ORIGINAL ARTICLE

Mechanism of leptin expression in breast cancer cells: role of hypoxia-inducible factor-1α

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We reported previously that the obesity hormone leptin is overexpressed in breast cancer biopsies. Here, we investigated molecular mechanisms involved in this process, focusing on conditions that are associated with obesity, that is, hyperinsulinemia and induction of hypoxia. By using quantitative real-time PCR, immunofluorescent detection of proteins and enzyme-linked immunosorbent assays, we found that treatment of MCF-7 breast cancer cells with high doses of insulin or the hypoxia-mimetic agent $CoCl_2$, or culturing the cells under hypoxic conditions significantly increased the expression of leptin mRNA and protein. Notably, the greatest leptin mRNA and protein expression were observed under combined hyperinsulinemia and hypoxia or hypoxia-mimetic treatments. Luciferase reporter assays suggested that increased leptin synthesis could be related to the activation of the leptin gene promoter. DNA affinity precipitation and chromatin immunoprecipitation experiments revealed that insulin, $CoCl_2$ and/or hypoxia treatments augmented nuclear accumulation of hypoxia-inducible factor-1α (HIF-1α) and increased its interaction with several upstream leptin regulatory sequences, especially with the proximal promoter containing four hypoxia-response elements and three GC-rich regions. By using reverse chromatin precipitation, we determined that loading of HIF-1α on the proximal leptin promoter concurred with the recruitment of p300, the major HIF coactivator, suggesting that the HIF/p300 complex is involved in leptin transcription. The importance of HIF-1α in insulin- and $CoCl_2$-activated leptin mRNA and protein expression was confirmed using RNA interference.
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Introduction

Excess body weight has been shown to (by 30–50%) increase significantly postmenopausal breast cancer risk (Klein *et al.*, 2002; Calle and Thun, 2004). A very recent report suggested that compared with non-obese breast cancer patients, obese subjects more frequently presented with advanced disease characterized by large, high grade, metastasizing tumors (Porter *et al.*, 2006). Although the molecular mechanism of obesity-related mammary carcinogenesis is not clear, significant role has been suggested for cytokines produced by adipose tissue, for example, leptin.

Leptin is a pleiotropic hormone whose major function is to regulate food intake and energy balance by interacting with satiety centers in the brain (Wauters *et al.*, 2000). Leptin also affects many peripheral organs, behaving as a mitogen, survival factor, metabolic regulator or angiogenic factor (Wauters *et al.*, 2000). Additionally, leptin appears to promote neoplastic processes, including breast carcinogenesis (Garofalo and Surmacz, 2006; Surmacz, 2007). In breast cancer cellular models, leptin has been shown to stimulate cell growth, survival and transformation and interfere with the action of antiestrogens (Garofalo and Surmacz, 2006). In addition, leptin can modulate major pathways involved in breast cancer progression. For instance, binding of leptin to its receptor (ObR) has been shown to transactivate the Her2/neu oncogenic receptor (Eisenberg *et al.*, 2004). Furthermore, leptin is able to induce the expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR2 in mammary tumors (Gonzalez *et al.*, 2006). In agreement with this finding, ObR peptide antagonists can inhibit mammary tumor growth (Gonzalez *et al.*, 2006).

Recently, others and we demonstrated that both leptin and ObR can be found in breast cancer biopsies, which suggests that leptin might affect breast cancer cells through autocrine and/or paracrine mechanisms. Importantly, the leptin system is overexpressed in breast cancer tissues, especially in high grade (G3) tumors, while it is absent or expressed at very low levels in normal mammary epithelium or benign tumors (Ishikawa *et al.*, 2004; Garofalo *et al.*, 2006). We also demonstrated that overexpression of leptin and ObR

mRNA in breast cancer could be induced by obesity-related stimuli, such as high concentrations of insulin or estrogen (Garofalo *et al.*, 2006). Overexpression of leptin and ObR on the protein and mRNA levels in breast cancer vs normal epithelium has been confirmed by other investigators (Ishikawa *et al.*, 2004; Revillion *et al.*, 2006).

The mechanism of leptin induction in breast cancer cells is unknown. Consequently, we investigated whether insulin and hypoxia-mimetic agents might regulate the expression of the leptin gene in this cell context. The human leptin gene promoter is ∼3000 long and contains several regulatory motifs, including AP2, SP-1, CREB, C/EBP, GRE, CRE elements (Mason *et al.*, 1998; Ahima and Flier, 2000; Melzner *et al.*, 2002; Meissner *et al.*, 2003). Most importantly, the promoter contains eight hypoxia-responsive elements (HRE) with the minimal core sequence 5′-RCGTG-3′ that can recruit hypoxia-inducible factor (HIF) (Grosfeld *et al.*, 2002; Meissner *et al.*, 2003). HIF is a master transcriptional factor in nutrient stress signaling and can induce an array of genes involved in energy metabolism, neovascularization, survival, cell migration and pH balance. In addition, HIF can promote metastatic processes by activating tumor neoangiogenesis, and increasing cell motility and invasion (Zhong *et al.*, 1999; Hirota and Semenza, 2006; Pouyssegur *et al.*, 2006).

