-HA-HIF-1α), a dominant negative form of HIF-1α (pcDNA3-HA-DN-HIF-1α), or wild-type HIF-1β (pcDNA3-HA-HIF-1β) have been described in Refs. 11 and 12.

Mutagenesis—A sequence contained within 0.146 kb of the leptin promoter sequence in the +146/luc construct was mutated by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Two distinct mutations were generated. The motif 5′-GCACGTCG-3′ spanning 121 to 114 was replaced by 5′-GCTTAATG-3′ or by 5′-GCAAACG-3′. The generated plasmids were designated p(146)lucmut1 and p(146)lucmut2, respectively. Each mutation was verified by direct sequencing, and two plasmid preparations isolated from distinct clones were tested in transfection.

Transient Transfection—LipoFectAMINE (Invitrogen)-mediated transfection of BeWo cells was performed as described previously (5). Briefly, 1 day before transfection, cells were plated into 35-mm 6-well dishes and transfected with 500 ng/well luciferase reporter construct and 60 ng/well pRSV-β-galactosidase expression vector to normalize for transfection efficiency. In some experiments, expression vectors encoding HIF-1α, HIF-1α wild-type, or HIF-1α mutant cDNA were co-transfected with the reporter constructs, as indicated in the figure legends. The total amount of transfected DNA was kept constant by adding the appropriate amount of pcDNA3 empty vector. Five h after transfection, the medium was changed, and the cells were cultured for 24 h in serum-free medium with or without addition of 100 μM CoCl2 (Sigma). Transfections were performed at least in triplicate. In each experiment, individual data were calculated as the mean of triplicates and expressed as the ratio of luciferase to β-galactosidase activity measured in the same cell lysate, as described previously (5).

Electrophoretic Mobility Shift Assays—HIF-1α and HIF-1β proteins were expressed by in vitro translation in rabbit reticulocyte lysates (Promega, Madison, WI). pcDNA3 empty vector, pcDNA3-HA-HIF-1α, or pcDNA3-HA-HIF-1β was used to transcribe the HIF-1α and HIF-1β probes. The electrophoretic mobility shift assays were performed using a double-stranded probe encompassing a sequence contained within the first 0.146 kb of the human leptin gene promoter. The sense strand sequence of the wild-type (wt) oligonucleotide is 5′-GTACTAGCACCGCCGCCGCAAGCACTTGGATTGTCAGGGGCCG-3′. Two oligonucleotides containing distinct mutations of the underlined sequence in the wt probe, 5′-GTACTAGACCCGCCGCCTTGGATTGTCAGGGGCCG-3′, were synthesized. The generated plasmids were designated p(146)lucmut1 and p(146)lucmut2, respectively. Each mutation was verified by direct sequencing, and two plasmid preparations isolated from distinct clones were tested in transfection.

Fig. 2. Deletion analysis of the 5′-flanking region of the leptin gene. BeWo cells were transiently transfected with 500 ng of one of seven luciferase reporter constructs containing 5′-deleted leptin promoter fragments, 60 ng of a β-galactosidase expression vector, 1 μg of pcDNA3-HA-HIF-1α, and either 1 μg of pcDNA3-HA-HIF-1α (dotted and black bars) or 1 μg of pcDNA3 empty vector (hatched bars). Five h after transfection, the medium was changed, and cells were cultured in serum-free medium in the absence (dotted bars) or presence (hatched and black bars) of 100 μM CoCl2 for 24 h. Cells were then lysed, and luciferase activity and β-galactosidase activity were measured in cell lysate. Transfections were performed at least in triplicate, and triplicate values were averaged to give the result of one experiment. The results show the fold increase in relative luciferase activity (ratio of luciferase over β-galactosidase activity) over basal cells. Data are the mean ± S.E. of three to seven independent transfections.
Transactivation of the Human Leptin Gene Promoter by HIF-1

Transfection of the BeWo cells with HIF-1α or CoCl2 transfected with a reporter plasmid containing 0.146 or 0.116 kb of the leptin gene promoter region. For each construct, the fold increase in luciferase activity was determined by the ratio of luciferase over β-galactosidase activity. Data are the mean ± S.E. of four independent experiments. *p < 0.05; **p < 0.01 (versus control cells).

