

# Roles for Peroxisome Proliferator-activated Receptor $\gamma$ (PPAR $\gamma$ ) and PPAR $\gamma$ Coactivators 1 $\alpha$ and 1 $\beta$ in Regulating Response of White and Brown Adipocytes to Hypoxia\*

Received for publication, February 8, 2012, and in revised form, March 31, 2012. Published, JBC Papers in Press, April 9, 2012, DOI 10.1074/jbc.M112.350918

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**Background:** What are the mechanisms regulating compensatory responses to hypoxia in adipocytes?

**Results:** Induction of hypoxia genes requires HIF-1 $\alpha$  and PPAR $\gamma$  in white adipocytes and PGC-1 cofactors in brown adipocytes.

**Conclusion:** Adipogenic factors and HIF-1 $\alpha$  regulate hypoxia responses in adipocytes.

**Significance:** Results suggest that obese adipose tissue is compromised due to defects in signaling pathways converging on HIF-1 $\alpha$ .

Obese white adipose tissue is hypoxic but is incapable of inducing compensatory angiogenesis. Brown adipose tissue is highly vascularized, facilitating delivery of nutrients to brown adipocytes for heat production. In this study, we investigated the mechanisms by which white and brown adipocytes respond to hypoxia. Brown adipocytes produced lower amounts of hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) than white adipocytes in response to low O<sub>2</sub>, but induced higher levels of hypoxia-associated genes. The response of white adipocytes to hypoxia required HIF-1 $\alpha$ , but its presence alone was incapable of inducing target gene expression under normoxic conditions. In addition to the HIF-1 $\alpha$  targets, hypoxia also induced many inflammatory genes. Exposure of white adipocytes to a peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) ligand (troglitazone) attenuated induction of these genes but enhanced expression of the HIF-1 $\alpha$  targets. Knockdown of PPAR $\gamma$  in mature white adipocytes prevented the usual robust induction of HIF-1 $\alpha$  targets in response to hypoxia. Similarly, knockdown of PPAR $\gamma$  coactivator (PGC) 1 $\beta$  in PGC-1 $\alpha$ -deficient brown adipocytes eliminated their response to hypoxia. These data demonstrate that the response of white adipocytes requires HIF-1 $\alpha$  but also depends on PPAR $\gamma$  in white cells and the PPAR $\gamma$  cofactors PGC-1 $\alpha$  and PGC-1 $\beta$  in brown cells.

Insulin resistance and the development of type 2 diabetes are linked to an increase in white adiposity, but the mechanisms responsible are not well understood. Expansion of white adipose tissue (WAT)<sup>2</sup> during obesity involves both hyperplasia and hypertrophy, leading to an increase in the number and size of individual adipocytes. Recent investigations suggest that obese WAT is hypoxic due to its failure to mount compensatory

angiogenesis (1–3). Hypoxic adipocytes secrete inflammatory cytokines such as TNF, IL-6, IL-1 and chemokines such as Ccl2, which attract macrophages, leading to inflammation and local insulin resistance (4, 5). Inflamed adipocytes undergo tremendous stress, resulting in alterations in fuel metabolism, as well as a reduction in the production of beneficial adipokines, including adiponectin and adipisin (6, 7). A reduction in the circulating levels of these factors contributes to systemic insulin resistance and its accompanying comorbidities (8). Another potential contributor to the deterioration of normal WAT function in obese individuals is fibrosis (9, 10). Recent investigations have suggested that enhanced production of fibrotic proteins, including components of a connective tissue extracellular matrix in obese WAT, is due to the accompanying hypoxia, possibly through the major hypoxia-inducible transcription factor, HIF-1 $\alpha$  (1). Understanding obesity-associated hypoxia and its stimulated fibrosis as opposed to the needed compensatory angiogenesis and identifying alternative means to induce vascular growth and development in hypoxic adipose tissue will aid in combating insulin resistance and type 2 diabetes.

The response of most cells to hypoxia is to activate a program of gene expression that functions to protect against the ensuing stress and stimulate blood vessel formation to provide an adequate supply of oxygen and nutrients (11). Transcription of this program of genes is regulated by HIFs, which are basic helix-loop-helix/PAS proteins consisting of an oxygen-regulated  $\alpha$ -subunit (HIF-1 $\alpha$ ) and a  $\beta$ -subunit (HIF-1 $\beta$ ) (12–14). These factors form heterodimers in response to hypoxia and activate transcription of a diverse group of genes by binding to hypoxia-responsive elements in the promoters/enhancers of the genes. The HIF target genes code for proteins participating in several physiological processes such as angiogenesis (*vegfa*) and glucose metabolism, which compensate for the deleterious effects of low oxygen (14). Under normoxic conditions in most tissues, HIF-1 $\alpha$  is hydroxylated on proline residues by prolyl hydroxylase domain-containing proteins (PHD1–3; also known as EglN2, EglN1, and EglN3, respectively), which facilitate its binding to the pVHL (von Hippel-Lindau protein)-ubiquitin E3 ligase, resulting in its rapid degradation by the proteasome (15).

\* This work was supported by United States Public Health Service Grants DK51586, DK58825, and DK086629 (to S. R. F.) and National Institutes of Health Training Grant 5T32AG000115-24 (to E. P.).

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<sup>2</sup> The abbreviations used are: WAT, white adipose tissue; HIF, hypoxia-inducible factor; BAT, brown adipose tissue; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; PGC, PPAR $\gamma$  coactivator; TZD, troglitazone.