HIF is a heterodimer of a constitutively expressed HIF-1β subunit and an oxygen-regulated, unstable HIF-1α subunit. HIF activation involves HIF-1α stabilization, its nuclear translocation, heterodimerization and interaction with other transcriptional regulators, of which the most important is p300, a histone acetyl transfersase (Ebert and Bunn, 1998; Gray *et al.*, 2005). HIF-1α expression is significantly increased under hypoxic conditions, but it can also occur in normoxia upon activation of the mTOR pathway (Pouyssegur *et al.*, 2006). We investigated whether in breast cancer cells, insulin, a potent inducer of mTOR pathway and hypoxia-mimetic agents can stimulate leptin expression through HIF-1α.

Results

Insulin, CoCl₂ and hypoxia enhance nuclear accumulation of HIF-1α in MCF-7 breast cancer cells

Previously, we reported that leptin is overexpressed in breast cancer and that this overexpression might be caused by obesity-related stimuli, such as hyperinsulinemia or hypoxia-mimetic agents (Garofalo *et al.*, 2006). Because the leptin promoter contains multiple sites that can recruit HIF, we hypothesized that increased leptin expression might be related to accumulation of nuclear HIF-1α, resulting in increased HIF loading on leptin regulatory sequences. Thus, we assessed if insulin alone or in combination with either hypoxia-mimetic agent CoCl₂ or physiological hypoxia could enhance nuclear HIF-1α expression in MCF-7 cells (Figure 1).

The levels of nuclear HIF-1α were significantly increased by insulin in a dose-dependent manner, with
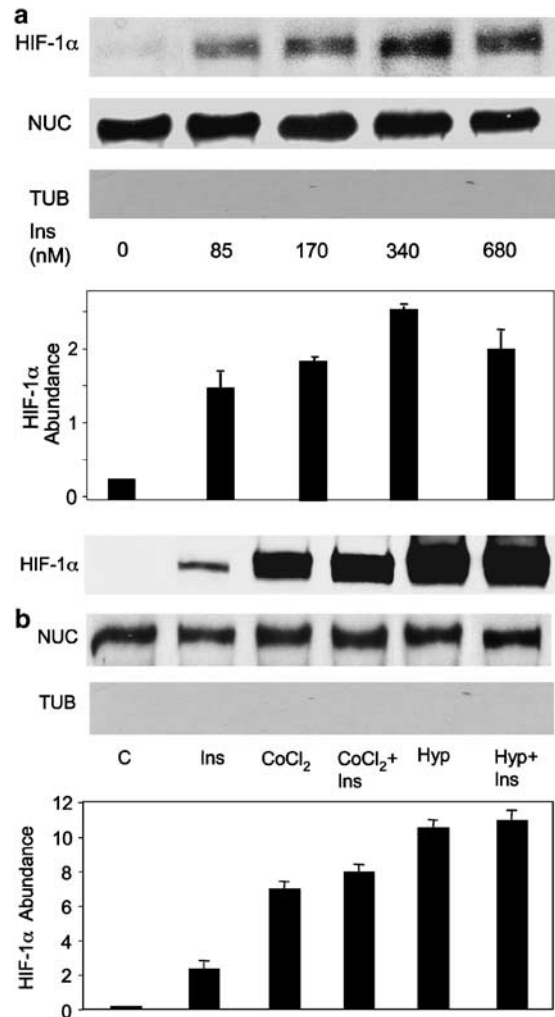
Figure 1 Insulin, CoCl₂ and hypoxia increase nuclear hypoxia-inducible factor (HIF)-1α levels. HIF-1α expression was studied by WB, as described in 'Materials and methods'. (**a**) HIF-1α expression in 100 μg of nuclear proteins obtained from cells treated for 4 h with 0–680 nM doses of insulin (Ins); (**b**) HIF-1α expression in 50 μg of nuclear proteins obtained from cells untreated (C), or treated for 4 h with 340 nM insulin (Ins), and/or CoCl₂ and/or hypoxia (Hyp). (**a** and **b**) Protein loading and purity of fractions were controlled by re-probing WB filters for the expression of a cytoplasmic protein β-tubulin (TUB) and a nuclear marker nucleolin (NUC). Relative HIF-1α expression normalized to NUC is presented in graphs; bars represent s.e.

the maximum effects (∼2.5-fold increase over control) produced by the 340 nM dose (Figure 1a). CoCl₂ and hypoxia treatments stimulated nuclear HIF-1α accumulation by ∼7- and ∼10-fold, respectively. The addition of insulin at 85–680 nM doses did not enhance the effects of CoCl₂ or hypoxia (Figure 1b and data not shown).
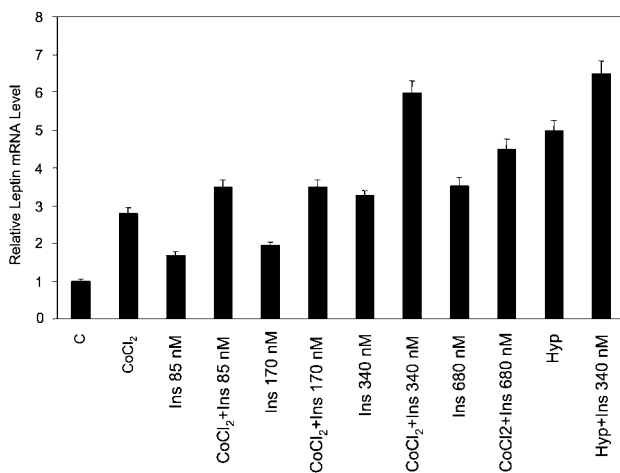
Insulin, CoCl₂ and hypoxia stimulate leptin mRNA expression

