Metabolic, Endocrine and Genitourinary Pathobiology

Aberrant Expression of Leptin in Human Endometriotic Stromal Cells Is Induced by Elevated Levels of Hypoxia Inducible Factor-1 α

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Elevated expression of leptin in endometriotic tissue results in an increase in stromal cell proliferation and may contribute to the development of endometriosis. However, the underlying mechanism responsible for aberrant expression of leptin is not known. We hypothesize that aberrant expression of leptin in endometriotic stroma may be regulated by increased levels of hypoxia-inducible factor-1 α (HIF-1 α), the master transcription factor that controls gene expression in response to hypoxia. Herein we show that the mRNA and protein levels of HIF-1 α were greater in ectopic endometriotic tissue compared with its eutopic counterpart. Exposure of eutopic endometrial stromal cells under hypoxic conditions or treated with desferrioxamine (DFO, chemical hypoxia) resulted in a time-dependent increase in leptin gene expression. A promoter activity assay demonstrated that HIF-1 α induced leptin promoter activity after DFO treatment. Chromatin immunoprecipitation assay further demonstrated that binding of HIF-1 α to leptin promoter was evident after DFO treatment. Finally, depletion of HIF-1 α by short interference RNA abolished leptin expression in ectopic endometriotic stromal cells. Taken together, our data demonstrate that aberrant expression of leptin in ectopic endometriotic stromal cells is induced, at least in part, by an elevated level of HIF-1 α in these cells, providing new insights into the etiology of endometriosis. (Am J Pathol 2007, 170:590–598; DOI: 10.2353/ajpath.2007.060477)

Leptin, a small peptide molecule produced from the obese (*ob*) gene product and synthesized and secreted mainly by adipocytes,¹ plays a major role in maintaining

energy homeostasis. It is suggested that leptin may also play important roles in endocrine, paracrine, and/or autocrine regulation of many physiological and pathological processes including angiogenesis, reproduction, and endometriosis.^{2–5} A significant increase of leptin levels in peritoneal fluid of patients with endometriosis was observed,⁶ but serum leptin levels were either elevated or not changed.^{6,7} A previous study in our laboratory has identified that leptin was markedly expressed in ectopic endometriotic lesions.³ Elevated leptin expression in endometriotic lesions further stimulates its own mRNA expression and enhances the proliferation of ectopic endometriotic stromal cells. However, peritoneal macrophages isolated from women with or without endometriosis did not express leptin. Our data suggest that ectopic endometriotic lesions may be important sites of leptin production, thereby elevating leptin concentration in the peritoneal fluid of women with endometriosis.

Although the up-regulation of leptin in endometriosis has been confirmed by several groups,^{6–8} mechanisms responsible for aberrant expression of leptin remain enigmatic. Recent reports demonstrated that expression of leptin could be induced by hypoxia in human placental tissue, skin dermal fibroblasts, and adipocytes.^{9–11} Hypoxia is a common pathophysiological feature that regulates gene expression and affects disease processes.^{12,13} Hypoxia-inducible factor (HIF) 1 is a master transcription factor that mediates the hypoxic effect. The HIF-1 protein is a heterodimeric transcriptional complex composed of HIF-1 α and HIF-1 β subunits. The HIF-1 β subunit is constitutively expressed; however, the level of the HIF-1 α subunit is specifically hypoxia-regulated.¹⁴ HIF-1 α is maintained at a low level in normoxic cells through rapid degradation by the ubiquitin-proteasome

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pathway.¹⁵ HIF-1 α stabilization can be induced in the presence of chemical reagents known to mimic hypoxic conditions such as desferrioxamine (DFO) and cobalt chloride. Stabilization and accumulation of HIF-1 α in hypoxic cells allows nuclear translocation and dimerization with HIF-1 β , causing target gene transcription by binding to a functional hypoxia response element (HRE) located in promoter or other untranslated regions of responsive gene.¹³

Hypoxia is considered a physiological stress response that leads to stimulation of angiogenesis in endometrial tissues during the premenstrual period. In the late secretory phase and menstruation, high expression of proinflammatory cytokines¹⁶ and the induction of hypoxia originating from vasoconstriction of the spiral arteries has been noted.¹⁷ The expression of HIF-1 α is also detected during these phases in normal endometrium.¹⁸ The intensity of HIF-1 α protein expression increases over the course of the secretory phase and is maximal during menstrual phase in glandular cells and stromal cells in the functional layer of endometrium. However, the expression of HIF-1 α in ectopic endometriotic tissues has not been previously reported. Consistent with the notion that HIF-1 β is ubiquitously expressed, the levels of HIF-1 β do not differ between eutopic endometrium and ectopic endometriotic tissue.¹⁹ Based on these clinical observations and the pathophysiological characteristics shown, we postulate that expression of HIF-1 α in endometriotic tissues may be up-regulated and thereby plays a role in the development of endometriosis.

