Insulin and Hypoxia Share Common Target Genes but Not the Hypoxia-inducible Factor-1α*

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Both hypoxia and insulin induce common target genes, including vascular endothelial growth factors and several glycolytic enzymes. However, these two signals eventually trigger quite different metabolic pathways. Hypoxia induces glycolysis, resulting in anaerobic ATP production, while insulin increases glycolysis for energy storage. Hypoxia-induced gene expression is mediated by the hypoxia-inducible factor-1 (HIF-1) that consists of HIF-1α and the aromatic hydrocarbon nuclear translocator (Arnt). Hypoxia-induced gene expression is initiated by the stabilization of the HIF-1α subunit. Here we investigated whether insulin-induced gene expression also requires stabilization of HIF-1α. Our results indicate that hypoxia but not insulin stabilizes HIF-1α protein levels, whereas both insulin- and hypoxia-induced gene expression require the presence of the Arnt protein. Insulin treatment fails to inactivate proline hydroxylation of HIF-1α, which triggers ubiquitination of the von Hippel-Lindau protein and oxygen-dependent degradation of HIF-1α. Insulin-induced gene expression is inhibited by the presence of the phosphoinositide (PI) 3-kinase inhibitor LY294002 and the dominant negative mutant of the p85 subunit of PI 3-kinase, whereas hypoxia-induced gene expression is not. Pyrroline dithiocarbamate, a scavenger of H₂O₂, reduces insulin-induced gene expression but not hypoxia-induced gene expression. Although both hypoxia and insulin induce the expression of common target genes through a hypoxia-responsive element- and Arnt-dependent mechanism, insulin cannot stabilize the HIF-1α protein. We believe that insulin activates other putative partner proteins for Arnt in PI 3-kinase- and H₂O₂-dependent pathways.

As oxygen levels decrease, cells generate ATP mainly from anaerobic glycolysis in the cytoplasm since the lack of oxygen diminishes the oxidative phosphorylation pathway in mitochondria. The hypoxic cells enhance glucose utilization by increasing transcription of glucose transporter proteins (Glut-1 and -3) and several glycolytic enzymes. Other genes involved in systemic responses to prolonged hypoxic stress include vascular endothelial growth factor (VEGF), which induces blood vessel formation at hypoxic sites; erythropoietin, which elevates the production of red blood cells; and inducible nitric oxide synthase, which induces vasodilation (1). These diverse target genes are induced by a common transactivator, hypoxia-inducible factor 1 (HIF-1). HIF-1 is composed of two subunits, HIF-1α and Arnt, both of which contain a basic-helix-loop-helix (bHLH) domain and PAS (Per-Arnt-Sim) domains.

Recent studies demonstrated that hydroxylation of HIF-1α at the 564 proline residue is catalyzed by HIF-1α proline hydroxylase using molecular oxygen as the substrate. The tumor suppressor von Hippel-Lindau (VHL) protein specifically interacts with hydroxylated HIF-1α and mediates the assembly of a complex that activates a ubiquitin-dependent proteasome. Ubiquitinated HIF-1α is degraded by the proteasome. When cells lack oxygen, proline is not hydroxylated, and therefore HIF-1α protein is accumulated (2–5). Stabilized HIF-1α protein translocates into nuclei and makes a heterodimer with its partner Arnt. The HIF-1α/Arnt heterodimer specifically contacts the hypoxia-responsive elements (HREs: -ANACGTGC-), recruits their coactivator p300/CBP, and increases transcription of their target genes. It is expected that the basic region of Arnt contacts CGTG sequences, whereas HIF-1α determines the half-site specificity of HRE (6).