## Hypoxia and Adipocytes

Under hypoxic conditions, hydroxylation does not occur because PHD proteins are inactive due to their high  $K_m$  for  $O_2$ . As a result, HIF-1 $\alpha$  is stabilized, leading to the formation of transcriptionally active HIF-1 $\alpha$ /HIF-1 $\beta$  heterodimers. The activity of PHD proteins requires  $\alpha$ -ketoglutarate and oxygen as cosubstrates and ascorbate and ferrous iron as cofactors. Hydroxylation of HIF-1 $\alpha$  is therefore responsive to reactive oxygen species, which oxidize the cofactors, and to succinate or fumarate, which competes with  $\alpha$ -ketoglutarate for binding to PHD proteins. In fact, the absence of the well established tumor suppressors succinate dehydrogenase and fumarate hydratase has been linked to tumor progression as a result of activation of the HIF pathway and angiogenesis (16, 17). Consequently, effectors that modulate the levels of succinate and/or fumarate have the potential to stimulate a pseudo-hypoxia response consisting of high HIF activity under normoxic conditions. Such effectors could be operating by altering mitochondrial activity that may include stimulation of the electron transport chain or an increase in mitochondrial density (18, 19). PHD proteins are also regulated by HIF-associated transcription of the corresponding genes, which mediate a feedback loop to modulate the hypoxia response by supplying more hydroxylases (20).

Adaptation to low oxygen by adipocytes in obese WAT and brown adipose tissue (BAT) could involve several different effectors and pathways. To gain insight into the response of white and brown adipocytes to hypoxia, we initiated this study to determine the involvement of HIF-1 $\alpha$  as well as other factors that might respond to novel effectors. The results show that the response of white adipocytes to low oxygen requires HIF-1 $\alpha$ , but its stabilization under normoxic conditions (pseudo-hypoxia) is incapable of inducing target gene expression. Hypoxia induces inflammatory genes as well as the classic HIF-1 $\alpha$  targets. Treatment of white adipocytes with troglitazone attenuates inflammatory gene expression but enhances expression of the HIF-1 $\alpha$  targets in response to low  $O_2$ . These responses to hypoxia require peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) in white adipocytes and the PPAR $\gamma$  coactivators PGC-1 $\alpha$  and PGC-1 $\beta$  in brown adipocytes.

### EXPERIMENTAL PROCEDURES

**Materials**—Specific reagents were purchased as indicated: dexamethasone, 3-isobutyl-1-methylxanthine, insulin,  $CoCl_2$ , troglitazone, indomethacin, and triiodothyronine from Sigma-Aldrich; Halt protease inhibitor mixture from Thermo Scientific; DMEM from Mediatech, Inc. (Herndon, VA); FBS from Gemini Bio Products (West Sacramento, CA); and calf serum from Invitrogen. The HIF-1 $\alpha$  inhibitor YC-1 was obtained from Cayman Chemical Co. (Ann Arbor, MI).

**Cell Lines and Cell Culture**—Murine 3T3-L1 preadipocytes and Swiss 3T3 fibroblasts ectopically expressing PPAR $\gamma$  were cultured and maintained in DMEM supplemented with 10% calf serum. Differentiation was induced by exposure of 2-day post-confluent cells to DMEM containing 10% FBS, 1  $\mu M$  dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 1.67  $\mu M$  insulin. After 48 h, the medium was changed every 2 days to DMEM containing only 10% FBS. Primary brown preadipocytes isolated from newborn wild-type and PGC-1 $\alpha$  knock-out mice, immortalized via pBABE-SV40T antigen, were a kind

gift from Dr. Bruce Spiegelman and were described previously (21). Differentiation of brown preadipocytes was induced by treatment of confluent cells with medium containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 125 nM indomethacin, 1  $\mu M$  dexamethasone, 850 nM insulin, and 1 nM triiodothyronine (T3). After 48 h, cells were incubated in medium containing 10% FBS, 850 nM insulin, and 1 nM triiodothyronine. For cells differentiated in the presence of the PPAR $\gamma$  agonist, 5  $\mu M$  troglitazone was added to the medium from day 2 of differentiation until harvest on days 8–10.

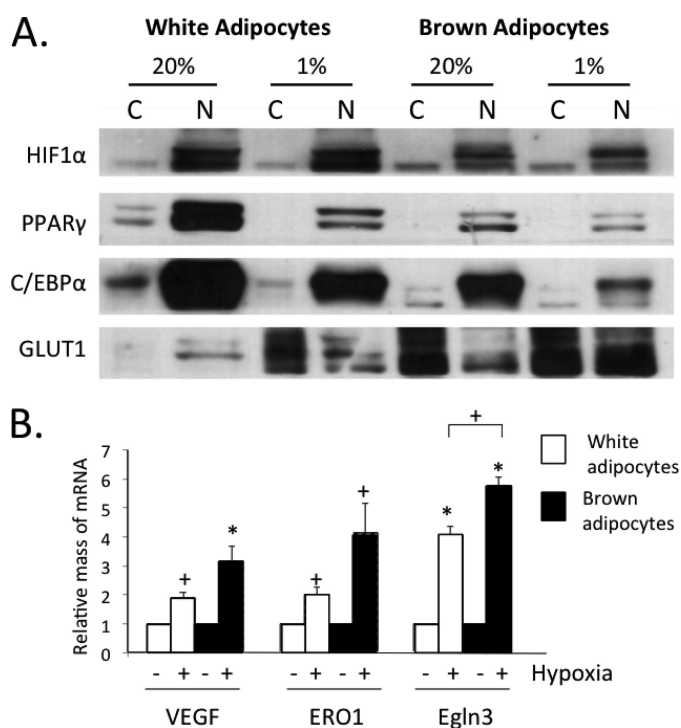
**Cell Extracts and Western Blot Analysis of Proteins**—Total and nuclear proteins of cultured cells were extracted into radio-immune precipitation assay buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM PMSF, 50  $\mu M$  leupeptin, and 5  $\mu M$  aprotinin). After the protein content was quantified using a BCA kit (Pierce), 40  $\mu g$  of protein from the supernatant of each sample was separated by SDS-PAGE and then transferred to a PVDF membrane (PerkinElmer Life Sciences). Following transfer, membranes were blocked in 5% nonfat dry milk in PBS and 0.1% Tween 20 and probed with primary antibodies. Horseradish peroxidase-conjugated secondary antibodies (Sigma) and an ECL substrate kit (PerkinElmer Life Sciences) were used in the detection of Western signals. Fractionation of cells into nuclear and cytoplasmic compartments and preparation of the corresponding extracts were performed using an NE-PER nuclear protein extraction kit (Thermo Fisher Scientific) as recommended by the manufacturer. The antibodies employed in the analysis were as follows: anti-CCAAT/enhancer-binding protein  $\alpha$  (Santa Cruz Biotechnology, Inc.), anti-Acrp30/adiponectin (Affinity BioReagents, Golden, CO), anti-aP2 and anti-PPAR $\gamma$  (Cell Signaling Technology, Inc.), and anti-HIF-1 $\alpha$  (Novus Biologicals, LLC, Littleton, CO; Abcam, Cambridge, MA).

