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Effects of Prolyl Hydroxylase Inhibitors on Adipogenesis and Hypoxia Inducible Factor 1 Alpha Levels Under Normoxic Conditions

Z. Elizabeth Floyd,* Gail Kilroy, Xiying Wu, and Jeffrey M. Gimble

Stem Cell Laboratory and Clinical Nutrition Research Unit, Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, Louisiana 70808

Abstract Adipocyte function is highly regulated in response to changing oxygen levels and physiological regulation of adipocyte formation involves factors originally identified as hypoxia-responsive proteins. Inhibition of adipogenesis at low oxygen tension is associated with activation of hypoxia inducible factor-1 alpha (HIF-1α), a transcription factor essential for cellular responses to decreased oxygen levels whose activity is regulated by prolyl hydroxylase (PHD) enzymes. However, HIF-1α RNA expression has been detected during the initial stages of adipocyte formation under aerobic conditions, suggesting a physiological role for HIF-1α during adipogenesis under a range of oxygen levels. Here we investigated the expression of HIF-1α during adipogenesis using the murine 3T3-L1 adipocyte model. Our results indicate the tissue-specific form of HIF-1α is upregulated during adipogenesis with maximal levels obtained within the first 24 h after induction. The increase in HIF-1α l.1 gene expression corresponds to increased nuclear HIF-1α protein, which gradually declines throughout adipogenesis under aerobic conditions. Each of the three HIF prolyl hydroxylases involved in regulating HIF-1α stability is expressed during adipogenesis. The prolyl hydroxylase domain 1 (PHD1) isoform of the HIF prolyl hydroxylases is expressed in early adipogenesis and the PHD2 and PHD3 isoforms are expressed during late adipogenesis. Pharmacological inhibition of PHD activity during the initial stages of adipogenesis abrogates the formation of adipocytes and inhibits gene expression of each of the PHD. However, inhibition of PHD activity does not consistently regulate HIF-1α l.1 expression or HIF-1α protein levels, suggesting that hydroxylation-independent mechanisms are involved in regulating HIF-1α expression in adipocytes under aerobic conditions. J. Cell. Biochem. 101: 1545–1557, 2007.

Key words: adipogenesis; prolyl hydroxylase; HIF-1 alpha; adipocyte

Adipocytes are highly specialized cells that play a major role in energy homeostasis. Fully differentiated adipocytes store energy in the form of lipid droplets and communicate with other peripheral tissues to regulate lipid and glucose metabolism in response to metabolic demands. Oxygen-sensing mechanisms are essential for maintaining energy homeostasis and accumulating data indicate that adipocyte function is highly regulated in response to changing oxygen levels [Yun et al., 2002; Grosfeld et al., 2002b; Lolmede et al., 2003; Hausman and Richardson, 2004; Swiersz et al., 2004]. These observations suggest coordination between oxygen-sensing mechanisms and regulation of energy storage in developing and fully differentiated adipocytes.

Many tissues normally encounter oxygen levels below what is considered to be the normal oxygen level of 21% in ambient air. Current studies indicate that tissue oxygen levels may be closer to 3–6% than 21%, suggesting a physiological role for cellular adaptation to moderate hypoxia [Giaccia et al., 2004]. This implies that oxygen-sensing in adipocytes may involve a complex set of interactions that operate under varying oxygen levels. Indeed,

Abbreviations used: ARNT, aryl hydrocarbon receptor nuclear translocator; DMOG, dimethyloxalylglycine; EDHB, ethyl-3,4-dihydroxybenzoate;HIF-1α, hypoxia inducible factor 1 alpha; PAS *domain*, per-ARNT-simpleminded domain; PHD, prolyl hydroxylase domain.

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*Correspondence to: Z. Elizabeth Floyd, PhD, Pennington Biomedical Research Center, 6400 Perkins Road, Baton Rouge, LA 70808. E-mail: floydez@pbrc.edu

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physiological regulation of adipogenesis involves transcription factors that were originally identified as hypoxia-responsive factors [Imagawa et al., 1999; Shimba et al., 2004].

Cellular adaptation to oxygen availability is mediated by the hypoxia inducible factor (HIF) transcription factor, a member of the basic helix-loop-helix-PAS superfamily. HIF-1 is a heterodimer of the oxygen-sensitive HIF-1α subunit and the constitutively expressed HIF-1β subunit, the aryl hydrocarbon receptor nuclear translocator (ARNT) [Wang and Semenza, 1995]. HIF-1α protein is maintained at low steady-state levels under normoxic conditions via hydroxylation by the HIF prolyl hydroxylases [Jaakkola et al., 2001]. The HIF prolyl hydroxylases are 2-oxoglutarate dioxygenases that are present in three forms in mammalian cells, designated PHD 1, PHD 2, and PHD 3 [Epstein et al., 2001]. PHD 2 is reported to be the primary form of the HIF prolyl hydroxylases responsible for regulating HIF-1α levels in normoxia [Berra et al., 2003] and is abundant in adipose tissue [Lieb et al., 2002]. PHD-dependent hydroxylation of HIF-1α results in rapid degradation of HIF-1α by the ubiquitin–proteasome system. In the presence of decreased oxygen levels, HIF-1α modification by the oxygen-sensitive prolyl hydroxylases (PHD) is substantially diminished. The stabilized HIF-1α translocates to the nucleus where it dimerizes with ARNT and becomes transcriptionally active (reviewed in [Maxwell, 2004]). This leads to activation of a range of genes encoding factors necessary for protection against cellular damage caused by hypoxia, including glycolytic enzymes and the vascular endothelial growth factor (VEGF) [Caro, 2001].

Although Yun et al. [2002] found that chronic hypoxia prevents adipogenesis via a HIF-1α-dependent pathway that inhibits PPARγ expression, HIF-1α is also transiently expressed within 3–12 h of induction of adipogenesis in 3T3-L1 adipocytes under normal (21%) oxygen conditions [Imagawa et al., 1999; Shimba et al., 2004]. This suggests a physiological role for oxygen sensing in the early stages of adipogenesis. Moreover, adipogenesis in 3T3-L1 adipocytes requires expression of endothelial PAS-1 (EPAS-1), a HIF family protein with structural similarity to HIF-1α [Shimba et al., 2004]. EPAS-1 (also known as HIF-2α) is expressed in the later stages of adipogenesis and is involved in basal and insulin-dependent glucose uptake. The connection between oxygen-sensing, adipogenesis and regulation of glucose metabolism is intriguing given other studies showing insulin-dependent regulation of HIF-1α under normal oxygen conditions [Zelzer et al., 1998; Treins et al., 2002].