Next, we used quantitative real-time–PCR (QRT–PCR) to measure leptin mRNA expression under different treatments in MCF-7 cells (Figure 2). Relative to basal levels, addition of CoCl₂ increased leptin mRNA by

~1.8. Culturing the cells under hypoxia for 4 and 16 h stimulated leptin mRNA by ~2.1- and ~3.9-fold, respectively. Insulin alone was most effective in increasing leptin mRNA at 340 and 680 nM doses. Remarkably, the highest (over sixfold) leptin mRNA expression was observed under combined insulin plus $CoCl_2$ or insulin plus hypoxia conditions (Figure 2).

Insulin, $CoCl_2$ and hypoxia induce leptin protein expression

The expression of intracellular leptin protein was studied using immunofluorescence and deconvoluted microscopy, while the abundance of secreted leptin was
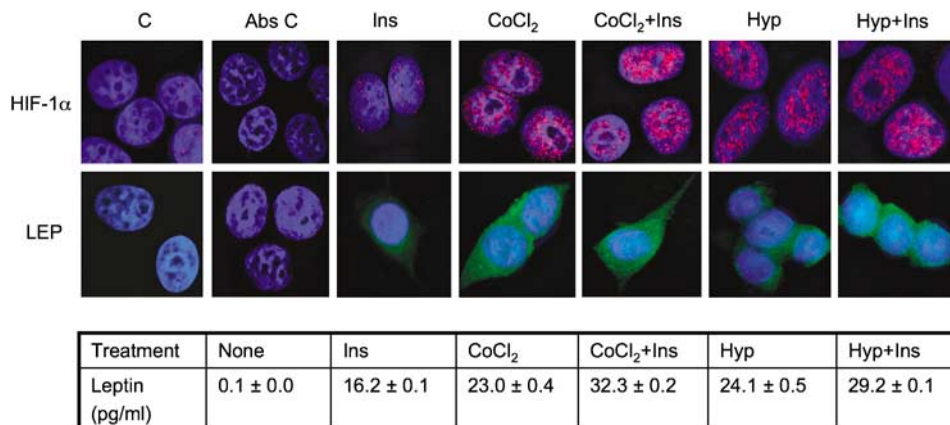

Figure 2 Insulin, $CoCl_2$ and hypoxia increase leptin mRNA expression. The abundance of leptin mRNA was studied with QRT–PCR in MCF-7 cells untreated (C) or treated for 4 h with different doses of insulin (Ins), and/or 100 μM $CoCl_2$ and/or hypoxia (Hyp), as described in 'Materials and methods'. The graph represents the increase of leptin mRNA relative to the increase of α-actin mRNA in the same sample ±s.e.

measured by enzyme-linked immunosorbent assay (ELISA; Figure 3). Cellular leptin staining was detectable in $15\pm1\%$ of cells treated with 340 nM insulin, $55\pm10\%$ of cells under $CoCl_2$ treatment and $67\pm4\%$ of cells under hypoxia. The combined insulin plus $CoCl_2$ or hypoxia treatments resulted in 55 ± 6 and $61\pm0\%$ cells positive for leptin staining, respectively (Figure 3). Parallel experiments revealed low HIF-1α expression in $40\pm4\%$ of cells stimulated with insulin, and abundant HIF-1α levels in 62 ± 4 and $80\pm7\%$ of cells treated with $CoCl_2$ and combined treatment, respectively (Figure 3). Under hypoxia and hypoxia plus insulin, high HIF-1α levels were detected in 80 ± 5 and $78\pm7\%$, respectively (Figure 3).

Next, we tested the effects of various treatments on bioactive leptin secretion. Insulin alone was most effective at 340 nM dose, stimulating synthesis of 16.2 ± 0.2 pg/ml leptin, while insulin treatment at 170 nM induced 14.2 ± 1.0 pg/ml leptin and 680 and 1000 nM induced 12.0 ± 0.5 and 5.0 ± 0.1 pg/ml leptin, respectively. The greatest leptin synthesis was seen under combined stimuli (340 nM insulin plus $CoCl_2$ or plus hypoxia) (Figure 3).

Leptin promoter is activated by insulin and hypoxia-mimetic treatment in breast cancer cells

To address the molecular mechanism by which insulin or hypoxia might activate leptin expression, we used the reporter plasmid, pGL3-OB1, containing the luciferase gene under the control of leptin regulatory sequences (−2924 to +31). Insulin and $CoCl_2$ as well as the combination of both stimuli significantly induced luciferase activity in MCF-7 cells transfected with pGL3-OB1 relative to cells transfected with an empty vector pGL3 (Figure 4). Specifically, insulin alone increased luciferase activity by 40%, $CoCl_2$ by 69% and the combination of both by 111%.