**Microarray Gene Chips**—The preparation of samples and the procedure for microarray analysis have been described previously (22).

**Gene Expression Analysis**—RNA was isolated from cultured cells using TRIzol reagent (Invitrogen) as instructed by the manufacturer. cDNA from cultured cells was produced from equivalent amounts of total RNA (2  $\mu g$ ) using a high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Analysis of gene expression was performed using Maxima SYBR Green qPCR Master Mix (Fermentas Inc., Glen Burnie, MD) in an ABI Prism 7300 sequence detector for an initial denaturation at 95 °C for 10 min, followed by 40 PCR cycles (with each cycle consisting of 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 30 s), and SYBR green fluorescence emissions were monitored after each cycle. mRNA expression was calculated relative to TATA-binding protein for murine samples. Amplification of specific transcripts was confirmed by melting-curve profiles (cooling the sample to 68 °C and heating slowly to 95 °C with measurement of fluorescence) at the end of each PCR.<sup>3</sup>

**Knockdown of HIF-1 $\alpha$  and PPAR $\gamma$  Using siRNA**—siRNA duplexes for mouse HIF-1 $\alpha$  and PPAR $\gamma$  were obtained from

<sup>3</sup> Primer sequences used for quantitative PCR are available upon request.



**FIGURE 1. In response to hypoxia, brown adipocytes produce lower amounts of HIF-1 $\alpha$  than white adipocytes but induce higher levels of hypoxia-associated genes.** 3T3-L1 and brown preadipocytes were differentiated until day 7, at which stage cells were exposed to 20% or 1% O<sub>2</sub> for 18 h. *A*, nuclear (N) and cytoplasmic (C) cell fractions were prepared, and extracts were subjected to Western blot analysis. *B*, cells were also harvested for RNA isolation and quantitative real-time PCR analysis. Results are means  $\pm$  S.E. ( $n = 3$ ). \*,  $p < 0.01$ ; +,  $p < 0.05$ . C/EBP $\alpha$ , CCAAT/enhancer-binding protein  $\alpha$ .

Integrated DNA Technologies, Inc. (Coralville, IA). Transfection of siRNAs was achieved by using a DeliverX Plus delivery kit (Affymetrix, Inc./Panomics, Santa Clara, CA) according to the manufacturer's instructions specific to 3T3-L1 adipocytes. Briefly, on days 6–8 of differentiation, 3T3-L1 adipocytes cells were trypsinized and replated at 50–70% confluence 18 h prior to transfection. 48–72 h post-transfection, cells were harvested for isolation of protein for Western blot analysis or for isolation of RNA for gene expression analysis.

**Statistical Analyses**—Data are expressed as means  $\pm$  S.D. from three independent experiments, which were performed in duplicate. Data were analyzed using Student's *t* test, and statistically significant differences are reported when  $p$  was  $< 0.05$ .

## RESULTS

Other studies have shown that overexpression of HIF-1 $\alpha$  in WAT induces fibrosis instead of its usual targets such as the compensatory angiogenesis factor *vegfa*. Because of the fact that BAT is highly vascularized compared with avascular WAT, we hypothesized that brown adipocytes might respond more favorably to hypoxia than white cells by stimulating production of greater quantities of HIF-1 $\alpha$ . However, Fig. 1*A* demonstrates a slightly lower amount of HIF-1 $\alpha$  in brown versus white adipocytes. Interestingly, there was only a slight increase in the abundance of nuclear localized HIF-1 $\alpha$  in response to hypoxia in both white and brown adipocytes. The dramatic increase in GLUT1, an HIF-1 $\alpha$  target, in the cytoplasm of the white cells indicates that they responded to the low O<sub>2</sub>. Normoxic brown

adipocytes already expressed abundant amounts of GLUT1 that were equivalent to those levels expressed in hypoxic white adipocytes. There was some GLUT1 detected in the nuclear fraction of brown adipocytes, which is accounted for by some contamination from the cytoplasmic fraction (data not shown). The data in Fig. 1*B* demonstrate that both cells responded to hypoxia by inducing expression of known HIF-1 $\alpha$  targets, including *vegfa*, *Ero1a*, and *Egln3*. The small increase in HIF-1 $\alpha$  levels in hypoxic white and brown adipocytes suggests that induction of hypoxia genes might require nuclear factors in addition to HIF-1 $\alpha$ . To determine the importance of HIF-1 $\alpha$ , its expression was knocked down in mature white adipocytes using an appropriate siRNA. Fig. 2*A* demonstrates the effectiveness of the knockdown, resulting in an  $\sim 70\%$  decrease in HIF-1 $\alpha$  levels and leading to a significantly reduced induction of GLUT1 by exposure to 1% O<sub>2</sub>. The quantitative PCR analysis shown in Fig. 2*B* demonstrates that the knockdown also attenuated the hypoxic induction of *vegfa*, *Egln1*, and *Ndr1* mRNAs. To gain additional insight into the importance of HIF-1 $\alpha$ , white adipocytes were treated with YC-1 (an HIF-1 $\alpha$  inhibitor) during their exposure to hypoxia or with CoCl<sub>2</sub> (an HIF-1 $\alpha$  mimetic) under normoxic conditions. The data show that the hypoxic response was similarly attenuated by reduction of HIF-1 $\alpha$  using YC-1 (Fig. 3). Interestingly, treatment of normoxic cells with CoCl<sub>2</sub> failed to enhance the expression of GLUT1 protein and the HIF-1 $\alpha$  target genes (Fig. 3). These data suggest that HIF-1 $\alpha$  does facilitate the induction of its target genes in white adipocytes but likely requires additional factors that are activated only under hypoxic conditions.