To examine the possible role of HIF-1α expression during adipogenesis under normoxic conditions, we employed the 3T3-L1 murine model of adipogenesis along with specific pharmacologic inhibitors of the PHD enzymes. Our current results demonstrate that expression of nuclear HIF-1α protein occurs at low levels in the preadipocytes, increases substantially within 24 h of induction of adipogenesis, and is maintained until late adipogenesis. Each of the three PHD genes is expressed during adipogenesis and PHD activity within the first 24 h of induction is required for adipogenesis. Surprisingly, inhibition of adipogenesis in the presence of PHD inhibitors does not correlate with increased HIF-1α gene expression or increased HIF-1α protein levels although lower concentrations of each inhibitor did increase HIF-1α protein levels. Our data suggest a complex relationship between PHD activity and regulation of HIF-1α during adipogenesis under normoxic conditions in which expression of HIF-1α does not inhibit adipogenesis.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's Media (DMEM) was purchased from MediaTech (Herndon, VA) Bovine and fetal bovine serums (FBS) were obtained from Hyclone (Logan, UT). Insulin, IBMX, dexamethasone, and ethyl-3, 4-dihydroxybenzoate (EDHB) were purchased from Sigma-Aldrich (St. Louis, MO). The PPARγ antibody was a mouse monoclonal antibody (#E-8, sc-7273) purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The HIF-1α antibody was a rabbit polyclonal antibody (#A300-286A) from Bethyl Laboratories (Montgomery, TX). Horseradish peroxidase (HRP) conjugated secondary antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). MG132 was purchased from BostonBiochem (Cambridge, MA). Dimethyloxalylglycine (DMOG) was purchased from Frontier Scientific (Logan, UT).

Cell culture. Murine 3T3-L1 preadipocytes were plated and grown for 2 days post-confluence

in DMEM with 10% bovine serum. Medium was changed every 48 h. Cells were induced to differentiate by changing the medium to DMEM containing a standard induction cocktail of 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1.7 μM insulin (MDI). After 48 h this medium was replaced with DMEM supplemented with 10% FBS and cells were maintained in this medium. Normoxic conditions corresponded to room air with 5% CO_2 in a standard tissue culture incubator. Five percent oxygen levels were obtained by regulating the nitrogen concentrations according to the desired oxygen level (5% oxygen, 90% nitrogen, and 5% CO_2) in a NAPCO incubator. In each case, the 3T3-L1 cell line was maintained at 37°C.

Preparation of whole cell extracts. Cell monolayers were rinsed with phosphate-buffered saline (PBS) and harvested in a non-denaturing buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton-X 100, 0.5% Nonidet P-40, 1 μM PMSF, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, and 2 mM sodium vanadate. Samples were extracted for 30 min on ice and centrifuged at 15,000 rpm at 4°C for 15 min. Supernatants containing whole cell extracts were analyzed for protein concentrations using a BCA kit (Pierce) according to the manufacturer's instructions.

Preparation of nuclear extracts. Cell monolayers were rinsed with PBS and harvested in a nuclear homogenization buffer containing 20 mM Tris (pH7.4), 10 mM NaCl, and 3 mM $MgCl_2$. Nonidet P-40 was added to a final concentration of 0.15%, and cells were homogenized with 16 strokes in a Dounce homogenizer. Homogenates were centrifuged at 600*g* at 4°C for 5 min to obtain nuclear and cytoplasmic fractions. The pelleted nuclei were suspended in a non-denaturing buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 1 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, 10 μM MG132, and 2 mM sodium vanadate. The samples were analyzed for protein concentrations using a BCA kit (Pierce) according to the manufacturer's instructions.

DMOG and EDHB treatment of 3T3-L1 adipocytes. The PHD inhibitors dimethyloxalyglycine (DMOG) and ethyl-3,4-dihydroxybenzoate (EDHB) were added to developing and fully differentiated 3T3-L1 adipocytes at the indicated times and concentrations. DMSO was used as a solvent for both DMOG and EDHB and was present in the control incubations at 1 μl/ml media, which corresponds to the volumes of DMOG and EDHB added.

Gel electrophoresis and immunoblotting. Proteins were separated in polyacrylamide (National diagnostics) gels containing sodium dodecyl sulfate (SDS) according to Laemmli [1970] and transferred to nitrocellulose (BioRad) in 25 mM Tris, 192 mM glycine, and 20% methanol. Following transfer, the membrane was blocked in 4% milk for 1 h at room temperature. The membranes were incubated with mouse monoclonal anti-PPARγ (1:125 dilution) or rabbit polyclonal anti-HIF-1α (1:1000 dilution) as indicated for 1–2 h. Following extensive washes, the results were visualized with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Pierce).

RT-PCR and real-time RT-PCR. Total RNA was purified from cultured cells using Tri-Reagent (Molecular Research Center) according to the manufacturer's instructions. RNA (2 μg) was reverse transcribed using Moloney Murine leukemia virus reverse transcriptase (Promega) with oligo dT at 42°C for 1 h for all genes except HIF-1α l.1 and HIF-1α l.2. For HIF-1α l.1 and HIF-1α l.2, the reverse transcriptase reaction was conducted using random primers at 37°C for 1 h. Primers for genes of interest were designed using Primer Express (Applied Biosystems). The primer sequences were: *mPHD1* forward (5′-TGCCTTGCATGCGGTACTATG-3′) and reverse (5′-CCGTCGGTCAGACCAGAAAAT-3′); *mPHD2* forward (5′-TGTCCGTCACGTTGATAACCC-3′) and reverse (5′-CCTTGTTTCGTGTCCAGATGG-3′); *mPHD3* forward (5′-CATCATGAGGCTGGATCTGGA-3′) and reverse (5′-TCATAGCGTACCTGGTGGCAT-3′), *mHIF-1α l.1* forward (5′-TCCTGTAAGCAAGGAGCCAGA-3′) and reverse (5′-CACCTTCCACGTTGCTGACTT-3′); *mHIF-1α l.2* forward (5′-TAGCCAGGCCTTGACAAGCTA-3′) and reverse (5′-CACCTTCCACGTTGCTGACTT-3′); *mARNT* forward (5′-CTCCCGACACAACATTGAAGG-3′) and reverse (5′-TGCTGTGTTCTGATCCTGCAC-3′); *mCyclophilin B* forward (5′-GGTGGAGAGCACCAAGACAGA-3′) and reverse (5′-CGGCCTCAGCTGTTACTAC-3′). PCR was carried out under universal cycling

conditions (35 cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 35 s followed by 72°C for 1 min) using GoTaq polymerase master mix (Promega). Real-time PCR was performed with SYBR Green PCR master mix (Applied Biosystems) using the 7900 Real-time PCR system (Applied Biosystems) using universal cycling conditions (50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min; followed by 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s). When the PCR fragment was greater than 400 base pairs, the cycling conditions were 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s, 55°C for 1 min, and 72°C for 1.5 min. In each case, a dissociation program of 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s was included. All results were normalized to a *Cyclophilin B* expression control.