Hypoxia-inducible factors play important roles in phenotypic adaptation to low oxygen levels via transcriptional regulation. Leptin gene expression is induced by hypoxia through the HIF-1 transcriptional pathway,^{9,20} but potential links between these two gene expressions have not been examined in endometriosis. Endometrial stromal cells show a greater response to hypoxia than do epithelial cells, such as increases in IL-6 expression.²¹ Considering that leptin can promote cell proliferation and be regulated by hypoxia, we hypothesized that low cellular oxygen tension in endometriotic stromal cells might up-regulate leptin expression to increase the proliferation of ectopic endometrial cells and thus increase the possibility of implantation of endometriotic lesions. Herein we provide compelling evidence to demonstrate that hypoxia induces the expression of leptin in eutopic endometrial stroma, which normally does not express leptin. Hypoxia-induced leptin gene expression is mediated via HIF-1 α binding to the functional HRE in the leptin promoter. Our findings provide a new etiological insight into the development of endometriosis as well as a molecular framework for designing new therapeutic strategies for this disease.

Materials and Methods

Patients

Patients of reproductive age (26 to 40 years old) who were treated at the Department of Obstetrics and Gynecology of National Cheng Kung University Hospital were recruited for this project. None received any hormone treatment within 6 months before gynecologic surgery. This study was approved by the institutional review board of National Cheng Kung University Medical Center, and informed consent was obtained from each patient. The stages of endometriosis were determined visually according to the revised American Society of Reproductive Medicine classification (1997). During the procedure of laparoscopy and laparotomy, normal endometria from endometriosis-free patients ($n = 16$) and ectopic endometriotic lesions including pelvic endometriosis and ovarian endometrioma from patients with endometriosis ($n = 14$) were collected. All samples were histologically confirmed by pathologists. In addition, five sets of endometrial biopsy samples were also obtained from the patients with advanced stages of endometriosis.

Isolation and Culture of Stromal Cells

The procedure used to isolate stromal cells from eutopic uterine endometrium and ectopic endometriotic lesions was described previously.²² In brief, after the tissues were minced and digested with type IV collagenase, stromal cells were separated from epithelial glands by nylon meshes. Filtrated stromal cells were then cultured in culture medium [Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% fetal bovine serum and antibiotics] at 37°C until 70% confluence was reached. After serum starvation for 12 hours, the cells were cultured under hypoxic condition (1% O₂ or DFO incubation). For mRNA isolation (2×10^4 cells/well), cells were directly lysed in the well using lysis buffer. The concentration and quality of mRNA were determined by absorption at both 260 and 280 nm. For protein isolation (5×10^5 cells/dish), cells were lysed and collected in the dish using radioimmunoprecipitation assay (RIPA) buffer [1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS) buffer]. Purity of the cells was immunostained with vimentin (stromal cell-specific) and cytokeratin (epithelial cell-specific), and originality of endometriotic stromal cells was confirmed by prolactin production after induced decidualization.²³

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from eutopic endometria and ectopic endometriotic lesions using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA samples (500 ng of total RNA) were reverse-transcribed at 42°C for 60 minutes, and 2 μ l of RT products were then subjected to PCR amplification using a thermal cycler (Applied Biosystems, Foster City, CA). Simple RT-PCR was performed to detect HIF-1 α and leptin mRNA expression. Sequences of primers used were 5'-ATGGAGGGCGCCGCGCGAG-3' and 5'-GTAACTTGATCCAAAGCTCTGAG-3' for HIF-1 α and 5'-GAACCCTGTGCGATTCTT-3' and 5'-CAGCTGCCA-

CAGCATGTC-3' for leptin. The primer sequences for GAPDH were reported previously.²⁴ The cycling conditions were 95°C for 10 minutes, followed by 30 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds.

Enzyme-Linked Immunosorbent Assay (ELISA)

The commercial leptin ELISA kit (Cayman Chemical, Ann Arbor, MI) was used for quantification of the protein levels of leptin in cell-free supernatant of culture media according to the manufacturer's protocol. All samples for leptin evaluation were analyzed in the same assay based on a double-antibody sandwich technique. The culture media (50 μ l) were added to microtiter wells precoated with polyclonal antibody specific for human leptin and incubated at room temperature for 60 minutes. The plate was then washed three times with washing buffer and then horseradish peroxidase-conjugated anti-leptin antibody was added into each well for 60 minutes. After washing away excess reagents, the amount of conjugate bound to each well can be quantified spectrophotometrically by measuring the absorbance at 450 nm, with a reference at 630 nm.

Western Blotting

The stromal cells were homogenized under RIPA buffer and centrifuged at 600 $\times g$ for 30 minutes at 4°C to remove debris. Nuclear and cytoplasmic extracts were prepared according to the manufacturer's instructions. Total protein extracts were also prepared, and equal amount of protein was loaded into each lane, separated on a 8% SDS-polyacrylamide gel electrophoresis, and transferred onto a PVDF membrane. Western blotting was performed as described previously.^{3,25} Blots were probed overnight at 4°C with a 1:1000 dilution of mouse anti-human HIF-1 α or a 1:1000 dilution of goat anti-human HIF-1 β (BD Biosciences, San Jose, CA).