Treatment	None	Ins	$CoCl_2$	$CoCl_2$+Ins	Hyp	Hyp+Ins
Leptin (pg/ml)	0.1 ± 0.0	16.2 ± 0.1	23.0 ± 0.4	32.3 ± 0.2	24.1 ± 0.5	29.2 ± 0.1

Figure 3 Insulin, $CoCl_2$ and hypoxia increase hypoxia-inducible factor (HIF)-1α and leptin protein expression. MCF-7 cells were treated for 16 h with 340 nM insulin (Ins), and/or $CoCl_2$ and/or hypoxia (Hyp), as described in 'Materials and methods'. The expression of HIF-1α (red fluorescence) and leptin (LEP, green fluorescence) was assessed by immunostaining with specific antibodies (Abs) and deconvoluted microscopy, as detailed in 'Materials and methods'. Cell nuclei were identified with DAPI (blue fluorescence). In control experiments (Abs C), primary Abs were omitted. In parallel, the abundance of secreted leptin (pg/ml) in conditioned medium of untreated and treated cells was determined by ELISA, as described in 'Materials and methods'.

HIF-1α binds multiple HRE motifs in the leptin promoter in vitro

To address molecular mechanisms of leptin promoter activation in breast cancer cells, we assessed whether increased nuclear accumulation of HIF-1α correlates with elevated HIF-1α binding to HRE motifs. We selected three HRE-containing regions (HRE-1, proximal to ATG, HRE-2, in the middle of the leptin promoter and HRE-3, most distant from ATG). By using DNA affinity precipitation assay (DAPA), we found that $CoCl_2$ and the combined $CoCl_2$ plus insulin treatment increased HIF-1α binding to all three HREs

Figure 4 Insulin and $CoCl_2$ stimulate leptin promoter activity in breast cancer cells. Leptin promoter activation was tested using luciferase assays, as described in 'Materials and methods'. The cells transfected with the control plasmid (pGL3) or the leptin promoter-luciferase plasmid (OB1) were treated with 340 nM insulin (Ins), 100 μM CoCl2 or left untreated (C). Luciferase activity was calculated as relative fold induction vs untreated cells. Bars represent s.e., asterisks (*) indicate statistically significant differences between pGL3 and OB1 transfections.

(Figure 5). The greatest HIF-1α binding was seen with HRE-1 and HRE-2, and the least with HRE-3. Insulin stimulated significant HIF-1α interaction with HRE-1, but not with HRE-2 or HRE-3. Combined insulin plus $CoCl_2$ treatments produced results statistically similar to that obtained with $CoCl_2$ alone (Figure 5).
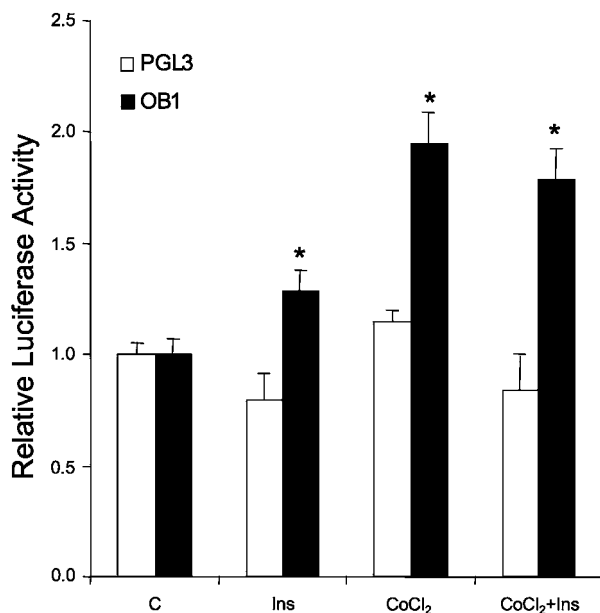
$CoCl_2$ and insulin stimulate association of the HIF-1α/p300 complex with the leptin promoter in vivo