Oil Red O staining. Oil Red O staining was performed as described by Green and Kehinde [1975]. Lipid accumulation was calculated using Un-Scan-It version 6.1 software (Silk Scientific) using the HSB Color intensity mode and reported as arbitrary units based on average pixels. The total number of pixels in each well was divided by a standard total area to obtain the average pixels.

RESULTS

Although relatively little is known about the effect of oxygen levels on adipose tissue, accumulating data indicate adipocyte function is highly regulated in response to changing oxygen levels [Grosfeld et al., 2002a; Grosfeld et al., 2002b; Lolmede et al., 2003; Hausman and Richardson, 2004]. Our studies using the murine 3T3-L1 adipocyte model are in agreement with previous studies [Yun et al., 2002; Swiersz et al., 2004] indicating that the formation of adipocytes is inhibited at lower oxygen levels (Fig. 1, control). The earlier studies also demonstrated that inhibition of adipogenesis under hypoxic conditions occurs in a HIF-1α-dependent manner [Yun et al., 2002]. Oxygen-dependent expression of HIF-1α is regulated by the PHD enzymes [Jaakkola et al., 2001] and use of DMOG, a PHD inhibitor, resulted in inhibition of adipogenesis under room air conditions (Fig. 1, DMOG). DMOG-associated inhibition of adipogenesis occurred without the accompanying changes indicative of cellular stress that we observed (data not shown) in the presence of hypoxia mimics such as $CoCl_2$, which stabilize HIF-1α protein and enhance HIF-1α-dependent signaling [Wang and Semenza, 1993].

These results suggest HIF-1α is downregulated during adipogenesis at 21% oxygen levels. However, as shown in Figure 2A, induction of adipogenesis in 3T3-L1 preadipocytes under normoxic conditions is associated with increased levels of nuclear HIF-1α early in adipogenesis. Nuclear HIF-1α levels then gradually decline until HIF-1α levels at Day 6 post-induction are comparable to the steady-state level observed pre-induction. The increase in HIF-1α protein corresponds to an increase in HIF-1α gene expression (Fig. 2B). Expression of the mouse HIF-1α gene is driven by two different promoters located 5′ to alternative first exons, designated HIF-1α l.1 and HIF-1α

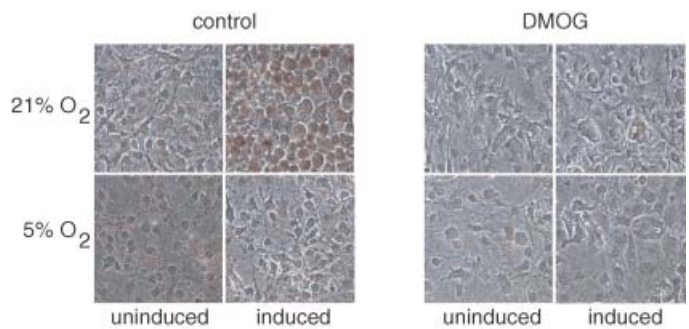

Fig. 1. Effect of prolyl hydroxylase inhibition on adipogenesis at ambient air. 3T3-L1 preadipocytes were maintained at confluence in the presence of DMEM containing 10% calf serum (uninduced) or were induced to undergo adipogenesis at 5% or room air oxygen levels in the presence of insulin (1.7 μM), isobutylmethylxanthine (0.5 mM), and dexamethasone (1 μM) in DMEM containing 10% fetal bovine serum (induced). The prolyl hydroxylase inhibitor, DMOG (1 mM) or the solvent DMSO (control) were added to the uninduced and induced preadipocytes at the time of induction. Lipid accumulation was assessed using Oil Red O staining of neutral lipids at 96 h post-induction. (*DMOG*, dimethyloxalylglycine). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