We have recently shown that activation of PPAR $\gamma$  with synthetic ligands in white adipocytes in mice and in culture induces expression of a brown adipose phenotype. This is characterized by an increase in mitochondrial biogenesis and function as well as suppression of several genes coding for adipokines associated with insulin resistance, which are highly expressed in visceral WAT (22, 23). Importantly, the troglitazone-associated “browning” of white adipocytes also included a 2–5-fold enhancement in expression of many HIF-1 $\alpha$  targets (Table 1) as well as suppression of many inflammatory genes (see below). We therefore questioned whether PPAR $\gamma$  could be contributing to the hypoxic response in white adipocytes. Fig. 4*A* demonstrates that induction of the HIF-1 $\alpha$  targets by troglitazone (TZD) was modest compared with their dramatic induction by hypoxia; however, TZD did appear to enhance the hypoxic response. It is interesting that TZD prevented the induction of inflammatory genes in response to hypoxia (Fig. 4*B*).

These data show that TZD activation of PPAR $\gamma$  has both a positive and negative effect on the response of white adipocytes to hypoxia. To determine whether PPAR $\gamma$  plays a critical role in these responses, we diminished its expression in mature 3T3-L1 adipocytes using siRNA technology. Fig. 5*A* shows an almost complete knockdown of PPAR $\gamma$  along with an accompanying reduction in CCAAT/enhancer-binding protein  $\alpha$  and adiponectin expression. Exposure of control cells to 1% O<sub>2</sub> for 18 h induced expression of select HIF-1 $\alpha$  targets (*Fos11*, *PAIL*, *Eno2*, and *Ndr1*) (Fig. 5*B*) and attenuated expression of CCAAT/enhancer-binding protein  $\alpha$  and adiponectin (Fig. 5*A*) as demonstrated previously by others (6). More importantly,



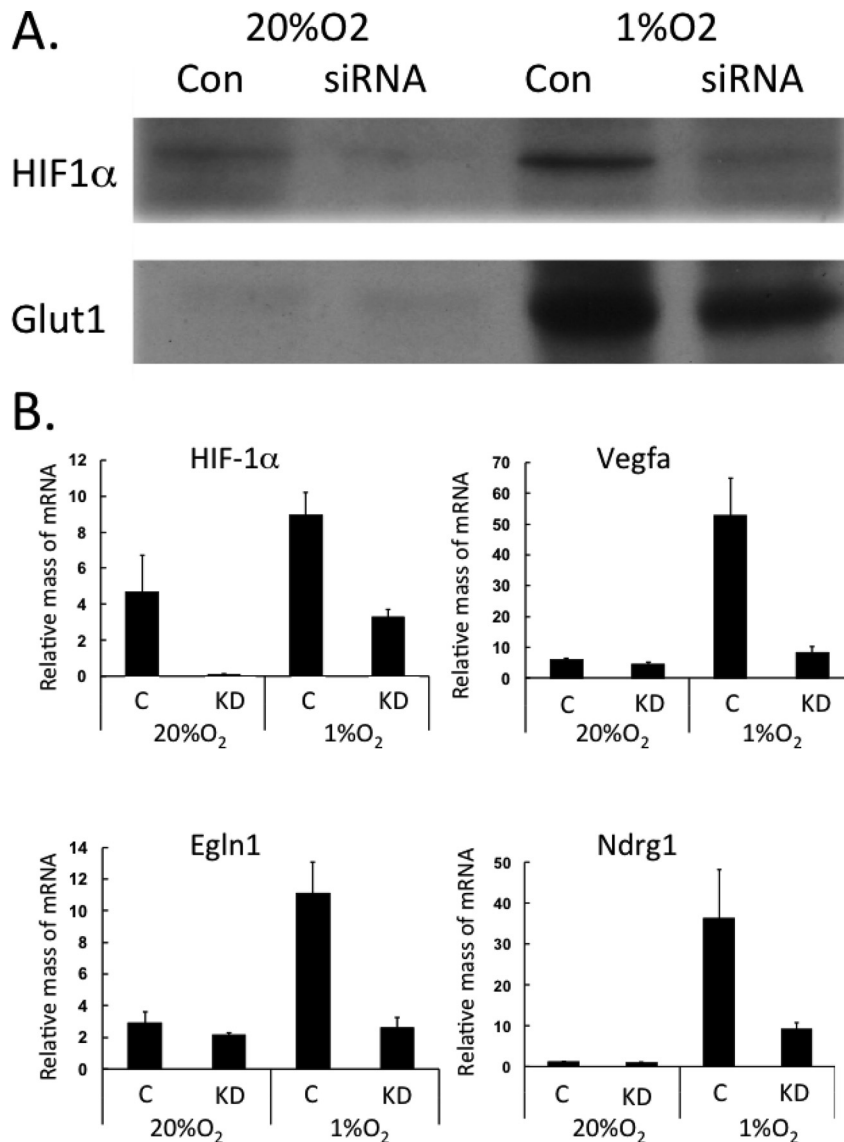
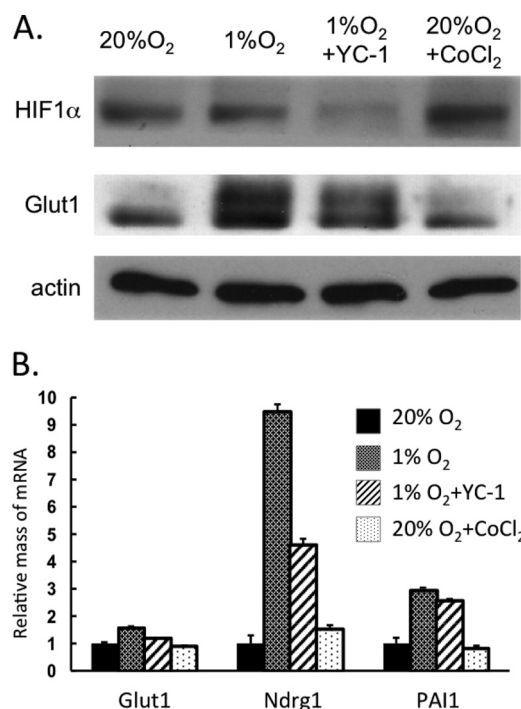