Next, we tested using chromatin immunoprecipitation (ChIP) assays if $CoCl_2$ and insulin stimulate HIF-1α interaction with the leptin promoter in vivo. All treatments significantly increased HIF-1α binding to the proximal leptin promoter region HRE-A. This region contains four HRE sites, including described above HRE-1 and HRE-2 as well as multiple GC-rich sequences that have been shown to improve HIF activity (Miki et al., 2004) (Figure 6). The greatest HIF-1α binding to HRE-A was detected under insulin plus $CoCl_2$ treatments, while the lowest under insulin alone. By using reverse ChIP (re-ChIP), we demonstrated that in all cases, the HIF-1 complex interacting with HRE-A contained the major HIF coactivator p300 (Figure 6).
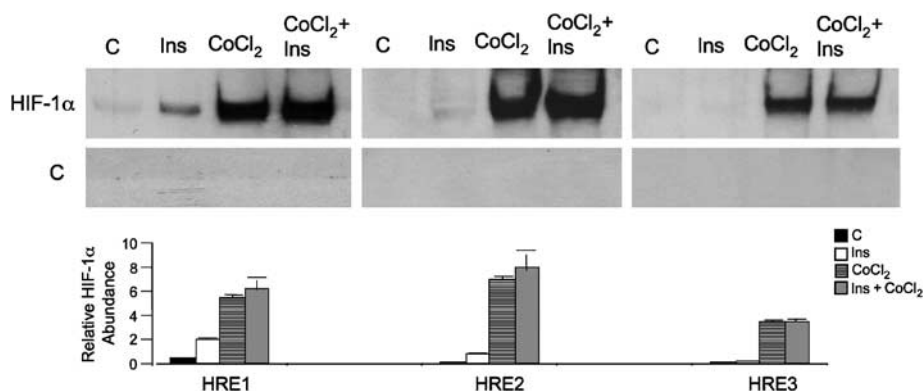
The HIF-1α/p300 complex was also detected on the distal HRE-B region that contains two HRE sites, but in this case the amounts of HIF-1α under all treatments were significantly lower than that seen with HRE-A. Furthermore, no synergistic effects of $CoCl_2$ and insulin were noted (Figure 6). Neither HIF-1α nor p300 was detected on the GAPDH promoter (which does not contain HREs) under treatments used (data not shown).

$CoCl_2$- and insulin-induced expression of leptin in breast cancer cells requires HIF-1α

The requirement for HIF-1α in the activation of leptin expression in breast cancer cells was studied using RNA interference. We found that ~90% HIF-1α knockdown was paralleled by ~70% inhibition of leptin mRNA synthesis (data not shown) and similar inhibition of cellular leptin expression under all treatment conditions (Figure 7).
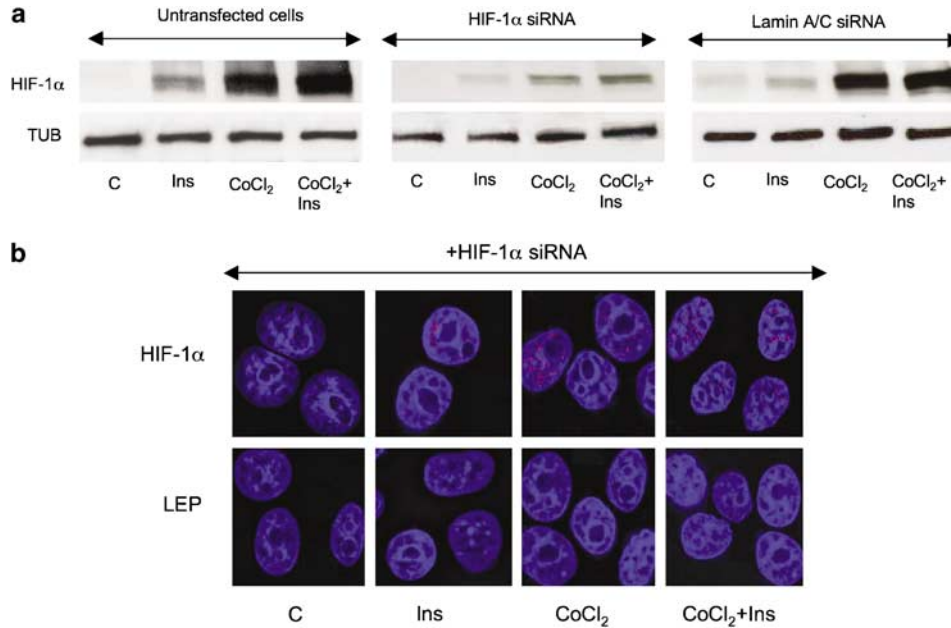

Figure 5 Hypoxia-inducible factor (HIF)-1α binds several hypoxia-responsive elements (HRE) motifs in the leptin promoter. The binding of HIF-1α to three different leptin promoter regions was assessed in vitro by DAPA, as described in 'Materials and methods'. Each studied region contains one HIF motif, specifically, (−120) ACGTC in HRE-1; (−627) GCGTG in HRE-2 and (−1501) ACGTG in HRE-3. In control experiments (C), HIF-1α-HRE interactions were tested in the presence of excess unlabeled probes. The graphs represent HIF abundance on HREs under different conditions ± s.e.
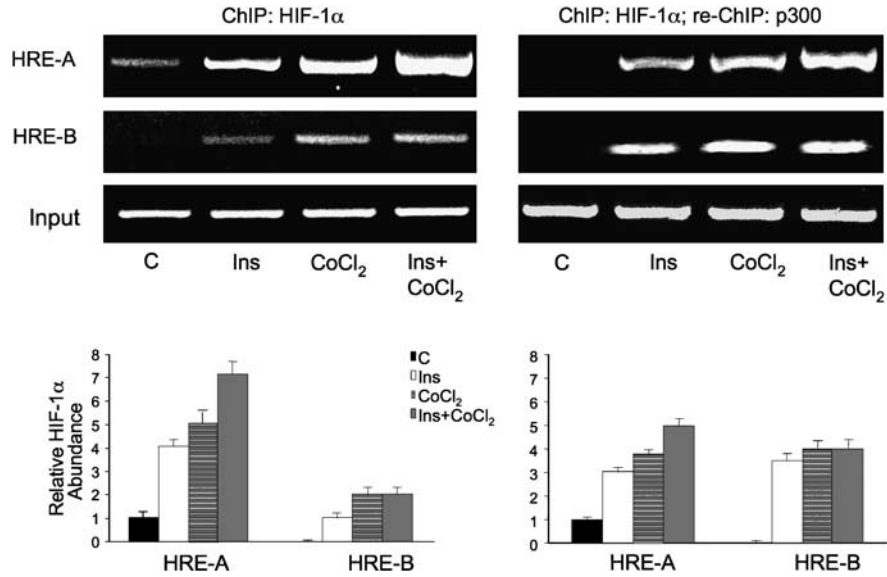
Figure 6 Hypoxia-inducible factor (HIF)-1α and p300 are corecruited to hypoxia-responsive elements (HRE)-containing regions in the leptin promoter. The binding of HIF-1α and corecruitment of p300 to different leptin promoter regions was tested by chromatin immunoprecipitation (ChIP) and reverse ChIP (re-ChIP) assays, as described in 'Materials and methods'. The HRE-A region encompasses four potential HRE binding sites, including those present in HRE1 and HRE2; the HRE-B region contains two potential HIF binding sites, including that present in HRE3. DNA input in PCR reactions was assessed using HRE-A (not shown) and HRE-B primers on samples before IPs. The graph represents relative abundance of HIF-1α or p300 on HREs under different conditions ±s.e.