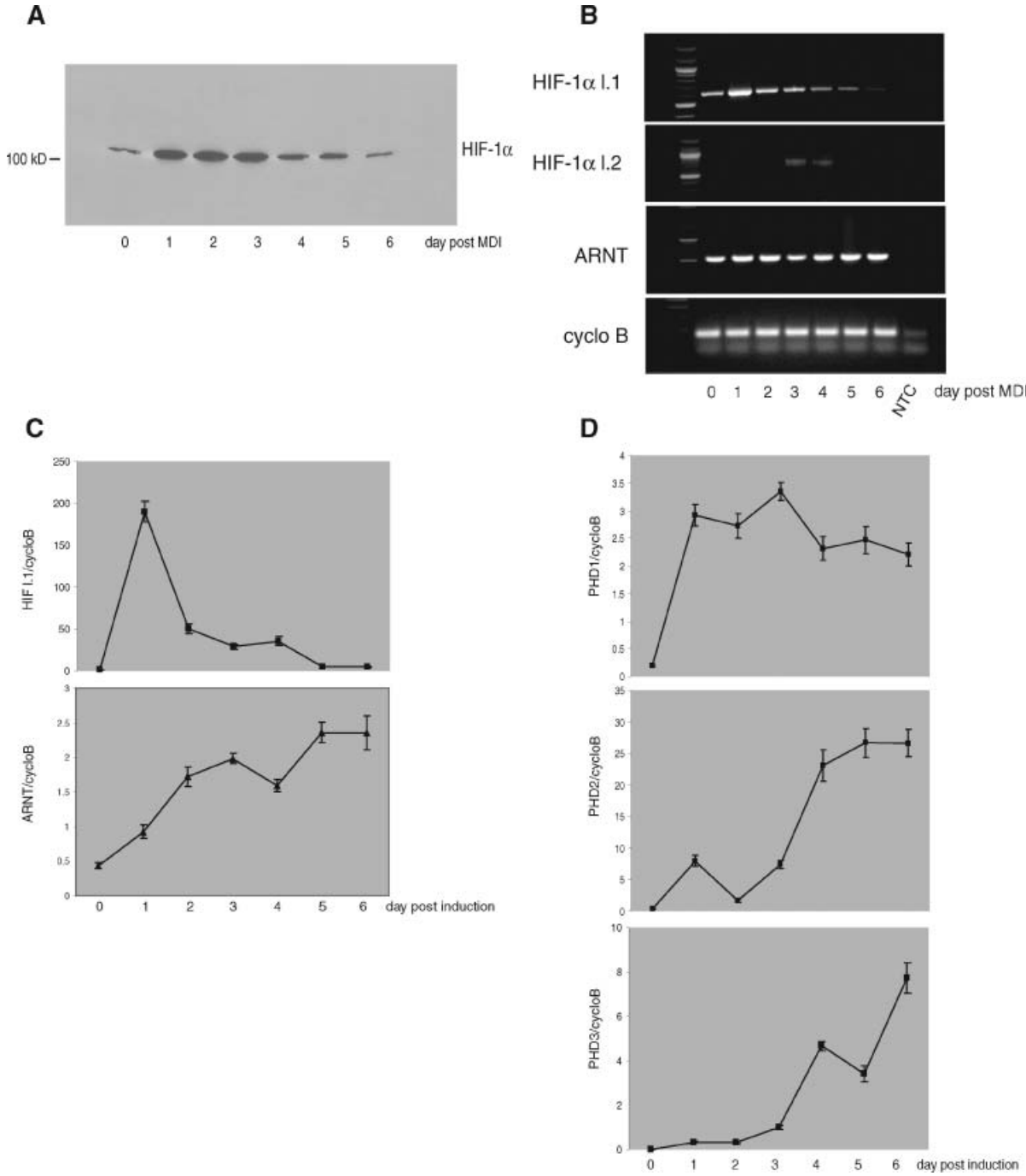

Fig. 2. Expression of HIF-1α/ARNT and the HIF prolyl hydroxylases during adipogenesis. **A**. Nuclear extracts were obtained at the indicated time points during differentiation of the 3T3-L1 adipocytes at room air oxygen. Thirty micrograms of each nuclear extract was separated by SDS–PAGE, transferred to nitrocellulose, and assayed for HIF-1α protein levels by Western blot analysis. **B**, **C**. Total RNA was isolated at the indicated time points during adipogenesis using TriReagent according to the manufacturer's instructions (Molecular Research Center) and subjected to standard RT-PCR as described in Materials and Methods. Cyclophilin B (cyclo B) was included in each assay as a loading control. NTC is a ''no template control''. **B**. HIF-1α l.1 is the tissue-specific form of HIF-1α and HIF-1α l.2 is the constitutive form of HIF-1α; ARNT is the aryl hydrocarbon receptor nuclear translocator. **C**. HIF-1α and ARNT gene expression were further assayed at each time point during adipogenesis using SYBR Green-based quantitative real-time RT-PCR and reported as the ratio of HIF-1α or ARNT/cyclophilin B. **D**. Expression of PHD1, PHD2, and PHD3 was assayed during adipogenesis using real-time RT-PCR as described in **C**. Prolyl hydroxylase domain (PHD) 1, 2, and 3 correspond to HIF prolyl hydroxylase 3, 2, and 1. (*ARNT*, aryl hydrocarbon receptor nuclear translocator; *cyclo B*, cyclophilin B; *HIF-1α*, hypoxia inducible factor-1alpha; *PHD*, prolyl hydroxylase domain).

l.2. HIF-1α l.2 encodes a protein predicted to contain an additional 12 amino acids at the N-terminus. In addition, HIF-1α l.1 is expressed in a tissue-specific manner while HIF-1α l.2 is constitutively expressed [Wenger et al., 1998]. Our results show the tissue-specific form of HIF-1α is expressed throughout adipogenesis with maximal expression at Day 1 post-induction (Fig. 2B, C) with the full extent of the induction appreciated when assayed by real-time PCR (Fig. 2C). The level of HIF-1α l.1 expression declines thereafter and is present at low levels in the fully differentiated adipocytes (Day 6 post-MDI). Interestingly, the constitutive form of HIF-1α is expressed only at low levels on Days 3 and 4 post-induction (Fig. 2B). This indicates differential regulation of HIF-1α gene expression during formation of adipocytes with the constitutive form present for a short period during adipogenesis and not detected in the fully differentiated adipocytes. HIF-1α protein levels correspond to the gene expression pattern observed for the tissue-specific form of HIF-1α, suggesting the shorter form of HIF-1α is the predominant form of HIF-1α protein expressed during adipogenesis. Our data also shows the heterodimeric partner of HIF-1α, ARNT [Wang and Semenza, 1995] is not expressed coordinately with HIF-1α during adipogenesis. We observed a slight increase in ARNT gene expression during adipogenesis (Fig. 2B) that is more apparent when assayed using real-time RT-PCR (Fig. 2C).

Under normoxic conditions, PHD enzymes modify HIF-1α, increasing ubiquitin–proteasome-dependent degradation of HIF-1α. Under hypoxic conditions, HIF-1α modification by the PHD enzymes is greatly diminished, resulting in a stabilized nuclear form of HIF-1α that is transcriptionally active [Jaakkola et al., 2001]. To begin examining the role of the PHD enzymes during adipogenesis, we assayed the gene expression of PHD 1, 2, and 3 at induction and on days one through six post-induction. As shown in Figure 2D, each of the PHD genes is expressed during adipogenesis and at Day 6 post-MDI in the fully differentiated adipocytes. The expression levels of all three PHD genes increase throughout adipogenesis with the PHD2 isoform becoming more abundant than PHD1 or PHD3 at late adipogenesis. PHD1 increases to maximal levels in early adipogenesis while PHD2 and PHD3 do not increase substantially until mid to late adipogenesis. This pattern contrasts with previous studies demonstrating upregulation of PHD3 and constitutive expression of PHD1 and PHD2 during myocyte differentiation [Lieb et al., 2002].

To explore the possible role of PHD enzyme activity during adipogenesis under normoxic conditions, we examined the effect of inhibition of PHD activity using two structurally independent inhibitors: 2, 3-ethyl dihydroxybenzoate (EDHB) and DMOG. EDHB has been characterized as a competitive inhibitor that binds to the ascorbate and 2-oxoglutarate (α-ketoglutarate) binding sites of 2-oxoglutarate-dependent dixoygenases, including the HIF prolyl hydroxylases [Majamaa et al., 1986; Sasaki et al., 1987]. DMOG is a 2-oxoglutarate analog that also competitively inhibits 2-oxoglutarate dioxygenases [Cunliffe et al., 1992]. Each inhibitor was added at induction and maintained in the media during adipogenesis until the adipocytes were harvested at Day 6 post-MDI. A dose curve response indicates (Fig. 3) increasing concentrations of either inhibitor results in inhibition of adipogenesis. Maximal decreases in neutral lipid accumulation in the presence of EDHB were observed at 50–100 μM as judged by Oil Red O staining (Fig. 3A, B). At concentrations greater than 100 μM EDHB, the decreases in lipid accumulation were accompanied by elongation and loss of cells (see phase contrast, Fig. 3A). In the presence of DMOG, inhibition of lipid accumulation occurred at greater than 100 μM without a corresponding elongation or loss of cells. Maximal inhibition of lipid accumulation occurred at 500–1000 μM DMOG (Fig. 3A, B). These results indicate PHD activity is required for the formation of adipocytes.