FIGURE 2. **Response of white adipocytes to hypoxia requires HIF-1 $\alpha$ .** 3T3-L1 preadipocytes were differentiated until day 8, at which time cells were exposed to DeliverX siRNA reagent alone (C, Con) or with oligonucleotides specific to HIF-1 $\alpha$  (KD). 48 h later, cells were exposed to 20% or 1% O<sub>2</sub> for 18 h. The cells were immediately harvested in lysis buffer for protein isolation and Western blot analysis (A) or in TRIzol reagent for RNA isolation and quantitative real-time PCR analysis (B). Results are means  $\pm$  S.E. ( $n = 3$ ).

the extent of induction of the HIF-1 $\alpha$  target genes by hypoxia as well as by TZD was significantly blunted in the absence of PPAR $\gamma$  (Fig. 5B). In the case of the inflammatory genes, the absence of PPAR $\gamma$  had a minimal effect on their expression in response to hypoxia, but it did significantly reduce their suppression by TZD (Fig. 5C).

In addition to promoting the browning of white adipocytes, including enhanced production of many HIF-1 $\alpha$  target genes, activation of PPAR $\gamma$  by TZD induces expression of PGC-1 $\beta$  (24). Other studies have shown that PGC-1 $\alpha$  facilitates induction of *vegfa* and compensatory angiogenesis in ischemic skeletal muscle in an HIF-1 $\alpha$ -independent manner (25). To begin to dissect the molecular mechanisms controlling the responses of adipocytes to hypoxia, we questioned whether PGC-1 $\alpha$  and/or PGC-1 $\beta$  collaborate with PPAR $\gamma$  in regulating hypoxia-associated gene expression. We chose to address this question in brown adipocytes because they express both forms of the

PGCs. Data of others demonstrated that PGC-1 $\alpha$  is dispensable for maintenance of the brown phenotype but is required for cold-induced thermogenesis (21). Importantly, loss of brown-specific gene expression occurred only following knockdown of PGC-1 $\beta$  along with PGC-1 $\alpha$  (Fig. 6A). These data highlight the importance of PGC-1 $\beta$  in regulating the brown adipocyte program. When exposed to 1% O<sub>2</sub> for 18 h, control brown adipocytes responded by significantly enhancing expression of *vegfa*, *EglN3*, and *Ero1* (Fig. 6B). The response of PGC-1 $\alpha$ -deficient cells, however, was blunted by  $\sim$ 50% compared with control cells, whereas cells deficient for both PGC-1 cofactors were incapable of responding to hypoxia (Fig. 6B). These data suggest that PGC-1 cofactors facilitate expression of hypoxia-responsive genes in brown adipocytes as well as skeletal muscle cells as observed by others (21). We also tested whether the synthetic PPAR $\gamma$  agonist TZD impacts hypoxia gene expression via PGC-1 $\alpha$  or PGC-1 $\beta$ . Interestingly, exposure of control



**FIGURE 3. HIF-1 $\alpha$  alone is incapable of inducing its target genes without accompanying exposure to hypoxia.** 3T3-L1 preadipocytes were differentiated until day 8, and then cells were exposed to 20% or 1% O<sub>2</sub> for 18 h. Some cells were also exposed to 200  $\mu$ M YC-1 under hypoxia for 18 h or to 200  $\mu$ M CoCl<sub>2</sub> under normoxia for 6 h. The cells were immediately harvested for protein isolation and Western blot analysis (A) or for RNA isolation and quantitative real-time PCR analysis (B). Results are means  $\pm$  S.E. ( $n = 3$ ).

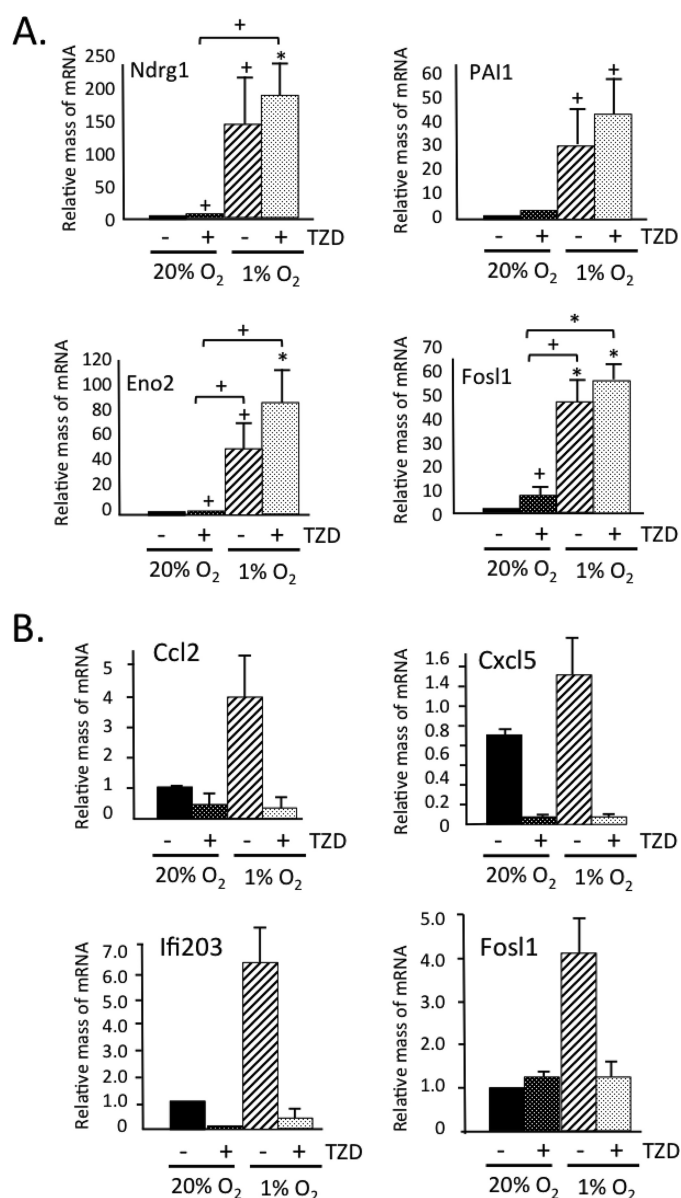
**TABLE 1**

**List of genes induced by exposure of adipocytes to troglitazone**

Swiss mouse fibroblasts ectopically expressing PPAR $\gamma$  were differentiated until day 10 with and without 5  $\mu$ M TZD and then immediately harvested in TRIzol for RNA isolation and Affymetrix microarray analysis.