Figure 7 Hypoxia-inducible factor (HIF)-1α knockdown reduces leptin expression. (a) MCF-7 cells were transfected with HIF-1α siRNA, as described in 'Materials and methods', or were left untransfected. In control experiments, the cells were transfected with lamin A/C siRNA. The expression of HIF-1α was assessed by WB in whole cell protein lysates. The expression of β-tubulin (TUB) was probed as control of protein loading. (b) The expression of HIF-1α and leptin (LEP) in MCF-7 cells transfected with HIF-1α siRNA was studied by immunofluorescence and deconvoluted microscopy as described in Figure 3 legend.

Discussion

Although recent evidence suggests that the leptin/ObR system is significantly overexpressed in breast cancers and related to tumor progression (Ishikawa et al., 2004; Garofalo et al., 2006; Revillion et al., 2006; Snoussi et al.,

2006), molecular mechanisms underlying this phenomenon have not been delineated. Here, we demonstrated that leptin expression in breast cancer cells can be induced by hyperinsulinemia, hypoxia and/or hypoxia-mimetic treatments, the conditions that can be associated with obesity (Losso and Bawadi, 2005; Garofalo et al., 2006).

First, we noted that the above treatments alone or in combination increased nuclear expression of HIF-1α, the HIF subunit that is known to be stabilized by hypoxic conditions as well as by activation of the insulin-sensitive mTOR pathway (Majumder et al., 2004; Treins et al., 2005; Pore et al., 2006). In MCF-7 cells, nuclear accumulation of HIF-1α in response to insulin was dose-dependent and maximal with the 340 nM dose. The greatest nuclear HIF-1α levels under physiological hypoxia were seen at 16 h, and under 100 nM CoCl$_2$ treatment at 4 h (Figure 1). Similar conditions stimulated maximal leptin mRNA and leptin protein expression. Notably, the highest leptin mRNA and protein expression were observed under combined insulin and hypoxia or CoCl$_2$ treatments (Figures 2 and 3). The same conditions and with similar dynamics appeared to stimulate the leptin gene promoter (from −2924 to +31; Figure 4). Although the same promoter has previously been shown to respond to glucocorticoids in 3T3-L1 cells (De Vos et al., 1998), and to insulin and hypoxia in BeWo cells (Grosfeld et al., 2002; Meissner et al., 2003), its activity in breast cancer cells has never been described.

Because the leptin promoter contains multiple HRE motifs, we tested whether high levels of insulin and hypoxic conditions can stimulate leptin expression through HIF. We demonstrated that in MCF-7 cells nuclear HIF-1α is able to associate with several leptin promoter domains containing HREs (Figures 5 and 6). The highest HIF-1α binding was detected on the proximal promoter (HRE-A), which contains four HRE motifs as well as three GC-rich regions that can enhance HIF activity (Miki et al., 2004). We also found that the HIF-1α complex interacting with the proximal promoter included p300 histone acetyltransferase, the major HIF coactivator (Figure 6). In ChIP assays, we noted that combined insulin plus CoCl$_2$ treatment induced slightly better HIF-1α loading on the proximal HRE-A promoter region, compared with single treatments (Figure 6). These synergistic effects were not detected using DAPA, possibly because this technique probes for protein binding to short DNA fragments and cannot fully reflect association of HIF-1α to native chromatin.

The above results are in agreement with data obtained in chorioncarcinoma cell model, where increased leptin expression was observed upon 500 nM insulin, hypoxia and combined treatments (Meissner et al., 2003). However, we are the first to show that both stimuli induce leptin in breast cancer cells acting through a common pathway involving HIF-1α. Most studies on leptin promoter activation relied on luciferase and other in vitro assays. Our report is first to demonstrate in vivo that the HIF-1α/p300 complex interacts with and activates the proximal leptin promoter in breast cancer cells. Furthermore, we established that HIF-1α is not only involved, but also required for insulin- and CoCl$_2$-stimulated leptin expression in MCF-7 cells.