Plasmids, Transfection, and Promoter Activity Assays

The upstream region (–490/–1) of the human leptin promoter was cloned to pCDNA vector containing luciferase reporter system. Site-directed mutagenesis of putative HRE constructs was generated from the –490/–1 plasmid using PCR amplification approach. A commercial plasmid containing CMV-driven *Renilla* reporter system was purchased from Promega Corp. (Madison, WI). Cells were plated on 24-well plates for the luciferase/*Renilla* assays. Plasmids were transfected using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA). Transfections were followed by rinsing and incubation in serum-free DMEM/F12 medium for 12 hours. After the medium was changed, cells were then treated with a hypoxia condition for another 12 hours. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System according to the manufacturer's instruc-

tions (Promega Corp. Madison, WI). Briefly, 100 μ l of luciferase substrates were added to 20 μ l of lysate, and luciferase activity was measured using a 20/20 luminometer (Turner Designs, Sunnyvale, CA). Each luciferase assay experiment was performed in triplicate.

Chromatin Immunoprecipitation (ChIP) Assay

The protocol used was as described previously.^{26,27} In brief, proteins (HIF-1 α) and DNA in normoxia-, hypoxia-, or DFO-treated cells (1×10^6 cells) were cross-linked by incubation for 10 minutes at 37°C with a final concentration of 1% formaldehyde. Cells were washed twice with ice-cold PBS containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride and 1 μ g/ml each of aprotinin and pepstatin A) and scraped into a conical tube, centrifuged for 5 minutes at 500 $\times g$ at 4°C, resuspended in 400 μ l of lysis buffer (1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 8.1), and placed on ice for 10 minutes. Genomic DNA was sheared to lengths of 0.5 to 1 kb by sonicating the cell lysate. Cell debris was removed by centrifugation, and the supernatant was diluted with 1 ml of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-HCl, 16.7 mmol/L NaCl, and proteinase inhibitors, pH 8.0). Two percent of the diluted lysate was kept for input control. Anti-HIF-1 α antibody or mouse IgG (for negative control) was added to the supernatant fraction and the mixture incubated overnight at 4°C with rotation, and then with 60 μ l of salmon sperm DNA/Protein A agarose slurry for 1 hour at 4°C with rotation. The pellet was then washed sequentially (5 minutes per wash) on a rotating platform with washing buffers and 1 $\times$ TE buffer. After the final wash, the pellet was eluted by resuspension in freshly made elution buffer (1% SDS and 50 mmol/L NaHCO₃) followed by centrifugation. The DNA was released from protein-DNA complex and subjected to 35 cycles of PCR amplification using primers that amplify 191 bp of human leptin promoter (5'-TGGGAGGTACCCAAGG-3' and 5'-GGCCCGATCACAACTT-3').

Short Interference RNA (siRNA)

The sequences and procedure used for performing siRNA against human HIF-1 α was reported previously.²⁷ For negative control, two ribonucleotides with scrambled sequences 5'-r(AGUUAACGACCAGUAGUC)d(TT)-3' and 5'-r(GACUACUGGUCGUUGAACU)d(TT)-3' were also synthesized. The ribonucleotides were dissolved in the siRNA suspension buffer (Qiagen), heated to 90°C for 1 minute, and incubated at 37°C for 60 minutes. The annealed siRNA (5 μ g) was used to transfect cells according to the manufacturer's instructions (Qiagen). After transfection, cells were incubated with or without 10 mmol/L DFO for 16 hours and subjected to RNA isolation and RT-PCR analysis.

Statistical Analysis

The results were expressed as means $\pm$ SEM. Data were analyzed by one-way analysis of variance using Prism

Figure 1. Expression of HIF-1 α mRNA in stromal cells obtained from eutopic endometrium and ectopic endometriotic lesions. **A:** A representative RT-PCR gel picture shows HIF-1 α mRNA levels in stromal cells isolated from normal endometria and endometriotic tissues. GAPDH was used as an internal control; N1–N3, eutopic endometria from disease-free patients (normal control); E1–E3, ectopic endometriotic lesions; NC, negative control. **B:** Mean HIF-1 α to GAPDH mRNA ratios in stromal cells isolated from eutopic endometria in disease-free patients (Normal; $n = 9$) and ectopic endometria in patients with endometriosis (Endo; $n = 9$). **Asterisk** indicates significant difference between these two groups ($P < 0.05$ with Student's t -test analysis).

statistical software version 4.02 (GraphPad Software Inc., San Diego, CA), and multiple comparisons were followed by Tukey's test if significant differences were found. Alternatively, data were analyzed by unpaired Student's t -test if only two groups were compared. Significant differences were accepted when two-tailed analysis yielded $P < 0.05$.