Gene symbol	TZD vs. control	Gene name
<i>Chchd10</i>	18.77	Coiled coil-helix-coiled coil-helix domain 10
<i>Pdk4</i>	12.01	Pyruvate dehydrogenase kinase, isoenzyme 4
<i>Egf21</i>	11.64	Fibroblast growth factor 21
<i>PAI1</i>	4.82	Plasminogen activator 1
<i>Cox7a1</i>	4.58	Cytochrome oxidase c subunit 7a1
<i>Acot2</i>	4.50	Acyl-CoA thioesterase 2
<i>Acot1</i>	4.36	Acyl-CoA thioesterase 1
<i>Egln3 (PHD3)</i>	4.20	HIF prolyl hydroxylase 3
<i>Ero11</i>	3.54	ERO1-like ( <i>Saccharomyces cerevisiae</i> )
<i>Ndrgr1</i>	3.46	N-myc downstream regulated gene 1
<i>Trib3</i>	3.40	Tribbles homolog 3 ( <i>Drosophila</i> )
<i>GLUT1</i>	3.09	Glucose transporter 1
<i>Scd3</i>	2.94	Stearoyl-CoA desaturase 3
<i>Ghrh</i>	2.64	Growth hormone-releasing hormone
<i>Angptl4</i>	2.60	Angiopoietin-like 4
<i>Itm2a</i>	2.53	Integral membrane protein 2A
<i>Angptl6</i>	2.40	Angiopoietin-like 6
<i>Ddit3</i>	2.16	DNA damage-inducible transcript 3
<i>Hmox1</i>	1.67	Heme oxygenase (decycling) 1
<i>vegfa</i>	1.50	Vascular endothelial growth factor A

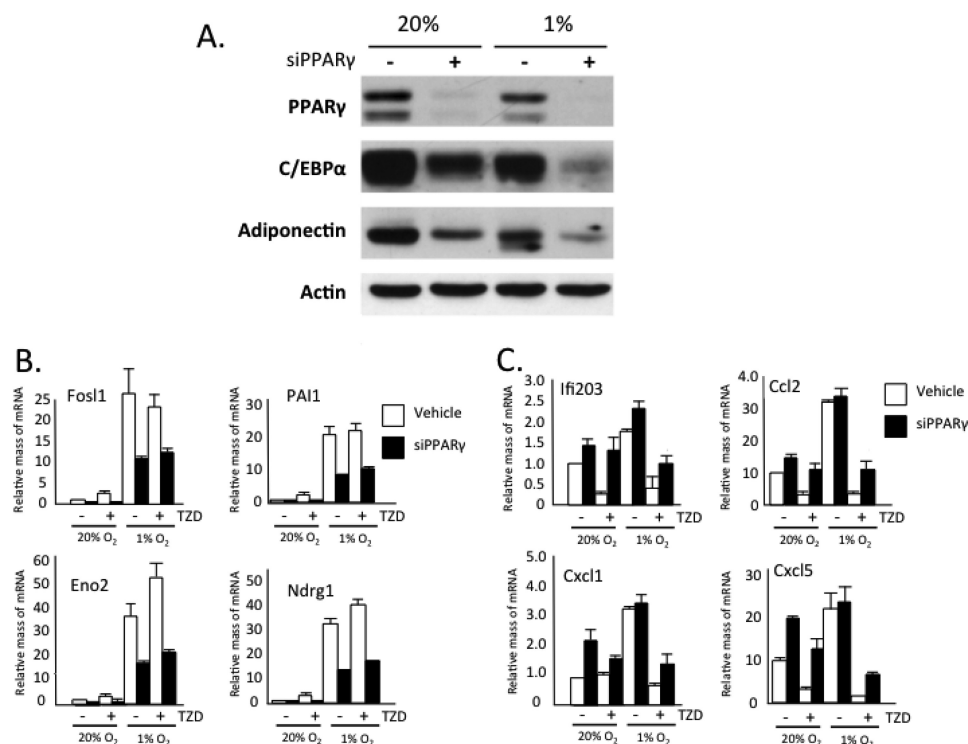
cells to the thiazolidinedione (TZD) enhanced expression of the HIF-1 $\alpha$  targets by  $\sim$ 2–5-fold compared with untreated cells (Fig. 6B). As observed for the response of these genes to hypoxia, their induction by TZD was blunted in the absence of PGC-1 $\alpha$  and completely eliminated when both PGC-1 factors were eliminated. These data suggest that the PGC-1 cofactors are mediators of the action of PPAR $\gamma$  in regulating expression of hypoxia genes in brown adipocytes.



**FIGURE 4. TZD suppresses hypoxia-associated induction of inflammatory genes and enhances expression of HIF-1 $\alpha$  targets.** 3T3-L1 preadipocytes were differentiated until day 8 with either 5  $\mu$ M TZD or Me<sub>2</sub>SO vehicle added to the medium. On day 8, cells were exposed to 20% or 1% O<sub>2</sub> for 18 h. The cells were immediately harvested in TRIzol reagent for RNA isolation and quantitative real-time PCR analysis for HIF-1 targets (A) or inflammatory genes (B). Results are means  $\pm$  S.E. ( $n = 3$ ). \*,  $p < 0.01$ ; +,  $p < 0.05$ .