To confirm the effect of PHD inhibition on adipogenesis, we assayed the expression of PPARγ proteins as a marker of fat cell formation [Rosen and Spiegelman, 2001] at various time points during adipogenesis. DMOG (1 mM) or EDHB (1 mM) were added at induction of adipogenesis and were maintained in culture until the cells were harvested at the indicated time point. As shown in Figure 4A, the higher molecular weight PPARγ2 protein is not detectable at 48, 72, or 96 h after induction in the presence of DMOG or EDHB. Only low levels of PPARγ1, which lacks 30 amino acids found at the N-terminus of PPARγ, can be detected at 48 and 96 h post-MDI in the presence of DMOG, but not in the presence of EDHB. Interestingly, inhibition of PHD activity must occur within the

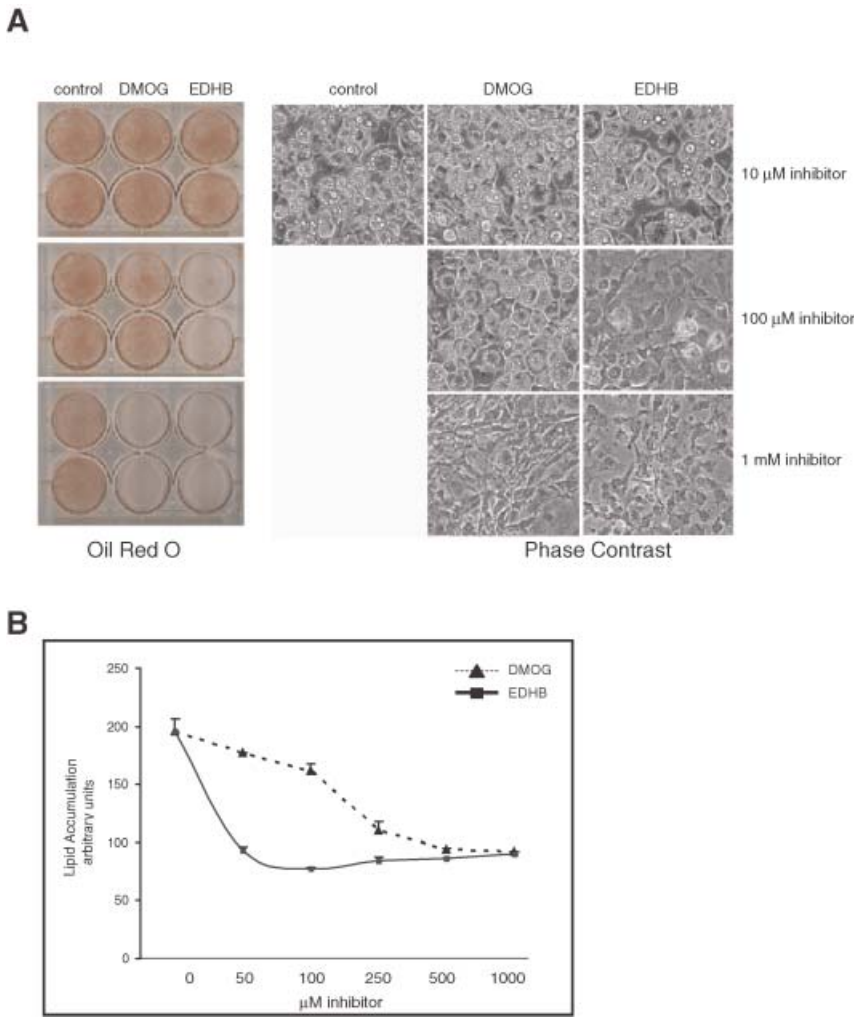

Fig. 3. Prolyl hydroxylase inhibitor dose curve response during adipogenesis. **A**. 3T3-L1 preadipocytes were induced to undergo adipogenesis at 21% oxygen in the absence (control) or presence of the prolyl hydroxylase inhibitors DMOG or ethyl-3,4-dihydroxybenzoate (EDHB) at the indicated concentrations. The cultured cells were assayed for cell morphology (phase contrast) or lipid accumulation (Oil Red O) on Day 6 post-induction. **B**. Lipid accumulation was quantitated using UnScan-It version 6.1 software (Silk scientific) and reported in arbitrary units as the mean −/+ standard deviation. (*EDHB,* ethyl-3,4-dihydroxybenzoate). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

first 24 h of induction in order to inhibit PPARγ protein expression. As shown in Figure 4B, addition of 1 mM DMOG at induction (t = 0), 12 or 24 h post-induction results in a substantial decrease in PPARγ protein levels in adipocytes harvested at 96 h post-induction. However, addition of DMOG (1 mM) at 48–72 h post-induction does not inhibit expression of PPARγ2 protein, which is expressed primarily in adipocytes [Rosen, 2005], indicating adipogenesis is unaffected by PHD inhibition at the later time points. This suggests PHD activity is critical to adipocyte development within the first 12–24 hours of differentiation. This period corresponds to induction of PPARγ gene and protein expression (reviewed in [Farmer, 2005]) as well as maximal HIF-1α protein and gene expression (Fig. 2A–C) and the initial increase in PHD 1 expression (Fig. 2D).

Inhibition of the PHD enzymes by DMOG and EDHB is expected to correspond to an increase in the steady-state level HIF-1α protein. We isolated nuclear extracts on Day 6 post-MDI and assayed the levels of HIF-1α protein in response to increasing concentrations of each PHD inhibitor. As shown in Figure 5, inhibition of the PHD enzymes by DMOG results in an increase in HIF-1α protein at the lower DMOG levels. However, HIF-1α protein is decreased at 1 mM DMOG, when lipid accumulation has