Results

Expression of HIF-1 α in Eutopic Endometrium and Ectopic Endometriotic Lesions

To compare the expression of HIF-1 α in normal endometrium and endometriotic lesions, tissues were collected and processed immediately after surgery. Stromal cells purified from normal endometrium and endometriotic lesions were directly lysed and total RNA was isolated. RT-PCR results showed that basal concentration of mRNA encoding for HIF-1 α in stromal cells isolated from ectopic lesions of patients with endometriosis was greater than that in stromal cells derived from eutopic endometria of disease-free women (Figure 1A). Results from nine endometriosis-free patients and nine patients with endometriosis revealed that HIF-1 α mRNA was two- to threefold greater in stromal cells isolated from endometriotic tissues than in those from normal controls (Figure 1B). Because protein function would require the presence of HIF-1 α , we used Western blot analysis to evaluate the expression of HIF-1 α protein. Expression of HIF-1 α protein was elevated in endometriotic tissues compared with endometria collected from disease-free women (Figure 2A). To avoid interference due to patient heterogeneity, paired samples obtained from individuals with endometriosis were used for comparison. Significant elevation of HIF-1 α protein in ectopic endometriotic stro-

Figure 2. Expression of HIF-1 α protein in samples obtained from eutopic endometria and ectopic endometriotic lesions. **A:** A representative Western blot shows expression of HIF-1 α protein in nuclear extracts of stromal cells isolated from normal endometrium (N1 and N2) and ectopic endometriotic lesion (E1 and E2). **B:** A representative Western blot shows levels of HIF-1 α protein in nuclear extracts of stromal cells isolated from paired eutopic (Eu) and ectopic (Ec) endometria of two patients with endometriosis. **C:** Means of HIF-1 α to β -actin protein ratio in eutopic and ectopic stromal cells ($n = 5$) similar to that shown in **B**. **Asterisk** indicates significant difference at $P < 0.05$ with Student's t -test.

mal cells was observed compared with its eutopic counterpart (Figure 2, B and C).

Hypoxia Induces Leptin mRNA and Protein Expression in Endometrial Stromal Cells

To determine whether HIF-1 α is able to induce leptin expression in normal endometrial stromal cells, hypoxic stress (1% O₂) was applied to cells, and expression of leptin was evaluated. Accumulation of HIF-1 α was observed at 4 and 24 hours in endometrial stromal cells cultured under hypoxic condition (Figure 3A). Concomitant with the elevation of nuclear HIF-1 α , expression of leptin mRNA was induced at 24 hours after hypoxia treat-

Figure 3. Induction of leptin mRNA by true and chemically mimicked hypoxia. **A:** A representative Western blot shows expression of HIF-1 α and HIF-1 β protein in stromal cells cultured under normoxia (N) or hypoxia (H). N4, 4 hours normoxia; H4, 4 hours hypoxia; N24, 24 hours normoxia; H24, 24 hours hypoxia. This experiment was repeated four times with different batches of cells, and the results were similar. **B:** A representative RT-PCR gel picture shows expression of leptin mRNA in endometrial stromal cells cultured under normoxia or hypoxia for 24 hours. Norm, normoxia; Hypo, hypoxia. This experiment was repeated four times with different batches of cells, and the results were similar. **C:** A representative Western blot shows expression of HIF-1 α and HIF-1 β protein in stromal cells cultured under normoxia treated with or without 10 mmol/L DFO for indicated time. This experiment was repeated four times with different batches of cells and the results were similar. **D:** A representative RT-PCR gel picture shows expression of leptin (leptin) and 18S ribosomal RNA (18S) mRNA in normal endometrial stromal cells treated with or without DFO for indicated time. Means and standard errors from five independent experiments using different batches of cells were combined and shown in the bottom panel. Asterisks indicate significant differences between control (Con; without DFO treatment) and DFO-treated (DFO) groups at $P < 0.05$ using Student's *t*-test at each time point.

ment in normal endometrial stromal cells (Figure 3B). Likewise, treatment with DFO (10 mmol/L) to mimic hypoxia significantly increased nuclear HIF-1 α accumulation, which was evident by as early as 4 hours (Figure 3C). Expression of leptin mRNA was induced by DFO treatment at 16 hours after treatment, although not significantly, due to the large variation among subjects (Figure 3D). Significant increase in leptin mRNA was observed at 24 hours after DFO treatment, and the level was further increased at 48 hours after treatment (Figure 3D). Concordant with this result, treatment of ectopic endometriotic stromal cells with hypoxia (1% O₂) for 24 hours also resulted in elevation of leptin mRNA (data not shown). There was discordance in the timing between HIF-1 α and leptin gene expression. The elevation of HIF-1 α concentration by true hypoxia and DFO treatment precedes the increase in leptin mRNA expression (Figure 3D). This is understandable, since activation of a latent gene normally takes several steps. These include the binding of transcription factor to the target promoter, recruiting co-activators to form an initiation complex, causing chromatin remodeling, and the formation of transcriptional complex. Nevertheless, the expression of leptin mRNA following HIF-1 α accumulation indicates that HIF-1 α can induce leptin mRNA expression in eutopic and ectopic endometrial stromal cells.