Even though HIF seems to be a major factor regulating leptin mRNA and protein expression in our cell model, we acknowledge that it cannot be the exclusive mediator of leptin expression. This is suggested by the fact that addition of insulin to hypoxia or CoCl$_2$ increased leptin mRNA and protein synthesis relative to single treatments but did not elevate nuclear HIF-1α levels and did not increase HIF-1α loading on distant HREs (Figures 1b, 6 and 7). Furthermore, reduction of HIF-1α expression by 90% was not sufficient to produce similar inhibition of leptin expression under combined treatments (Figure 7). This suggest that leptin must be regulated by other insulin-sensitive transcriptional mechanism, as suggested before (Meissner et al., 2003). In fact, we found that in some breast cancer cells, insulin upregulates leptin expression predominantly via SP-1-dependent mechanisms (data not shown).

Cumulatively, our results indicate that one of the mechanisms of leptin overexpression in breast tumors might involve HIF-1α, a component of HIF transcriptional factor that can be upregulated by hypoxia and hyperinsulinemia. Notably, HIF-1α is often overexpressed in invasive breast cancer (Vleugel et al., 2005) and is a predictive marker of chemotherapy failure (Generali et al., 2006). Thus, molecular targeting of HIF-1α might help in the treatment of leptin-overexpressing tumors.

Materials and methods

Cell culture and treatments
MCF-7 cells were grown as described before (Garofalo et al., 2004). A total of 70% confluent cultures were treated with 340 nM insulin (Sigma, St Louis, MO, USA), 100 μM CoCl$_2$ (Sigma) or a combination of both stimuli. Hypoxia was accomplished by culturing the cells in a hypoxic chamber (Coy Laboratories, Grass Lake, MI, USA) with 1% O$_2$, 94% N$_2$, 5% CO$_2$ at 37°C.

Western blotting
The cells were stimulated with 340 nM insulin and/or 100 μM CoCl$_2$ for 4 h. The expression of proteins was analysed in 50–100 μg of nuclear cell lysates obtained as described before (Morelli et al., 2004). The following antibodies (Abs) were used: HIF-1α mAb (B&D systems, Minneapolis, MN, USA), β-tubulin H235 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and nucleolin mAb (Santa Cruz Biotechnology). The intensity of bands was measured as described before (Morelli et al., 2004).

Luciferase reporter assays
MCF-7 cells were grown in 24-well plates were transfected for 6 h with 0.5 μg DNA mixture/well using Fugene 6 (Roche, Indianapolis, IN, USA). The transfection mixtures contained 0.3 μg of the leptin promoter reporter plasmid pGL3-Ob1 (or an empty vector pGL3) and 50 ng of a plasmid encoding Renilla luciferase (RI Luc) (Promega, Madison, WI, USA). The pGL3-Ob1 plasmid encodes the firefly luciferase (Luc) cDNA under the leptin promoter (from −2924 to +31) (Miller et al., 1996; De Vos et al., 1998). Upon transfection, the cells were treated with 340 nM insulin and/or 100 μM CoCl$_2$ for 4 h, or left untreated. Luciferase activity (Luc and RI Luc) were measured as described before (Morelli et al., 2004).

Immunofluorescence/deconvoluted microscopy
A total of 5×10^4 MCF-7 cells were plated in 2-well Permanox chamber slides (Nunc, Rockester, NY, USA). After 24 h, the

cells were treated with 340 nM insulin and/or 100 μM CoCl$_2$, and/or hypoxia for 4 h (for HIF-1α detection) or 16 h (for leptin detection). Then the cells were washed with PBS and fixed for 20 min at 4°C in 4% paraformaldehyde for HIF-1α staining, or for 10 min at −20°C in methanol for leptin staining. Next, the cells were permeabilized with 0.2% Triton X-100, and unspecific binding was blocked in 7.5% BSA fraction V for 1 h at room temperature. HIF-1α expression was detected using 200 μg/ml HIF-1α pAb H-184 (Santa Cruz Biotechnology) and rhodamine-conjugated donkey anti-rabbit immunoglobulin G (IgG) (1:500, Santa Cruz Biotechnology). Leptin expression was probed with 200 μg/ml Ob pAb A-20 (Santa Cruz Biotechnology) and donkey anti-rabbit IgG-FITC (1:500, Santa Cruz Biotechnology). In control experiments, primary Abs were replaced by non-immune serum. The slides were covered with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) to allow visualization of cell nuclei. The abundance of nuclear HIF-1α and leptin was assessed using Olympus IX81 deconvoluted microscope and Slidebook software. HIF-1α and leptin expression were quantified by determining percentage of positive cells in at least 10 viewing fields.