Besides hypoxia, the nutritional state and hormones of the cell also regulate transcription of many glycolytic enzymes required for maintaining metabolic homeostasis. Insulin plays a central role in regulating the metabolic pathways associated with energy storage and utilization. It triggers the conversion of glucose into glycogen and triglycerides and inhibits gluconeogenesis. Insulin has been known to modulate cellular metabolism by modifying the activity or changing the cellular location of preexisting enzymes. Recently the regulation of gene expression by insulin has been recognized as a major function of this hormone (7). Insulin increases the transcription of fatty-acid synthase and acetyl-CoA synthetase, both of which are involved in lipogenesis. Insulin also increases the transcription of Glut-1 and phosphoglycerate kinase-1 (PGK-1), proteins involved in glucose uptake and glycolysis. However, insulin inhibits de novo synthesis of phosphoenolpyruvate carboxykinase and pyruvate kinase, enzymes involved in gluconeogenesis (8). Some of these genes direct the synthesis of enzymes that have a well established metabolic connection to insulin, while others do not even though they are up-regulated by insulin. These genes include insulin-like growth factor; PAS; Period/Aryl hydrocarbon receptor/Single-minded; PGK-1, phosphoglycerate kinase-1; PI, phosphoinositide; PDTC, pyrrolidinedithiocarbamate; Sim, Single-minded; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau; CBP, cAMP-response element-binding protein (CREB)-binding protein; PPAR, peroxisome proliferator-activated receptor; MEM, minimum Eglı’s medium; DTT, dithiothreitol; GST, glutathione S-transferase; TOR, target of rapamycin; IPAS, inhibitory PAS domain protein.

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§ The abbreviations used are: Glut-1, glucose transporter-1; Arnt, aryl hydrocarbon receptor nuclear translocator; bHLH, basic-helix-loop-helix; HIF-1, hypoxia-inducible factor-1; HRE, hypoxia-responsive element; IGFl, insulin-like growth factor; PAS, Period/Aryl hydrocarbon receptor/Single-minded; PGK-1, phosphoglycerate kinase-1; PI, phosphoinositide; PDTC, pyrrolidinedithiocarbamate; Sim, Single-minded; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau; CBP, cAMP-response element-binding protein (CREB)-binding protein; PPAR, peroxisome proliferator-activated receptor; MEM, minimum Eglı’s medium; DTT, dithiothreitol; GST, glutathione S-transferase; TOR, target of rapamycin; IPAS, inhibitory PAS domain protein.
genes represent major secretory proteins, such as VEGFs (9), erythropoietin (10), nerve growth factor (11), endothelin-1 (12), and transcription factors such as peroxisome proliferator-activated receptors (PPARs) and CCAAT/enhancer-binding protein δ (13).

In contrast to other hormone response elements, consensus cis-acting elements and their unique trans-acting factors have not been discovered among these diverse genes affected by insulin. Strikingly most hypoxia-inducible genes are also induced by insulin. These genes include VEGF, erythropoietin, endothelin-1 (14), p85α checkpoint enzyme, and glucose transporters. Zelzer et al. (15) demonstrated that insulin increases the transcription of these target genes through an HRE by inducing the formation of an HRE-protein complex. Arnt is required for both insulin- and hypoxia-induced gene expression. Although hypoxia and insulin share common target genes, they have opposite effects on metabolism: energy consumption versus energy conservation (1, 16, 17).

In this study, we investigated how two stimuli, hypoxia and insulin, share signaling pathways to induce the same set of target genes. Our results demonstrate that the phosphoinositol (PI) 3-kinase pathway and reactive oxygen species are involved in insulin- but not in hypoxia-induced gene expression. Insulin treatment induces HRE-dependent gene expression without increasing levels of the HIF-1α protein, implying that insulin-induced HRE-responsive transcription functions independently of HIF-1α.

**EXPERIMENTAL PROCEDURES**

**Materials and Plasmids**—The anti-HIF-1α antibody was obtained from Transduction Laboratories (H72320). The immunogenic region of this antibody is located within amino acids 610–727 of human HIF-1a.

The anti-Arnt antibody was obtained from Transduction Laboratories. The p[HRE]luc reporter plasmid contains four copies of the erythropoietin hypoxia-responsive element (5′-GATCCGCTACTAGCT-GTCTCA-3′, nucleotides 3449–3470), the SV40 promoter, and the firefly luciferase gene (18). Plasmid Δp85 encodes a dominant negative mutant of the p85 subunit of PI 3-kinase (Δp85), which abolishes the activity of this regulatory subunit to bind to the 110-kDa catalytic subunit of PI 3-kinase (19). LY294002 and Wortmannin were obtained from Calbiochem-Novabiochem. CoCl2 and pyrrolidinedithiocarbamate (PDTC) were obtained from Sigma.

**Cell Cultures and Treatments**—Wild-type mouse Hepa1c1c7 cells and Arnt-defective Hepa1c1c7 cells were cultured in α-MEM supplemented with 10% fetal bovine serum. 48 h after transfection, cell extracts were prepared and analyzed by a luminometer (Berthold Lumat LB9501) using the luciferase assay system (Promega). Luciferase activity was normalized for total protein concentration as determined by the Bradford assay using bovine serum albumin as a standard. The trend was monitored by measuring cotransfection of the β-galactosidase-encoding plasmid (pCH110) and β-galactosidase activity.