We further used ELISA to evaluate the leptin protein expression under hypoxic stress (Figure 4). A significant increase in leptin protein expression was observed at 48 hours after hypoxia treatment in eutopic stromal cells (37.5 ± 5.3 pg/ml). It was not possible to compare the difference of leptin protein concentration between nor-

moxia and hypoxia in stromal cells derived from eutopic endometria because these cells did not express leptin under normoxic condition. In contrast, stromal cells derived from endometriotic lesions expressed high amounts of leptin (44.3 ± 11.8 pg/ml) even under normoxic condition. Likewise hypoxic treatment significantly increased leptin protein concentration in stromal cells obtained from ectopic endometriotic lesions (191.5 ± 31.4 pg/ml). These results indicate that hypoxia can induce leptin

Figure 4. Levels of leptin protein expression in cell-free supernatant of culture media measured by ELISA method in the paired eutopic and ectopic endometrial stromal cells from the same patients ($n = 3$). The leptin was undetectable in eutopic stromal cells under normoxia conditions. However, increased leptin expression was noted in both eutopic and ectopic stromal cells under hypoxic (1% O₂) conditions. Asterisk indicates significant difference ($P < 0.05$) within the ectopic endometriotic stromal cells between normoxia and hypoxia conditions using Student's *t*-test.

Figure 5. DFO-induced leptin promoter activity is mediated by proximal hypoxia response element (HRE). **A:** A schematic drawing of the 5'-regulatory region of human leptin promoter. Two putative HREs (HRE1 and HRE2) analyzed by bioinformatic methods were indicated in boxes with mutated sequences shown under the HRE. **Arrows** indicate the direction of HRE. **B:** Human endometrial stromal cells were transiently transfected with reporter plasmid containing empty vector (pGL3), human leptin promoter construct with wild-type HRE (a fragment corresponding to human leptin gene promoter -490/-1), or constructs with site-directed mutated HREs. Transfected cells were then treated with or without DFO (10 mmol/L) for 16 hours and luciferase activity was determined. WT, wild type; m, mutated; dm, double mutated; HRE, hypoxia response element; RLU, relative luciferase activity. **Asterisks** indicate significant differences ($P < 0.05$) in luciferase activity between normoxia and hypoxia under different transfected conditions using analysis of variance.

protein expression, both in eutopic and ectopic endometriotic stromal cells.

Transcriptional Regulation of Leptin Gene Expression by HIF-1 α

So far, we have shown that expression of leptin in eutopic and ectopic endometrial stromal cells is associated with elevation of nuclear HIF-1 α . To demonstrate that HIF-1 α up-regulates leptin gene expression directly through transcription, promoter activity was assayed, using the 5'-regulatory region of human leptin gene, in eutopic endometrial stromal cells. Sequence analysis of the human leptin promoter using a bioinformatic method revealed two putative HRE motifs, including HRE1 (-201~-197) and HRE2 (-120~-116), which are present within the 500 bp upstream of transcription start site (Figure 5A). Thus, a fragment corresponding to human leptin gene promoter (-490/-1) was cloned into the pGL3 basic plasmid reporter system and designed as the wild-type construct. Site-directed mutagenesis was performed to mutate either one or both HREs in the human leptin promoter construct (Figure 5A). When the

Figure 6. HIF-1 α binds to human leptin promoter and regulates its activity. **A:** A representative PCR gel from eutopic stromal cells using the ChIP assay demonstrates a single band amplified by PCR using HIF-1 α antibody precipitated genomic DNA obtained from DFO-treated cells as template ($n = 5$). C, control (without DFO treatment). D, DFO-treated group. Ig, normal rabbit IgG. This experiment was repeated four times using different batches of cells and the results were identical. **B:** A representative gel picture shows transfection of siRNA targeted at HIF-1 α (SiHIF-1) abolished DFO-induced leptin mRNA expression in ectopic endometriotic stromal cells. The sequence-scrambled siRNA (SrHIF-1) was not effective. This experiment was repeated three times using different batches of cells, and the results were similar.

expression of luciferase activity was compared in wild-type constructs, DFO treatment significantly up-regulated human leptin gene promoter activity by 2.5-fold. Mutation of distal HRE (HRE1) of the leptin promoter only partially attenuated the promoter activity induced by DFO (Figure 5B). In contrast, induction of the leptin promoter activity by DFO was abolished by mutating proximal HRE (HRE2) or HRE1 and HRE2 doubled mutated constructs. These results demonstrate that HIF-1 α directly up-regulates human leptin gene expression in eutopic endometrial stromal cells and that HRE2 is most important for HIF-1-regulated leptin gene activity.

Expression of Leptin in Endometriotic Stromal Cells Is HIF-1 α -Dependent

To verify that HIF-1 α indeed regulates leptin gene transcription in endometrial stromal cells under hypoxia, chromatin immunoprecipitation assay was performed to detect the physical binding of HIF-1 α to the leptin promoter *in vivo*. Results demonstrated that binding of HIF-1 α to the leptin promoter was evident after DFO

treatment (Figure 6A). The binding was specific, because the use of rabbit IgG to substitute for the primary antibody failed to precipitate any DNA/protein complex (Figure 6A).

Finally, we aimed to test whether aberrant expression of leptin in ectopic endometriotic stromal cells resulted from increased expression of HIF-1 α . Ectopic endometriotic stromal cells were transiently transfected with siRNA targeted at HIF-1 α (siHIF-1 α) or siRNA using scrambled HIF-1 α sequence (srHIF-1 α). Transfection with siHIF-1 α inhibited DFO-induced leptin expression, whereas transfection with scrambled HIF-1 α siRNA had no such effect (Figure 6B). These results demonstrate that HIF-1 α plays a pivotal role in controlling leptin expression in ectopic endometriotic stromal cells.