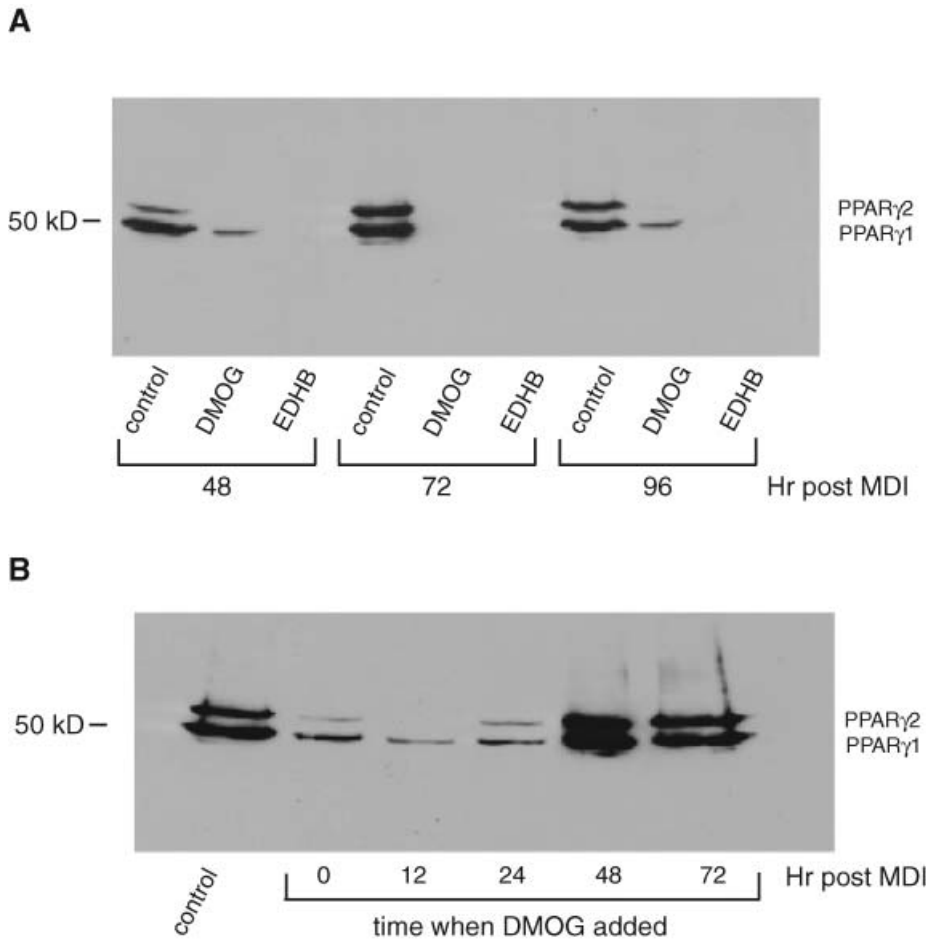

Fig. 4. Effect of prolyl hydroxylase inhibition on PPARγ proteins expression during adipogenesis. Whole cell extracts were harvested during 3T3-L1 differentiation at 21% oxygen. Thirty micrograms of each extract were separated by SDS–PAGE, transferred to nitrocellulose and subjected to Western blot analysis using a monoclonal anti-PPARγ antibody in which the carboxy-terminus antigenic epitope is detected in PPARγ1 and PPARγ2. **A**. The hydroxylase inhibitors dimethyloxalyglycine (1 mM) (DMOG) or ethyl-3,4-dihydroxybenzoate (1 mM) (EDHB) were added at induction and maintained throughout differentiation. DMSO was present as a vehicle control (control). The whole cell extracts were harvested at the indicated time post-induction (Hr post-MDI). **B**. Dimethyloxalyglycine (1 mM) (DMOG) was added at the indicated time during differentiation and whole cell extracts were harvested at Day 6 post-induction. DMSO was added at induction (control) as a vehicle control.

been substantially reduced. Thus, DMOG inhibition of PHD corresponds to increases in the steady-state level of HIF-1α up to 500 μM DMOG. However, stabilization of HIF-1α is not associated with the maximal inhibition of adipogenesis at Day 6 post-MDI. In addition, HIF-1α protein levels are not appreciably increased in the presence of EDHB. Indeed, HIF-1α protein is decreased at higher concentrations of EDHB, showing no correlation with lipid accumulation.

HIF-1α l.1 gene expression on Day 6 post-MDI in the presence of the PHD inhibitors follows the same trend as HIF-1α protein levels (Fig. 6A). HIF-1α l.1 gene expression increases substantially in the presence of EDHB up to 250 μM, but the levels are undetectable at higher concentrations (Fig. 6A). HIF-1α l.1 levels in the presence of DMOG decrease significantly only at 1 mM DMOG. The changes in HIF-1α l.1 gene expression correlate with HIF-1α protein levels, but do not correlate with the dose-dependent decline in lipid accumulation observed in the presence of PHD inhibition. Interestingly, ARNT gene expression (Fig. 6A), declined in the presence of both DMOG and EDHB except for the levels at 1 mM EDHB, which were unchanged from the control. This finding suggests expression of ARNT, the HIF-1β subunit, is regulated by PHD activity. Finally, we asked if inhibition of PHD enzyme activity affected gene expression of the PHD themselves. As shown in Figure 6B,

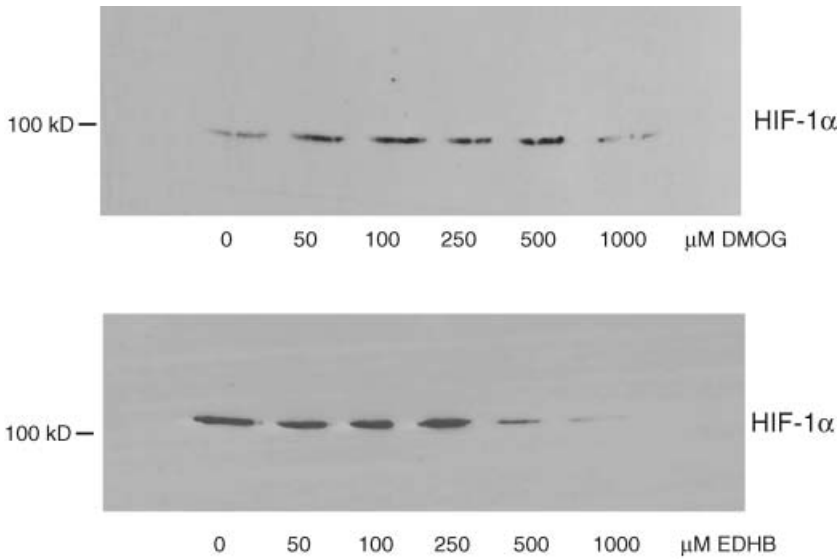

Fig. 5. Expression of HIF-1α protein in response to prolyl hydroxylase inhibition. Nuclear extracts were harvested at Day 6 post-induction of adipogenesis at room air oxygen. Thirty micrograms of each nuclear extract were separated by SDS–PAGE, transferred to nitrocellulose, and assayed for HIF-1α protein levels by Western blot analysis. The hydroxylase inhibitors dimethyloxalylglycine (DMOG) or ethyl-3,4-dihydroxybenzoate (EDHB) were added at the indicated concentration upon induction and were maintained throughout differentiation. DMSO was added as a vehicle control (0).

the gene expression of PHD 1, 2, and 3 was decreased in the presence of either DMOG or EDHB, suggesting PHD activity in adipocytes functions in a feedback mechanism controlling gene expression of the PHD enzymes.