**Western Analysis of HIF-1α and Arnt**—Cells were plated at 1 × 10^6 cells/well of a 12-well plate. 18 h later, transfection was carried out using the LipofectAMINE Plus reagent according to the manufacturer’s instructions (Invitrogen). 20–24 h prior to hypoxic treatment, transfected cells were serum-starved with medium containing 0.5% fetal bovine serum. 48 h after transfection, cell extracts were prepared and analyzed by a luminometer (β-galactosidase activity).

**In Vitro HIF-1α-VHL Interaction Assay**—HeLa cells were serum-starved for 48 h and then treated with insulin (100 nM) and hypoxia (0.1% oxygen) for 4 h prior to lysis preparation. Treated HeLa cells were harvested, collected by centrifugation, and washed once with ice-cold hypotonic buffer (10 mM Hepes-NaOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM DT). To make the S-100 cytoplasmic fraction, the cell pellet was resuspended at 10^6 cells/0.5 ml of hypotonic buffer. After incubation on ice for 10 min, the cells were disrupted by homogenizing 15 times in a Wheaton Dounce homogenizer using a type B pestle. The lysates were centrifuged at 3,300 × g for 15 min at 4 °C, resin-containing GST-HIF-1α-(401–603) was washed three times with ice-cold hypotonic buffer (10 mM Hapes-NaOH, pH 7.9, 1.4 mM KCl, 0.03 mM MgCl2) and added to the supernatant cytoplasmic fractions. This cytoplasmic fraction was centrifuged at 100,000 × g for 4 h (21). The resulting supernatant, the S-100 cytoplasmic fraction, was used for the following in vitro hydroxylation reaction.

**S-10**Methionine-labeled VHL protein was synthesized using an in vitro transcription and translation kit according to the instruction manual (Promega, catalog number L1170). Glutathione S-transferase (GST)-HIF-1α-(401–603) fusion protein was expressed in Escherichia coli and purified using glutathione uniflow resin according to the instruction manual (Clontech, Palo Alto, CA). All buffers and reagents, S-100 cytoplasmic fraction, GST-HIF-1α-(401–603) were preincubated with 200 μM of the S-100 cytoplasmic fraction (1,000 μg of total proteins) for 90 min at 30 °C. The reaction mixture was centrifuged and washed with NETN buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). The res insulin-GST-HIF-1α-(401–603) was mixed with 10 μl of S-10-labeled VHL. After 2 h at 4 °C, resin-containing GST-HIF-1α-(401–603) was washed three times. Proteins were eluted in 3× SDS sample buffer, fractionated by SDS-PAGE, and detected by autoradiography.

Reactions in hypoxia were performed in a 0.1% oxygen, 5% CO2, 85% N2, and 10% H2 atmosphere created using a work station within the anaerobic incubator (Model 1029, Forma Scientific, Inc.). All buffers and reagents, S-100 cytoplasmic fraction, GST-HIF-1α-(401–603) were preincubated in this hypoxic atmosphere for 16–18 h on ice. S-10-labeled in vitro translated VHL was preincubated in a hypoxic atmosphere for 4 h on ice prior to the hydroxylation reaction (3, 5, 22).

**Preparation of Nuclear Extracts**—Cells were serum-starved by incubation in α-MEM containing 0.5% fetal bovine serum for 40–48 h and then incubated in 0.1% O2 for 6 h. Nuclear extracts were prepared as described previously (45). 70% confluent cells in 100-mm tissue culture plates were washed twice with cold phosphate-buffered saline, resuspended in 4 packed cell volumes of buffer A (10 mM Tris- HCl, pH 7.8, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.4 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM Na3VO4), and incubated on ice for 10 min. Subsequently the cells were homogenized by 15 strokes with a Dounce type B pestle. The nuclei were pelleted by centrifugation at 33,000 × g for 15 min at 4 °C and resuspended in 2 packed nuclei volumes of buffer B (20 mM Tris-
RESULTS