Discussion

Leptin was originally identified as an adipose tissue-derived hormone that is also expressed in a variety of peripheral tissues with different physiological effects.²⁸ We have previously shown that leptin gene expression is increased in ectopic endometriotic tissues.³ Aberrant expression of leptin in human endometriotic tissue leads to an increase in stromal cell proliferation, which may be linked to the pathogenesis of endometriosis. Concordantly, it was reported that leptin levels in peritoneal fluid were positively correlated with the stage of endometriosis and with endometriosis-associated pain.⁸ These findings suggest that leptin may exert a local autocrine effect in the ectopic endometrium. However, the underlying mechanism leading to aberrant expression of leptin is not clear. In the current report, we found that aberrant leptin gene expression in ectopic endometriotic cells may result from prolonged exposure to hypoxic stress in the peritoneal cavity through HIF-1-dependent transactivation of leptin promoter. Our findings provide a molecular mechanism to explain, at least in part, aberrant expression of leptin in ectopic endometriotic lesions.

HIF-1 α is a master transcription factor that mediates hypoxic effects such as angiogenesis, erythropoiesis, glucose transport, glycolysis, iron transport, cell survival, and proliferation.²⁹ The discovery of elevated HIF-1 α mRNA and protein in ectopic endometriotic tissues provides new clues to understanding mechanisms that might contribute to the etiology of endometriosis. To our knowledge, this is the first report to examine the expression of HIF-1 α in normal and endometriotic tissues. In addition, we used paired samples collected from the same individual to eliminate the potential influence of different genetic backgrounds. The level of HIF-1 α is regulated at the protein level by polyubiquitination-mediated degradation. In addition, it has been shown that the level of HIF-1 α mRNA is elevated by hormonal or other stimulation.^{30–33} For example, norepinephrine and thyroid hormone can induce the expression of HIF-1 α mRNA.^{32,33} Activation of nuclear factor- κ B by the proinflammatory cytokine IL-1 β also leads to up-regulation of HIF-1 α mRNA in normal human cytotrophoblast cells.^{31,34} In this study, we demonstrated that HIF-1 α mRNA and protein levels were both

elevated in ectopic endometriotic tissue compared with its eutopic counterpart. This result suggests that elevated concentrations of HIF-1 α in ectopic endometrial tissues, potentially regulated at the post-translational level, may also be due to consistent stimulation by proinflammatory cytokines such as IL-1 β and/or prostaglandins, which have been shown to be elevated in the peritoneal fluid of women with endometriosis.³⁵ Increased HIF-1 α expression provides greater availability of this transcription factor, which in turn may regulate many hypoxic responsive genes necessary for the survival and/or maintenance of endometriotic cells.

Aberrant expression of leptin in ectopic endometriotic lesions contributes significantly to the pathological process of endometriosis including stimulation of stromal cell proliferation and dysmenorrhea.^{3,8} By identifying the elevation of HIF-1 α in ectopic endometriotic tissues, we hypothesized that elevated levels of HIF-1 α in these lesions may play a critical role in regulating leptin gene expression. To test this hypothesis, we tested whether hypoxia can induce leptin expression in normal endometrial stromal cells that usually do not express leptin. Treatment with DFO or culturing normal endometrial stromal cells under hypoxic condition (1% O₂) was sufficient to induce the expression of leptin in these cells, thus providing evidence for the importance of HIF-1 α in regulating leptin gene expression. Further studies using a promoter activity assay demonstrated that HIF-1 α induced leptin expression at the transcriptional level by binding to the proximal HRE of the leptin promoter. Based on bioinformatic analysis, there are two putative HRE motifs in the upstream promoter region of the leptin gene. The proximal HRE-2 seems to play a more important role in response to hypoxia compared with the distal HRE-1 motif, using a site-directed mutagenesis method. Our results are consistent with previous report by Grosfeld et al,²⁰ who demonstrated that the consensus HRE site located at –116 in the proximal promoter is more responsive to hypoxia than the one located between –171 and –213. Further investigation is needed to clarify the different functions of these two HREs.

Although our data support the notion that elevation of leptin gene expression in ectopic endometriotic stroma is regulated by HIF-1 α , it is not clear whether this is due to a direct or indirect pathway. To establish that hypoxia-induced leptin gene expression is directly regulated by the transcription factor HIF-1 α , ChIP assay was performed to demonstrate the physical binding of HIF-1 α on leptin gene promoter. Our data provide strong evidence to support that hypoxia-induced leptin gene expression is an effect mediated directly by HIF-1 α rather than indirectly via other transcription factors such as nuclear factor- κ B. Furthermore, we used siRNA to deplete endogenous HIF-1 α , as previously reported,²⁷ and evaluated its effect on leptin mRNA expression. Our results demonstrated that expression of leptin in ectopic endometriotic stromal cells was inhibited by knocking down HIF-1 α . These results provided the first evidence that aberrant expression of leptin in endometriotic lesion might be under the regulation of hypoxia-mediated HIF-1 α . Our results are consistent with previous reports demonstrating

that hypoxia can induce leptin synthesis via a HIF-1-dependent mechanism in other cell types.^{9,20,36} Taken together, these results and our finding that HIF-1 α is elevated in ectopic endometriotic tissue provide direct evidence that the aberrant increase in leptin expression in endometriotic stromal cells is due to HIF-1-induced leptin gene expression.