ELISA
A total of 1.3×10^7 MCF-7 cells were left untreated or were treated for 16 h with 340 nM insulin, and/or 100 μM CoCl$_2$ and/or cultured under hypoxic conditions with or without insulin. Conditioned medium was collected and concentrated with Amicon centrifugal filter 10K (Millipore, Billerica, CA, USA) to final volume of 500 μl. Leptin abundance was measured using the ELISA low range leptin kit (R&D Systems, Minneapolis, MN, USA) following manufacturer's instructions.

Quantitative real-time–PCR
MCF-7 cells were treated for 4 h with 340 nM insulin, and/or 100 μM CoCl$_2$ and/or hypoxia. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA). A total of 3 μg of RNA was reverse transcribed using the TaqMan RT kit (Applied Biosystems, Foster City, CA, USA). A total of 2 μl of the RT products were used to amplify leptin sequences using the Hs00174877 A1 Lep TaqMan kit (Applied Biosystems). To normalize QRT–PCR reactions, parallel TaqMan assays were run on each sample for α-actin. Leptin mRNA content relative to α-actin mRNA was determined using a comparative CT method (Applied Biosystems User Bulletin no. 2). An average CT value for each RNA was obtained for replicate reactions. Similar method was used to evaluate leptin mRNA levels in HIF-1α siRNA-treated cells.

DNA affinity precipitation assay
Binding of nuclear HIF-1α to three HRE motifs (HRE-1: region −103 to −134; HRE-2: −611 to −641 and HRE-3: −1511 to −1541) was assessed using DAPA protocol described before (Cascio et al., 2007). Briefly, 70 μg of nuclear proteins obtained from cells stimulated with 340 nM insulin and/or 100 μM CoCl$_2$ for 4 h were mixed with 2 μg of specific biotinylated DNA HRE probes in 400 μl of Buffer D (Cascio et al., 2007) and then precipitated with 50 μl of strepta-vidin-agarose beads (Invitrogen). Upon removal of beads, supernatants were analysed for the presence and abundance of HIF-1α by western blot (WB).

The specific HRE probes were prepared by annealing a biotinylated sense oligonucleotide (HRE-1, -2 and -3) with the corresponding non-biotinylated antisense oligonucleotide. The pairs were HRE-1: 5′-Bio-CTAGCAGCCGCCCGGCACGT CGCTACCCTGA-3′ and 5′-TCAGGGTAGCGACGTGCC GGGCGGCTGCTAG-3′; HRE-2: 5′-Bio-TCCAGAGAGCG TGCACTCCCTGGGGTGCCA-3′ and 5′-TGGCACCCCA GGGAGTGCACGCTCTCTGGA; HRE-3: 5′-Bio-TATCTG GTGCCCAACGTGGGATACTGAGAT-3′ and 5′-ATCTC AGTATCCCACGTTGGGCACCAGATA. The specificity of probe–protein interactions was tested by addition of a 10-fold excess of unlabeled probes.

Chromatin immunoprecipitation and reverse ChIP
MCF-7 cells were stimulated with 340 nM insulin and/or 100 μM CoCl$_2$ for 4 h, or left untreated. Then, the cultures were cross-linked with 1% formaldehyde and soluble chromatin was obtained as described by us before (Morelli et al., 2004). To precipitate HIF-1α associated chromatin, we used HIF-1α pAb (Santa Cruz). A total of 5 μl sample of each HIF-1α ChIP product was used to detect specific HREs by PCR. The following primers were used: HRE-A (region from −992 to +377) forward 5′-GCGCAGTGGGGACCAGAA-3′, reverse 5′-CACCACCTCTGTGGAGTAG-3′; HRE-B (−1892 to −1403) forward 5′-TTGTGGTCAGACCAGTTTTCT-3′, re-verse 5′-GTTTGGTAATGCCCAAAAGCT-3′. The PCR conditions for HRE-A region were as follows: 1 min at 94°C, 1 min at 53°C, 1 min 20 s at 72°C. For HRE-B region: 1 min 94°C, 1 min at 60°C, 1 min at 72°C. The amplification of HRE regions was analysed after PCR 32 cycles. In control samples, the primary Abs were replaced with non-immune rabbit IgG.

Re-ChIP (Morelli et al., 2004) was used to test the presence of p300 associated with HIF-1α. HIF-1α ChIP pellets obtained as described above were eluted, precipitated with p300 pAb (Upstate Biotechnology/Millipore) and processed as for one-step ChIP.

HIF1α RNA interference
HIF-1α expression was inhibited using the following siRNA oligonucleotides: HIF siRNA I 5′-UGAGGAAGUACCAUU AUAUdTdT-3′ and HIF siRNA II 5′-UUAUGGUUCUCAC AGAUGAdTdT-3′ (Dharmacon, Lafayette, CO, USA). A total of 800 nM of HIF-1α siRNA I + II was mixed with the transfection agent RNAiFect (Qiagen, Valencia, CA, USA) (siRNA:RNAiFect ratio 1:3) and incubated for 15 min at room temperature. Then the mixture was transfected into 70% confluent cultures of MCF-7 cells for 6 h. After that, the cells were placed in fresh medium for 24 h and then treated with 340 nM insulin, and/or 100 μM CoCl$_2$. As controls, we used lamin A/C siRNA (Qiagen).

Acknowledgements

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