**DISCUSSION**

Obese WAT is susceptible to hypoxia, whereas BAT is less likely to become hypoxic due to its high vascular density. Recent studies have suggested that white adipocytes respond poorly to hypoxia due to their inability to activate HIF-1 $\alpha$ -associated compensatory angiogenesis (1, 2). The data presented here demonstrate that white and brown adipocytes have a limited ability to induce HIF-1 $\alpha$  in response to hypoxia, but white cells require HIF-1 $\alpha$  to maximally respond to hypoxia. It appears though that HIF-1 $\alpha$  alone is not sufficient because its induction under normoxic conditions (+CoCl<sub>2</sub>) is incapable of inducing its target genes. These data suggest that additional factors activated by low O<sub>2</sub> cooperate with HIF-1 $\alpha$  to facilitate

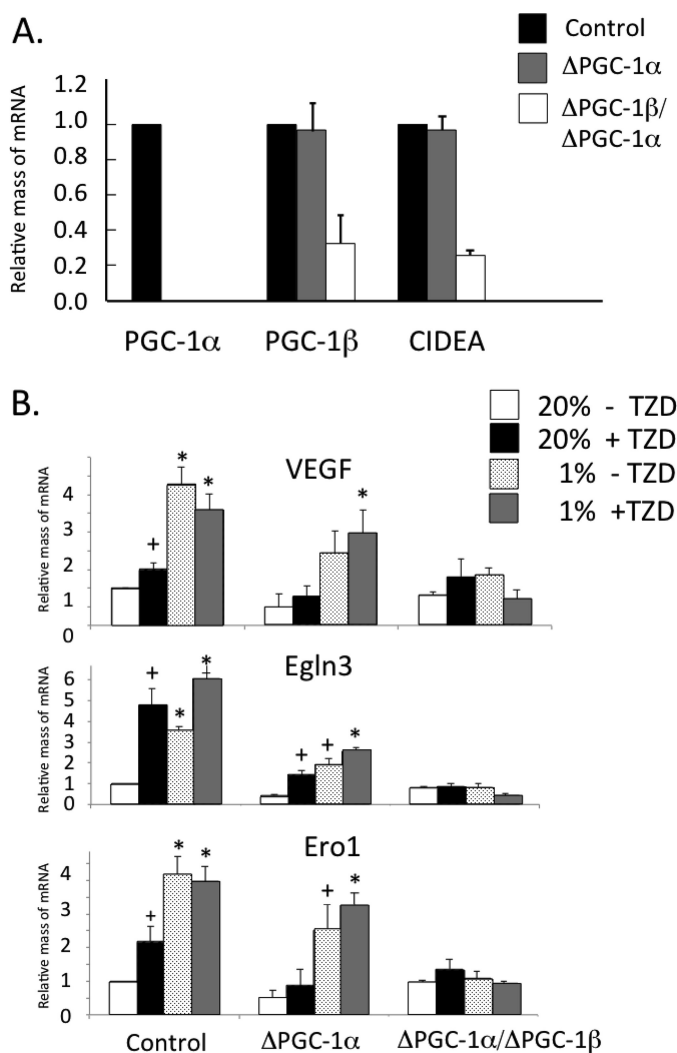


**FIGURE 5. PPAR $\gamma$  deficiency attenuates induction of hypoxia-responsive genes while increasing expression of inflammatory genes in mature hypoxic adipocytes.** 3T3-L1 preadipocytes were differentiated until day 4, and then either 5  $\mu$ M TZD or Me<sub>2</sub>SO vehicle was added to the medium until day 10. On day 8, cells were exposed to DeliverX siRNA reagent alone or with oligonucleotides specific to PPAR $\gamma$ . On day 9, cells were exposed to 20% or 1% O<sub>2</sub> for 18 h. The cells were immediately harvested in lysis buffer for protein isolation and Western blot analysis (A) or in TRIzol reagent for RNA isolation and quantitative real-time PCR analysis (B and C). Results are means  $\pm$  S.E. ( $n = 3$ ). siPPAR $\gamma$ , PPAR $\gamma$  siRNA.

the full response in white and brown adipocytes. In the case of white adipocytes, activation of PPAR $\gamma$  by TZD induced expression of HIF-1 $\alpha$  target genes and suppressed inflammatory genes that were also induced by 1% O<sub>2</sub>. Knockdown of PPAR $\gamma$  in 3T3-L1 white adipocytes demonstrated that induction of hypoxia genes was significantly attenuated in the absence of PPAR $\gamma$ , whereas induction of inflammatory genes was unaffected. Interestingly, previous studies by others demonstrated that induction of *vegfa* and angiogenesis in skeletal muscle and brown adipocytes by hypoxia is mediated by PGC-1 $\alpha$  via an HIF-1 $\alpha$ -independent mechanism (25). Our data show that the induction of multiple hypoxia-responsive genes requires PGC-1 $\alpha$  and PGC-1 $\beta$  in brown adipocytes. These data suggest a role for PPAR $\gamma$  and PGCs in regulating the response of white and brown adipocytes to hypoxia, respectively.

As discussed previously, obese WAT is hypoxic and responds by enhancing expression of fibrotic proteins as opposed to classic hypoxia-associated proteins, including those that regulate compensatory angiogenesis. In fact, fibrosis is a newly appreciated hallmark of the pathological alteration of human adipose tissue to obesity (10). Scherer and coworkers (1) have shown that transgenic expression of HIF-1 $\alpha$  in adipose tissue leads to an extensive induction of known fibrotic genes. These investigators proposed that development of fibrosis due to hypoxia activation of HIF-1 $\alpha$  in white adipose might precede progression to an insulin-resistant state (26). The data presented here are consistent with the suggestion that HIF-1 $\alpha$  is not the only mechanism by which adipocytes respond to low oxygen and that PPAR $\gamma$  and PGC-1 cofactors are also required presumably