By identifying the elevation of HIF-1 α in ectopic endometriotic lesions, we have opened a window for further investigation of previously unidentified mechanisms responsible for pathophysiological processes of endometriosis. For example, our results implicate a possible mechanism for survival of retrograded endometrial tissue before blood supply is available and for angiogenesis in implanted ectopic endometriotic lesions. Given that HIF-1 α is the master transcription factor that responds to oxygen deprivation and other physiological and pathological stimuli, it is reasonable to presume that many previously identified or as yet unidentified genes that play critical roles in the development/maintenance of endometriosis might be controlled by HIF-1 α . Furthermore, hypoxia due to lack of blood supply in shedding endometrium would putatively induce leptin gene expression, thereby enabling survival of endometriotic cells under hypoxic condition and/or providing an adaptation response for early implantation and development of ectopic endometriotic implants. Finally, the elevation of HIF-1 α in implanted ectopic tissues, probably induced by continuous stimulation of proinflammatory cytokines, would up-regulate leptin expression, thus providing an autocrine mitogen for the proliferation of ectopic tissues. Obviously, further studies are warranted to investigate the functional roles of hypoxia and HIF-1 α in the etiology of endometriosis. Our findings provide a novel molecular framework to consider for designing future therapeutic regimens against this disease by targeting HIF-1 α -regulated genes.

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References

- Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994, 372:425–432
- Kitawaki J, Kusuki I, Koshiba H, Tsukamoto K, Honjo H: Leptin directly stimulates aromatase activity in human luteinized granulosa cells. *Mol Hum Reprod* 1999, 5:708–713
- Wu MH, Chuang PC, Chen SM, Lin CC, Tsai SJ: Increased leptin expression in endometriosis cells is associated with endometrial stromal cell proliferation and leptin gene-upregulation. *Mol Hum Reprod* 2002, 8:456–464
- Matkovic V, Ilich JZ, Skugor M, Badenhop NE, Goel P, Clairmont A, Klisovic D, Nahhas RW, Landoll JD: Leptin is inversely related to age at menarche in human females. *J Clin Endocrinol Metab* 1997, 82:3239–3245
- Sierra-Honigsmann MR, Nath AK, Murakami C, Garcia-Cardena G, Papapetropoulos A, Sessa WC, Madge LA, Schechner JS, Schwabb MB, Polverini PJ, Flores-Riveros JR: Biological action of leptin as an angiogenic factor. *Science* 1998, 281:1683–1686
- Matarese G, Alvisi C, Sanna V, Howard JK, Lord GM, Carravetta C, Fontana S, Lechler RI, Bloom SR, De Placido G: Increased leptin levels in serum and peritoneal fluid of patients with pelvic endometriosis. *J Clin Endocrinol Metab* 2000, 85:2483–2487
- Mataliotakis IM, Koumantaki YG, Neonaki MA, Goumenou AG, Koumantakis GE, Kyriakou DS, Koumantakis EE: Increase in serum leptin concentrations among women with endometriosis during danazol and leuprolide depot treatments. *Am J Obstet Gynecol* 2000, 183:58–62
- Bedaiwy MA, Falcone T, Goldberg JM, Sharma RK, Nelson DR, Agarwal A: Peritoneal fluid leptin is associated with chronic pelvic pain but not infertility in endometriosis patients. *Hum Reprod* 2006, 21:788–791
- Ambrosini G, Nath AK, Sierra-Honigsmann MR, Flores-Riveros J: Transcriptional activation of the human leptin gene in response to hypoxia: involvement of hypoxia-inducible factor 1. *J Biol Chem* 2002, 277:34601–34609
- Mise H, Sagawa N, Matsumoto T, Yura S, Nanno H, Itoh H, Mori T, Masuzaki H, Hosoda K, Ogawa Y, Nakao K: Augmented placental production of leptin in preeclampsia: possible involvement of placental hypoxia. *J Clin Endocrinol Metab* 1998, 83:3225–3229
- Grosfeld A, Turban S, Andre J, Cauzac M, Chailier JC, Hauguel-de Mouzon S, Guerre-Millo M: Transcriptional effect of hypoxia on placental leptin. *FEBS Lett* 2001, 502:122–126
- Carmeliet P, Jain RK: Angiogenesis in cancer and other diseases. *Nature* 2000, 407:249–257
- Semenza GL: HIF-1 and human disease: one highly involved factor. *Genes Dev* 2000, 14:1983–1991
- Wang GL, Jiang BH, Rue EA, Semenza GL: Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci USA* 1995, 92:5510–5514
- Sutter CH, Laughner E, Semenza GL: Hypoxia-inducible factor 1 α protein expression is controlled by oxygen-regulated ubiquitination that is disrupted by deletions and missense mutations. *Proc Natl Acad Sci USA* 2000, 97:4748–4753
- von Wolff M, Thaler CJ, Strowitzki T, Broome J, Stolz W, Tabibzadeh S: Regulated expression of cytokines in human endometrium throughout the menstrual cycle: dysregulation in habitual abortion. *Mol Hum Reprod* 2000, 6:627–634
- Popovici RM, Irwin JC, Giaccia AJ, Giudice LC: Hypoxia and cAMP stimulate vascular endothelial growth factor (VEGF) in human endometrial stromal cells: potential relevance to menstruation and endometrial regeneration. *J Clin Endocrinol Metab* 1999, 84:2245–2248
- Critchley HO, Osei J, Henderson TA, Boswell L, Sales KJ, Jabbour HN, Hirani N: Hypoxia-inducible factor-1 α expression in human endometrium and its regulation by prostaglandin E-series prostanoid receptor 2 (EP2). *Endocrinology* 2006, 147:744–753
- Bulun SE, Zeitoun KM, Kilic G: Expression of dioxin-related transactivating factors and target genes in human eutopic endometrial and endometriotic tissues. *Am J Obstet Gynecol* 2000, 182:767–775
- Grosfeld A, Andre J, Hauguel-De Mouzon S, Berra E, Pouyssegur J, Guerre-Millo M: Hypoxia-inducible factor 1 transactivates the human leptin gene promoter. *J Biol Chem* 2002, 277:42953–42957
- von Wolff M, Stieger S, Lump K, Bucking J, Strowitzki T, Thaler CJ: Endometrial interleukin-6 in vitro is not regulated directly by female steroid hormones, but by pro-inflammatory cytokines and hypoxia. *Mol Hum Reprod* 2002, 8:1096–1102
- Tsai SJ, Wu MH, Lin CC, Sun HS, Chen SM: Regulation of steroidogenic acute regulatory protein expression and progesterone production in endometriotic stromal cells. *J Clin Endocrinol Metab* 2001, 86:5765–5773
- Wing LYC, Chuang PC, Wu MH, Chen HM, Tsai SJ: Expression and mitogenic effect of fibroblast growth factor-9 in human endometriotic implant is regulated by aberrant production of estrogen. *J Clin Endocrinol Metab* 2003, 88:5547–5554
- Tsai SJ, Wu MH, Chuang PC, Chen HM: Distinct regulation of gene expression by prostaglandin F_{2 α} (PGF_{2 α}) is associated with PGF_{2 α} resistance or susceptibility in human granulosa-luteal cells. *Mol Hum Reprod* 2001, 7:415–423
- Wu MH, Wang CA, Lin CC, Chen LC, Chang WC, Tsai SJ: Distinct regulation of cyclooxygenase-2 by interleukin-1 β in normal and endometriotic stromal cells. *J Clin Endocrinol Metab* 2005, 90:286–295
- Sun HS, Hsiao KY, Hsu CC, Wu MH, Tsai SJ: Transactivation of steroidogenic acute regulatory protein in human endometriotic stromal cells is mediated by the prostaglandin EP2 receptor. *Endocrinology* 2003, 144:3934–3942