Insulin and Hypoxia Share Common Target Genes—Both insulin and hypoxia are known to increase the de novo synthesis of several glycolytic enzymes, glucose transporter proteins, and VEGF in various cells. Our Northern analysis confirmed that both stimuli increase the expression of a common set of target genes in human Hep3B cells and preadipocyte 3T3-L1 cells. In these cells, hypoxia increases the mRNA level of VEGF, PKG-1, and Glut-1 as indicated in Fig. 1A. Similarly insulin stimulates the expression of the same target genes. The same intensities of 18 S/28 S rRNA in each lane confirmed that the same amount of RNA was loaded for each sample. Hypoxia-induced VEGF mRNA was identified as multiple bands that represent different size mRNAs of VEGF isomers, whereas insulin increases the mRNA of only the largest isomer. Hypoxia is also known to increase the mRNA level of VEGF by stimulating transcription and increasing the stability of VEGF mRNA.

Hypoxia-inducible gene expression is mediated by common cis-acting elements, HREs located in the regulatory region of several hypoxia-inducible genes. To confirm whether the HRE is also able to mediate insulin-induced gene expression, we transiently transfected Hep3B cells with HRE-driven reporter plasmids and then treated these cells with hypoxia and/or insulin (Fig. 1B). Our findings indicate that insulin and hypoxia significantly increase expression of the luciferase reporter gene. Dual treatment with insulin (100 nM) and hypoxia (0.1% O2) synergistically increases the expression of HRE-driven luciferase. In contrast, dual treatment with insulin (100 nM) and hypoxia (0.1% O2) fails to show an additive effect on induction of the endogenous target genes. Our finding that insulin and hypoxia synergistically increase HRE activity implies that the two stimuli activate HRE via different pathways.

Hypoxia Stabilizes the HIF-1α Protein but Insulin Does Not—Hypoxia-induced gene expression is mediated by the HIF-1α/Arnt heterodimeric transcription activator, which specifically binds to its cognitive cis-acting element, HRE. The responsiveness of the HIF-1 complex is due to the hypoxia-induced stabilization of the HIF-1α protein, which is otherwise degraded by a ubiquitin-dependent proteasome under normoxic conditions. We investigated whether insulin also stabilizes the HIF-1α protein as does hypoxia. Western analyses demonstrated that hypoxia or CoCl2 increases the protein level of HIF-1α in various human liver cells (including HepG2, Hep3B, SK-Hep1, and SNU368), the human breast cancer cell line (MCF7 cells), and the human kidney cell line (293 cells).
HIF-1α with VHL, we investigated whether proline hydroxylation of its proline residue (Pro-564) and interaction from all three lysates were inhibited upon exposure to a hypoxic medium containing 0.5% fetal bovine serum for 36–48 h and then treated with insulin (100 nM) and/or hypoxia (CoCl₂ (100 μM) or 0.1% O₂) for 6 h. 30 μg of whole cell lysates were separated by 8% SDS-PAGE and transferred to a Nytran membrane. Immunoblot analysis was performed using anti-human HIF-1α antibody (Transduction Laboratories) and visualized using a chemiluminescence-based system. B, Hep3B cells were treated with various doses of insulin or hypoxia for various times as shown above. Cells were lysed in RIPA buffer. Immunoblot analyses were performed using anti-human HIF-1α antibody. C, 293 cells were treated as shown. Immunoblot analyses were performed using anti-mouse HIF-1α antibody (Transduction Laboratories), anti-phospho-Akt (pAkt) antibody, or anti-Akt antibody.

(Fig. 2). In contrast, the protein levels of Arnt remain unchanged after treatment with either hypoxia or insulin. To test whether insulin increases the level of HIF-1α proteins, we treated Hep3B cells with different doses of insulin for various times. We could not detect stabilized HIF-1α in either Hep3B cells or any other cell lines that were treated with insulin (Fig. 2B). We confirmed that insulin (100 nM, 6 h) activates a downstream protein kinase by demonstrating phosphorylation of the Akt kinase in 293 cells (Fig. 2D). Our results suggest that hypoxia induces target genes, including VEGF, PGK-1, and Glut-1, by stabilizing HIF-1α but that insulin does not require the stabilization of HIF-1α for the induction of the same set of target genes.

Hypoxia but Not Insulin Reduces the Interaction between VHL and HIF-1α—Since degradation of HIF-1α is triggered by hydroxylation of its proline residue (Pro-564) and interaction with VHL, we investigated whether proline hydroxylation of HIF-1α decreases in insulin-treated cells. We prepared cell lysates from glycerol, PGK-1, and Glut-1, by stabilizing HIF-1α but that insulin does not require the stabilization of HIF-1α for the induction of the same set of target genes.