DISCUSSION

Recent studies have shown that decreased oxygen levels are associated with inhibition of PPARγ expression and adipocyte formation via a HIF-1α-mediated mechanism [Yun et al., 2002; Swiersz et al., 2004]. In the presence of hypoxia, inhibition of adipogenesis occurred via activation of DEC1/Stra13, a HIF-1α inducible transcriptional repressor involved in cell differentiation [Staal et al., 2001]. The current results indicate that HIF-1α gene and protein expression is upregulated during adipogenesis under normoxic conditions, suggesting a positive role for HIF-1α-mediated signaling in the formation of adipocytes. Upregulation of HIF-1α mRNA has been previously reported at 3–12 h post-induction [Imagawa et al., 1999] or at induction through Day 2 post-induction in 3T3-L1 adipocytes [Shimba et al., 2004]. Our data extend the earlier findings to show that the substantial upregulation of HIF-1α in the early stages of adipogenesis is attributed to induction of mRNA expression of the tissue-specific form of HIF-1α, HIF-1α l.1. In addition, the constitutive form of HIF-1α, HIF-1α l.2, is expressed at Day 3 and Day 4 post-induction, indicating differential regulation of HIF-1α gene expression throughout adipogenesis at normoxic conditions. Upregulation of HIF-1α l.1 mRNA corresponds to increased steady-state levels of nuclear HIF-1α protein. Increased steady-state levels of HIF-1α in the nucleus is typically associated with stabilization of HIF-1α in response to hypoxia as HIF-1α escapes degradation by association with the von Hippel–Lindau complex of the ubiquitin–proteasome system [Jaakkola et al., 2001].

However, there is accumulating evidence that HIF-1α is activated under non-hypoxic conditions by the degradation–independent mechanisms. In these instances, the usually labile HIF-1α protein is expressed at elevated levels in the presence of normal oxygen levels ([Dery et al., 2005] and references therein). In particular, insulin and insulin-like growth factor have been described as activators of HIF-1α/ARNT transcriptional activity [Zelzer et al., 1998; Treins et al., 2002; Yim et al., 2003; Doronzo et al., 2006] in the presence [Treins et al., 2002; Doronzo et al., 2006] or absence of increased HIF-1α protein levels [Yim et al.,

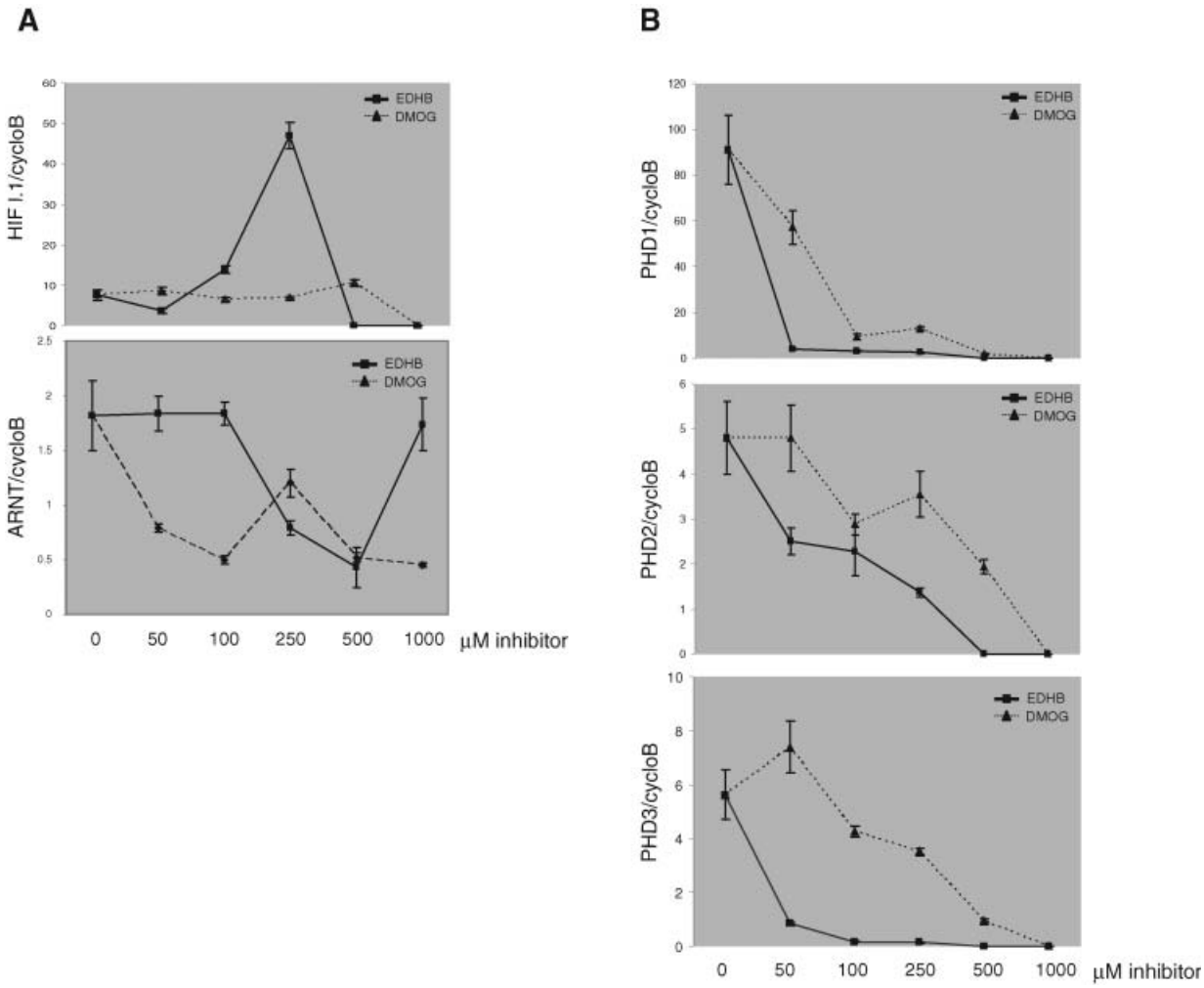

Fig. 6. Gene expression of HIF-1α l.1, ARNT, and HIF prolyl hydroxylases in response to prolyl hydroxylase inhibition. 3T3-L1 pre-adipocyte were induced to undergo adipogenesis in the presence of dimethyloxalyglycine (DMOG) or ethyl-3,4-dihydroxybenzoate (EDHB) at the indicated concentrations and DMSO was included as the vehicle control (0). Total RNA was isolated at Day 6 post-induction using TriReagent according to the manufacturer's instructions (Molecular Research Center) and subjected to SYBR Green-based semi-quantitative real-time RT-PCR and reported as the ratio of the gene of interest/cyclophilin B. **A**. HIF-1α l.1 is the tissue-specific form of HIF-1α and ARNT is the aryl hydrocarbon receptor nuclear translocator. **B**. Prolyl hydroxylase domain (PHD) 1, 2, and 3 corresponds to PHD 3, 2, and 1.