Effect of HRE site-specific mutation on hypoxia-induced leptin promoter activity. BeWo cells co-transfected with the p(116)luc reporter vector were unresponsive to CoCl2 on this region of the leptin promoter, BeWo cells were transiently transfected with 60 ng of a β-galactosidase expression vector, 0.5 μg of pCDNA3-HA-HIF-1α, and 500 ng of p(146)luc (A) or p(116)luc (B) luciferase reporter plasmids containing 0.146 or 0.116 kb of the leptin gene promoter region. The total amount of transfected DNA was kept at 5 μg by addition of pCDNA3 empty vector. Five h after transfection, the medium was changed, and cells were cultured in serum-free medium in the absence (Basal) or presence of 100 μM CoCl2 for 24 h. The cells were then lysed, and luciferase activity and β-galactosidase activity were measured. Transfections were performed at least in triplicate, and individual values were averaged to give the result of one experiment. Relative luciferase activity was determined by the ratio of luciferase over β-galactosidase activity. Data are the mean ± S.E. of four independent experiments. *p < 0.05; **p < 0.01 (versus control cells).

Deletion Analysis of the 5'-Flanking Region of the Leptin Gene—To determine the promoter region mediating activation by endogenous HIF-1α or CoCl2, BeWo cells were transiently transfected with reporter constructs containing various lengths of the leptin gene promoter region. For each construct, the fold increase in luciferase activity elicited by either HIF-1α overexpression, CoCl2 treatment, or a combination of both was determined over non-stimulated cells. As mentioned above, HIF-1β was routinely transfected, whatever the experimental condition. A similar pattern of reporter gene expression was observed for several constructs containing up to 0.146 kb of the leptin gene 5'-flanking region (Fig. 2). Both HIF-1α and CoCl2 individually stimulated luciferase activity by 5–7-fold. The effect of the two stimuli in combination was always greater than that elicited by HIF-1α or CoCl2 alone. However, when combined, these effects were never fully additive. In contrast to all other deleted constructs, the p(116)luc reporter vector was unresponsive to HIF-1α and/or CoCl2. This analysis revealed that the first 146 bp of the leptin promoter harbor a sequence responsive to CoCl2 and exogenous HIF-1α, which is missing or disrupted in the p(116)luc construct.
p(146)lucmut1 and p(146)luc mut2, were transfected in BeWo cells, and their capacity to respond to CoCl2 treatment and HIF-1 overexpression was tested. As shown in Fig. 4, the luciferase activity produced by both mutated leptin promoter fragments was not increased by these stimuli. This supports the hypothesis that this HRE consensus sequence is required for hypoxia-mediated induction of leptin promoter activity.

In Vitro Binding of HIF-1α and HIF-1β to the HRE located at −116 in the leptin promoter. A, radiolabeled oligonucleotides corresponding to wild-type (wt) or mutated (mut1 and mut2) HRE located at −116 in the leptin promoter were incubated with 2 μl of in vitro-translated HIF-1α, 2 μl of in vitro-translated HIF-1β, or both as indicated. Unprogrammed reticulocyte lysate was used as control and also to keep the total volume of lysate at 4 μl. B, competition assays were carried out by incubating the radiolabeled wt probe with in vitro-translated HIF-1α and HIF-1β and cold wt or mutated probes in 5-, 10- or 50-fold molar excess, as indicated. These autoradiograms are representative of four independent electrophoretic mobility shift assays.

Data demonstrate that HIF-1 binds to the consensus HRE present within the proximal region of the leptin gene promoter.