FIG. 2. HIF-1α protein in hypoxia- or insulin-treated cells. A, before stimulation, various human cell lines were serum-starved with medium containing 0.5% fetal bovine serum for 36–48 h and then treated with insulin (100 nM) and/or hypoxia (CoCl₂ (100 μM) or 0.1% O₂) for 6 h. 30 μg of whole cell lysates were separated by 8% SDS-PAGE and transferred to a Nytran membrane. Immunoblot analyses were performed using anti-human HIF-1α antibody (Transduction Laboratories) and visualized using a chemiluminescence-based system. B, Hep3B cells were treated with various doses of insulin or hypoxia for various times as shown above. Cells were lysed in RIPA buffer. Immunoblot analyses were performed using anti-human HIF-1α antibody. C, 293 cells were treated as shown. Immunoblot analyses were performed using anti-mouse HIF-1α antibody (Transduction Laboratories), anti-phospho-Akt (pAkt) antibody, or anti-Akt antibody.

or insulin but that it instantly loses its activity in the absence of its oxygen substrate (3). Insulin usually induces covalent modification of its target enzymes through intrinsic receptor tyrosine kinase-dependent signaling pathways. Our results rule out the possibility that insulin induces covalent modification of HIF-1α proline hydroxylase leading to persistent repression of its activity.

Arnt Is Required for the Induction of Genes by Both Hypoxia and Insulin—To test whether Arnt is also required for insulin-induced gene expression, we used variant Hapa1c1c7 cells that are defective in Arnt expression. Arnt-defective cells had been reconstituted with Arnt by retroviral infection using pMFG retroviral vector that has no selective antibiotic resistance gene (20). Thereby these reconstituted cells have heterogeneous populations in terms of Arnt protein expression. Although the collective expression level of Arnt protein in the reconstituted cells is greater than that of wild-type cells, the individual cell
has a different expression level of Arnt (Fig. 4A). For this reason, we suspect that these reconstituted cells could not demonstrate the exact expression profile of the wild-type cells. We treated three types of cells with either hypoxia or insulin and Northern analysis. The results shown in Fig. 4B indicate that the absence of Arnt significantly reduces hypoxia- and insulin-induced expression of VEGF and PGK-1. The intensity of 18S/28S rRNA was equivalent in all lanes. However, Arnt addition in Arnt-defective cells restored hypoxia- and insulin-induced expression of VEGF and PGK-1 in part (Fig. 4B).

Our findings emphasize that Arnt is essential for both hypoxia- and insulin-induced gene expression. To confirm that Arnt is essential for both hypoxia- and insulin-induced activity of HRE, we transiently transfected three types of Hepa1c1c7 cells with the HRE reporter plasmid (Fig. 4C). The results indicate that the presence of Arnt is required for both hypoxia- and insulin-induced HRE activity. Our results raise the question of how insulin increases the HRE-dependent gene expression without stabilizing HIF-1α and how Arnt is involved in insulin-induced gene expression without HIF-1α.

**The Differential Effect of the PI 3-Kinase Pathway on Insulin- and Hypoxia-induced Gene Expression**—Our findings imply that both hypoxia and insulin activate the same set of target genes in an Arnt-dependent manner, but they do so via different signaling pathways since the former involves stabilization of HIF-1α, while the latter does not. In this context, we investigated the differences between the signaling pathways for insulin- and hypoxia-induced gene expression. Insulin binds to a cell surface receptor and activates an intrinsic receptor tyrosine kinase that activates PI 3-kinase and potential downstream effectors, including the serine/threonine kinase Akt (23, 24) or reactive oxygen species production (25). We investigated how inhibition of PI 3-kinase affects hypoxia- or insulin-induced gene expression. Insulin binds to a cell surface receptor and activates an intrinsic receptor tyrosine kinase that activates PI 3-kinase and potential downstream effectors, including the serine/threonine kinase Akt (23, 24) or reactive oxygen species production (25). We investigated how inhibition of PI 3-kinase affects hypoxia- or insulin-induced gene expression. Insulin binds to a cell surface receptor and activates an intrinsic receptor tyrosine kinase that activates PI 3-kinase and potential downstream effectors, including the serine/threonine kinase Akt (23, 24) or reactive oxygen species production (25).
Hypoxia and Insulin Do Not Share HIF-1α