27. Chen KF, Lai YY, Sun HS, Tsai SJ: Transcriptional repression of human cad gene by hypoxia inducible factor-1alpha. *Nucleic Acids Res* 2005, 33:5190–5198
28. Ahima RS, Flier JS: Leptin. *Annu Rev Physiol* 2000, 62:413–437
29. Semenza G: Signal transduction to hypoxia-inducible factor 1. *Biochem Pharmacol* 2002, 64:993–998
30. Wiener CM, Booth G, Semenza GL: In vivo expression of mRNAs encoding hypoxia-inducible factor 1. *Biochem Biophys Res Commun* 1996, 225:485–488
31. Jung YJ, Isaacs JS, Lee S, Trepel J, Neckers L: IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. *FASEB J* 2003, 17:2115–2117
32. Nikami H, Nedergaard J, Fredriksson JM: Norepinephrine but not hypoxia stimulates HIF-1alpha gene expression in brown adipocytes. *Biochem Biophys Res Commun* 2005, 337:121–126
33. Hawkins DE, Niswender KD, Oss GM, Moeller CL, Odde KG, Sawyer HR, Niswender GD: An increase in serum lipids increases luteal lipid content and alters the disappearance rate of progesterone in cows. *J Anim Sci* 1995, 73:541–545
34. Qian D, Lin HY, Wang HM, Zhang X, Liu DL, Li QL, Zhu C: Normoxic induction of the hypoxic-inducible factor-1 alpha by interleukin-1 beta involves the extracellular signal-regulated kinase 1/2 pathway in normal human cytotrophoblast cells. *Biol Reprod* 2004, 70:1822–1827
35. Gazvani R, Templeton A: Peritoneal environment, cytokines and angiogenesis in the pathophysiology of endometriosis. *Reproduction* 2002, 123:217–226
36. Grosfeld A, Zilberfarb V, Turban S, Andre J, Guerre-Millo M, Issad T: Hypoxia increases leptin expression in human PAZ6 adipose cells. *Diabetologia* 2002, 45:527–530