to facilitate expression of the hypoxia-associated genes. Studies by Spiegelman and coworkers (25) showed that the angiogenic response to limb ischemia is mediated through a novel PGC-1 $\alpha$ /estrogen-related receptor pathway independent of the HIF-1 $\alpha$  pathway. Their study focused primarily on the induction of *vegfa* and angiogenesis in skeletal muscle and not on most of the other hypoxia-responsive genes. We show here that the PGC-1 cofactors are required for induction of a wide range of HIF-1 $\alpha$  target proteins that function to regulate different compensatory processes to counteract the stress of hypoxia. The fact that some of these proteins are induced during the TZD-mediated browning of WAT (Table 1) and are regulated by PGC-1 $\alpha$  suggests that they may contribute to the function of the brown adipocytes under normoxia. This possibility might explain why BAT is more highly vascularized than WAT. Interestingly, O'Hagan *et al.* (27) demonstrated that overexpression of PGC-1 $\alpha$  in skeletal muscle cells induces expression of a cohort of HIF-1 target genes under normoxic conditions through a mechanism involving stabilization of HIF-1 $\alpha$ . These authors suggested that PGC-1 $\alpha$  activity causes intracellular hypoxia due to elevated oxygen consumption resulting from PGC-1 $\alpha$ -associated mitochondrial biogenesis. It is conceivable that in brown adipocytes, PGC-1 $\alpha$ /PGC-1 $\beta$ , in addition to maintaining expression of brown-specific functions, including mitochondrial biogenesis, also facilitates the observed stabilization of HIF-1 $\alpha$  under normoxic conditions (Fig. 1). Consequently, metabolically active brown adipocytes under normoxia might express some hypoxia-associated proteins that contribute to the brown phenotype, including blood vessel for-



**FIGURE 6. PGC-1 cofactors facilitate expression of hypoxia-responsive genes in brown adipocytes.** WT, PGC-1 $\alpha$  knock-out ( $\alpha$ -KO), and PGC-1 $\alpha$  knock-out/PGC-1 $\beta$  knockdown ( $\alpha/\beta$ -KO) brown preadipocytes were differentiated until harvesting on day 8, at which stage total RNA was isolated using TRIzol reagent and analyzed by quantitative real-time PCR analysis (A). Results are means  $\pm$  S.E. ( $n = 3$ ). WT, PGC-1 $\alpha$  knock-out ( $\Delta$ PGC-1 $\alpha$ ), and PGC-1 $\alpha$  knock-out/PGC-1 $\beta$  knockdown ( $\Delta$ PGC-1 $\alpha$ /PGC-1 $\beta$ ) brown preadipocytes were differentiated until day 7, at which stage cells were exposed to 20% or 1% O<sub>2</sub> for 18 h. The cells were immediately harvested for RNA isolation and quantitative real-time PCR analysis (B). Results are means  $\pm$  S.E. ( $n = 3$ ). \*,  $p < 0.01$ ; +,  $p < 0.05$ . CIDEA, cell death-inducing DFFA-like effector A.

mation. It is possible that such a response is similar to that reported by Arany *et al.* (25), whereby PGC-1 $\alpha$  regulates expression of select HIF-1 $\alpha$  targets, independently of HIF-1 $\alpha$ . It is also likely that HIF-1 $\alpha$  is required for induction of the entire program of hypoxia-responsive genes in skeletal muscle and that the PGC-1 $\alpha$ /estrogen-related receptor pathway is unique to *vegfa* and other angiogenic regulators. A recent study demonstrated that PGC-1 $\alpha$  regulates Sirt3 expression by coactivating estrogen-related receptor  $\alpha$  bound to a corresponding response element in the promoter of the *Sirt3* gene (28). The fact that Sirt3 can influence the response of cancer cells to hypoxia (18, 19) suggests another potential link between PGCs and hypoxia-responsive gene expression. It is relevant to mention that exposure of mice to cold induced a browning of WAT as revealed by induction of UCP-1 and PGC-1 $\alpha$  but also acti-

vated angiogenesis along with *vegfa* expression in a hypoxia-independent manner (29). These data are therefore consistent with the notion that activation of the PGCs can induce angiogenesis under normoxic conditions, and this property might be a hallmark of the brown adipocyte phenotype.

In the case of white adipocytes, the fact that TZD induces expression of multiple HIF-1 $\alpha$  targets suggests that PPAR $\gamma$  and HIF-1 $\alpha$  pathways converge on each other. One possibility is that PPAR $\gamma$  directly activates the hypoxia target genes by interacting with factors bound to regulatory elements within the corresponding promoter/enhancer regions. In fact, the data suggest the involvement of additional factors that are induced by hypoxia because the extent of induction of these genes by TZD alone is significantly lower than that by low oxygen (Figs. 5B and 6B). We propose that the adipocyte senses low oxygen and activates a signaling pathway that converges on PPAR $\gamma$  and other associated nuclear factors to induce the entire program of hypoxia genes.

The inability of obese WAT to respond to hypoxia by inducing a set of compensatory processes might be due to the fact that HIF-1 $\alpha$  requires PPAR $\gamma$  and the PGC-1 cofactors for its full activity. It is known that expansion of WAT causes infiltration of macrophages and local secretion of inflammatory cytokines, which attenuate PPAR $\gamma$  activity on select target genes (30). Such a stress-induced decline in PPAR $\gamma$  activity could redirect HIF-1 $\alpha$  to regulate gene programs such as those coding for components of the extracellular matrix that have the potential to cause fibrosis. BAT might be protected from hypoxia-associated fibrosis because of a significantly lower level of cytokine-induced stress and consequently high levels of PPAR $\gamma$  activity. Clearly, additional investigations into the role of hypoxia-associated factors and adipogenic nuclear factors in regulating gene expression in hypoxic adipose tissue will provide important information concerning the development of insulin resistance in obese individuals.

*Acknowledgment*—We thank Dr. Bruce Spiegelman (Dana-Farber Cancer Institute and Harvard Medical School) for the kind gift of PGC-1 $\alpha$ - and PGC-1 $\alpha$ /PGC-1 $\beta$ -deficient brown preadipocytes.

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