3-kinase affects insulin- and hypoxia-induced gene expression, we cotransfected Hep3B cells with an HRE-driven reporter plasmid together with increasing amounts of plasmids that encode a dominant negative mutant of the 85-kDa regulatory subunit of the PI 3-kinase (Δp85) that cannot recruit the catalytic subunit of the PI 3-kinase (Δp110) (Fig. 5B). Overexpression of the dominant negative pΔ85 mutant reduced insulin-induced activation of HRE more dramatically than hypoxia-induced activation of HRE, implying that insulin leads to the activation of HREs via the PI 3-kinase pathway but that hypoxia-induced HRE activation is less sensitive to PI 3-kinase inhibitors (32). Forced expression of constitutively active Akt fails to increase protein levels of HIF-1α.

In this context, we tested how PDC and TCA, a thiol reductant scavenger of H2O2, affects hypoxia- or insulin-induced gene expression. Pretreatment with PDC (50 and 100 μM) dramatically reduces insulin-induced expression of PGK-1 and GLUT-1, whereas it does not affect hypoxia-induced expression of the same genes (Fig. 7). Our results indicate that insulin- but not hypoxia-induced gene expression shows sensitivity to H2O2.

**DISCUSSION**

Acute intensive insulin therapy increases VEGF expression in the retina of diabetic rats, leading to blood-retina barrier breakdown that causes a worsening of diabetic retinopathy. It was demonstrated that retinal nuclear extracts from insulin-treated rats contain increased levels of a protein that is detected by an anti-HIF-1α antibody (Sigma) and increased protein binding to HRE in the VEGF promoter (29). In the arising retinal pigment epithelial cell line-19 (ARPE-19) and human colon carcinoma cells (HCT116), insulin- or IGF-1-induced HIF-1α protein was detected using anti-HIF-1α antibody (clone H1106, Novus Biologicals, Inc.). The facts that insulin- and IGF-1-mediated increases in HIF-1α protein levels were blocked by inhibitors of PI 3-kinase or the target of rapamycin (TOR) and that IGF-1 increases phosphorylation of the translational regulatory proteins eIF-4B1, p70 S6 kinase, and eukaryotic initiation factor 4E suggested that IGF-1 treatment increases the synthesis rate of the HIF-1α protein through a PI 3-kinase/Akt/TOR pathway (30, 31).

Other studies suggest that activation of PI 3-kinase/Akt pathways by hypoxia is cell type-specific and is not required for hypoxic stabilization of HIF-1α (32). Forced expression of constitutively active Akt fails to increase protein levels of HIF-1α.
Fig. 6. Hypoxia- or insulin-induced HRE-protein complex. A, oligonucleotide W18 contains the HRE sequences in the erythropoietin enhancer region. M18 has three base pair substitutions that abolish its ability to interact with HIF-1. Hep3B cells were serum-starved with medium containing 0.5% fetal bovine serum for 48 h and were unstimulated (lane 1) or stimulated for 6 h with 0.1% O2 (lanes 5, 6, 7, and 8) or with 100 nM insulin (lanes 2, 3, 4, and 8). Nuclear extracts were incubated with radiolabeled W18 in the presence or absence of 100-fold molar excess of unlabeled W18 (lanes 3 and 6) and M18 (lanes 4 and 7). The mixtures were analyzed by electrophoretic mobility shift assay. Insulin-induced protein-HRE complexes are indicated with an arrow, and hypoxia-induced protein-HRE complexes are indicated with a closed triangle. B, serum-starved Hep3B cells were pretreated with the PI 3-kinase inhibitor LY294002 (10 or 25 μM in Me2SO) for 30 min prior to insulin (100 nM, 6 h) or hypoxia (0.1%, 6 h) treatment. Nuclear extracts were incubated with radiolabeled W18 from treated cells and subjected to electrophoretic mobility shift assay using the radiolabeled oligonucleotide W18 as described above. C, the nuclear extracts (10 μg) of Hep3B cells were mixed with radiolabeled W18 followed by incubation with either 1 μg of anti-HIF1α antibody (upper panel) or 1 μg of anti-Arnt antibody (lower panel) for 2 h at 4 °C prior to loading; N, normoxia; H, hypoxia; I, insulin.