**DISCUSSION**

We have shown previously that leptin gene expression is increased by hypoxia in a trophoblast-derived cell line (5). The present study provides functional evidence that HIF-1 mediates this effect via a HIF-1-responsive element located at −116 in the human leptin promoter. This conclusion is based on results obtained in experiments where the cellular level of HIF-1 was altered to induce or inhibit HIF-1 transcriptional activity. Consistent with the implication of HIF-1, exogenous overexpression of HIF-1α in the BeWo cells markedly activated the leptin promoter. Moreover, a similar amount of stimulation was produced by CoCl2 treatment, giving support to the idea that stabilization of endogenous HIF-1α by this agent mediates leptin promoter activation. The most compelling evidence for the implication of HIF-1 came from the use of a dominant negative form of HIF-1α, which totally abolished the effect of CoCl2. This demonstrates unequivocally that CoCl2-induced leptin promoter activity is driven by increased endogenous HIF-1α leading to the activation of HIF-1.

Sequence analysis reveals the presence of several putative...
HREs within the first 1.872 kb of the human leptin promoter. We have previously observed that two regions containing 1.87 and 1.20 kb of the promoter respectively conferred high and relatively lower responsiveness to hypoxia (5). These data suggested to us that a distal HRE located at −1.83 kb in the promoter could mediate the effect of hypoxia. However, this hypothesis was not confirmed by subsequent experiments performed in BeWo cells overexpressing HIF-1α. Indeed, we show here that both CoCl2- and HIF-1α-induced activation of the leptin promoter are of a similar magnitude for each 5′-deleted fragment extending from 1.872 to 0.146 kb. Therefore, it is possible that distinct hypoxia responsiveness was coincidentally associated with promoter length, although it cannot be excluded that low levels of endogenous HIF-1α have been instrumental in this effect. In the present study, deletion analysis and site-specific mutagenesis clearly implicate the most proximal HRE of the leptin promoter in HIF-1 responsiveness.

These observations add the human leptin gene to a list of genes activated by hypoxia via the HIF-1 pathway. After its initial discovery as a satiety factor, leptin has been subsequently implicated in a variety of functions, some of which are related to hypoxia (19). Consistent with a stimulatory effect of a local hypoxic environment on the leptin gene expression (15) and in the adipose tissue of rats submitted to 15% oxygen, we have recently observed that leptin gene expression is increased in human PAZ6 adipose cells in response to cellular hypoxia (15) and in the adipose tissue of rats submitted to hypobaric hypoxia. If leptin also exerts a proangiogenic effect in this tissue, it can be anticipated that a local effect of leptin would be to stimulate vasoconstriction during normal or pathological adipose tissue growth. Interestingly, the angiogenic capacity of adipose tissue has been used clinically to promote wound healing and revascularization of ischemic tissues (16, 17). This effect could be mediated, at least in part, by leptin, in concert with other angiogenic factors such as vascular endothelial growth factor (18). Besides the placenta, leptin is produced in various non-adipose tissues, including the stomach (19). Consistent with a stimulatory effect of a local hypoxic environment produced at wound sites, leptin gene expression is increased in gastric ulcers (20, 21). This suggests that leptin might participate in the mechanisms leading to ulcer healing in the stomach because the hormone has been shown to promote skin wound re-epithelialization (22, 23). These observations favor the idea that up-regulation of leptin production by hypoxia is physiologically relevant not only in the placenta but also in other leptin-producing tissues, including adipose tissue.

Hypoxia is not the only condition that stabilizes HIF-1α and activates HIF-1 transcriptional activity. Several hormones and growth factors, including insulin and insulin-like growth factor I (24), angiostatin II, thrombin and platelet-derived growth factor (11), and, more recently, endothelin-1 (25), have been shown to increase the level of HIF-1α in various cell types. In addition, inflammatory cytokines, such as interleukin 1β and tumor necrosis factor α, also induce HIF-1 activity in normoxic cells (26–28). It would be of interest to know whether these factors could regulate leptin gene expression in some cell types by activating hypoxia-independent HIF-1 pathways. Interestingly, this could be the case in the hypoxic placenta during preeclampsia, in which increased inflammatory cytokine production has been described (29, 30).

In conclusion, the data presented here are consistent with the leptin gene being a genuine hypoxia-inducible gene. Moreover, they show that hypoxia mediates increased leptin gene expression via HIF-1α and HIF-1-dependent transcriptional activity, as described for several other genes regulated by low oxygen availability.

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