2003]. Insulin-dependent stimulation of HIF activity is associated with increased HIF-1α protein expression that is dependent on phosphatidylinositol 3-kinase (PI3K) signaling to increase translation of HIF-1α protein [Treins et al., 2002; Doronzo et al., 2006]. Translation of HIF-1α is independent of oxygen levels [Gorlach et al., 2000] and insulin-mediated increases in HIF-1α protein translation may be sufficient to increase the steady-state levels of nuclear HIF-1α during adipogenesis.

Insulin plays an essential role in regulating adipogenesis in the 3T3-L1 murine model system [Guller et al., 1988] as well as in modulating metabolic pathways involved in energy storage and usage. Oxygen-sensing is a fundamental requirement for regulating metabolism and insulin induces transcription of a number of genes that are also induced in hypoxia by HIF-1α· For example, insulin-dependent formation of adipocytes is associated with a substantial increase in glycolytic enzymes [Soukas et al., 2001]. Genes encoding glycolytic enzymes are also induced by HIF-1α in response to hypoxia [Seagroves et al., 2001] as well as in tumor cells where aerobic glycolysis is upregulated (reviewed in [Semenza, 2003] and references therein). Kietzmann et al. have postulated cross-talk between hypoxia and glucose via HIF-1α [Kietzmann et al., 2002].

Our results are consistent with potential cross-talk between insulin-dependent pathways and HIF-1α activation within the first 24 h of insulin-dependent induction of adipogenesis, coincident with upregulation of glycolytic enzymes. Moreover, recent studies have established a role for glycolytic metabolites such as pyruvate, lactate, and oxaloacetate in aerobic activation of HIF-1α [Lu et al., 2002, 2005]. Finally, formation of the HIF-1α/ARNT complex is essential in insulin-mediated regulation of HIF-1α activity [Zelzer et al., 1998]. Our studies indicate that regulation of HIF-1α and ARNT during adipogenesis is not coordinated, although ARNT expression is apparent throughout adipogenesis, supporting the notion that transcriptionally active HIF-1α is involved in insulin-mediated regulation of adipogenesis under normoxic conditions.

There are other possible explanations for the induction of HIF-1α during adipogenesis in the 3T3-L1 model system under aerobic conditions. Others have observed induction of HIF-1α in confluent cell culture conditions where PHD-dependent modification of HIF-1α was inhibited [Paltoglou and Roberts, 2005]. In our experiments adipogenesis was induced 2 days post-confluence. HIF-1α protein was expressed at low levels in the confluent culture prior to induction and was substantially increased at 24 h post-induction and declined thereafter to pre-induction levels. This suggests confluent cell culture conditions do not contribute substantially to the induction of HIF-1α we observed under normoxic conditions during adipogenesis. Moreover, the confluent cultures could be induced to undergo adipogenesis at room air oxygen, but not at 5% oxygen levels, suggesting hypoxia associated with confluent cell culture conditions cannot account for the induction of HIF-1α during adipogenesis under normoxic conditions. Finally, we did not observe expression of PHD2 and PHD3 prior to induction of adipogenesis although hypoxia has been noted to induce expression of both PHD2 and PHD3 [Berra et al., 2001]. An additional possibility is the inclusion of dexamethasone as a component of the adipogenesis induction cocktail. The glucocorticoid receptor is expressed at high levels during adipogenesis [Floyd and Stephens, 2003] and activation of the glucocorticoid receptor regulates HIF-1α gene expression and activity [Kodama et al., 2003]. However, glucocorticoid receptor regulation of HIF-1α expression and activity occurred under hypoxic conditions, but not in the presence of 21% oxygen. Activation of the glucocorticoid receptor did not affect HIF-1α protein levels, in contrast to the increase in HIF-1α gene and protein expression we observed during adipogenesis under normoxic conditions. Hence, our results remain consistent with insulin-dependent regulation of HIF-1α expression during adipogenesis under normoxic conditions.

Our further studies examined PHD gene expression and the effect of PHD enzyme inhibition. Each of the three HIF prolyl hydroxylase genes is expressed during adipogenesis and our data indicate PHD activity is required for formation of adipocytes at normal oxygen levels. PHD activity is essential for adipogenesis during the initial 24 h after induction, but is dispensable at later time points. This critical period for PHD activity corresponds to induction of an array of transcription factors that are essential for adipocyte formation, including PPARγ gene and protein expression (reviewed in [Farmer, 2005]). During this period, PHD1 expression is increased substantially while PHD2 and PHD3 levels increase in late adipogenesis. This observation suggests PHD1 is the PHD isoform associated with the essential PHD activity in early adipogenesis. Interestingly, early adipogenesis also corresponds to maximal HIF-1α l.1 gene expression and HIF-1α protein expression. Though the two events are related temporally, the increased protein levels of HIF-1α under normoxia suggest prolyl hydroxylation of HIF-1α by PHD1 or the other HIF prolyl hydroxylases is not the dominant regulatory mechanism involved in determining HIF-1α levels at that time point. In addition, the role of PHD activity in regulating adipogenesis appears to be unrelated to HIF-1α stabilization, but is associated with regulation of the expression of each of the three PHD genes. Nevertheless, our studies cannot rule out the possibility that PHD activity is involved in modulating HIF-1α stability or activity during adipogenesis. Earlier studies using siRNA silencing of each of the PHD in HeLa cells showed that silencing of PHD2, but not PHD1 or PHD3, affected HIF-1α stability under normoxic conditions [Berra et al., 2003]. The earlier results are consistent with our findings in that PHD2 expression is inversely related to HIF-1α protein levels during adipogenesis under normoxic conditions.

The current results reveal a complex relationship between PHD activity and HIF-1α expression under aerobic conditions in which prolyl hydroxylation regulates adipogenesis, but does not control the steady-state levels of HIF-1α protein. Adipogenesis in the 3T3-L1 model involved differential regulation of each of the three PHD genes as well as the tissue-specific and constitutive forms of HIF-1α, providing evidence supporting a role for the PHD enzymes and HIF-1α during the formation of adipocytes under a range of oxygen levels.

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