Fig. 7. The effect of the reactive oxygen species inhibitor PDTC on insulin- and hypoxia-induced gene expression. Hep3B cells were serum-starved with medium containing 0.5% fetal bovine serum for 36–48 h. Cells were pretreated with the PDTC doses as described above for 30 min prior to 16-h treatment with insulin (100 nM) or hypoxia (0.1% O2). Total RNA was isolated and separated on a 1% formaldehyde-agarose gel. RNA was transferred onto a Nytran membrane and hybridized with α-32P-labeled cDNA of PGK-1 or Glut-1. mRNA levels were visualized by exposure to x-ray film.

in the murine pro-B cell line FL5.12, indicating that Akt-mediated downstream signaling is not enough to increase HIF-1α protein levels in these cell lines (33). In contrast, it was observed that forced expression of a hydroxysteroidoxigen-regulatable form of Akt in the absence of insulin induces Glut-1 mRNA to levels comparable to those induced by insulin in hepa1c1c7 cells, suggesting that activation of the Akt cascade is sufficient to induce glut-1 gene expression (34). In HepG2 cells, other studies have demonstrated that IGF treatment activates phosphorylation of Akt but fails to increase levels of a protein that is detected by anti-HIF-1α antibody (Transduction Laboratories) (32).

Hypoxia stabilizes HIF-1α almost instantaneously, and reoxygenation reduces DNA binding of HIF-1 within 2 min in HeLa S3 cells (35). These kinetic analyses show the quickness of HIF-1α proline hydroxylase and VHL-dependent degradation. To accumulate HIF-1α protein, the rate of its synthesis must exceed the rate of its degradation. Our results demonstrate that neither hypoxia nor insulin treatments could persistently inhibit the activity of HIF-1α proline hydroxylase; rather the absence of its substrate, oxygen, instantly limits the enzyme reaction of lysates from both hypoxia- and insulin-treated cells.

The following previous observations suggested that both hypoxia and insulin may increase HIF-1α protein levels. (i) Insulin and hypoxia share common target genes, such as VEGF, PGK-1, and Glut-1; (ii) insulin induces novel protein interactions on HRE; and (iii) both insulin and hypoxia fail to induce expression of VEGF and glycolytic enzymes in the absence of Arnt. However, we could not detect the HIF-1α protein in insulin-induced cells using an anti-HIF1α antibody that recognizes amino acids 610–727 of human HIF-1α (Transduction Laboratories). It has been shown that the same antibody could also not detect HIF-1α protein in IGFinduced HepG2 cells (32). These results suggest that the insulin-induced protein is not identical to hypoxia-induced HIF-1α in HepG2 cells.

Although insulin and hypoxia share common target genes such as VEGF, erythropoietin, and other glycolytic enzymes, these two signals eventually manifest opposite effects in terms of energy conservation and cell differentiation. Therefore, insulin and hypoxia must also have unique target genes. Several studies demonstrated that insulin increases gene expression of PPARs and other genes necessary for adipogenesis and lipogenesis (36, 37). In contrast, hypoxia inhibits lipogenesis and adipogenesis resulting from energy surplus. Yun et al. (38) demonstrated that hypoxia suppresses PPARγ2 expression and adipogenesis in a HIF-1α-dependent manner. In addition, hypoxia down-regulates the expression of PPARβ/δ in a HIF-1α-dependent manner. Hypoxia induces protein binding at the HRE site in the regulatory region of the PPARα gene (39). If insulin also activates HIF-1α, it is unclear how insulin increases the expression of PPARs and adipogenesis in the presence of the
negative regulator HIF-1α. In the eye, corneal epithelia and rods and cones of retina specifically express the inhibitory PAS domain protein (IPAS) that is known to interact with HIF-1α and complex formation between HRE and the HIF-1α heterodimer. Therefore, IPAS in the cornea and retina represses angiogenesis and maintains an avascular phenotype even under hypoxic conditions. Acute intensive insulin treatment often causes retina-blood barrier breakdown by increasing VEGF expression in retina. In this context, any insulin-induced HIF-1α protein might escape the inhibitory effects of IPAS to induce expression of VEGF in retinas.

Based on these observations, we believe that insulin activates a novel Arnt partner protein that is similar but not identical to HIF-1α. The question is how different bHLH-PAS combinations recognize the same DNA sequences. Several studies have suggested that different bHLH-PAS combinations recognize the same DNA sequences. Besides HIF-1α, Arnt forms a dimer with AhR, Sim, Trh, or Arnt itself suggesting that the Arnt protein has a broad range of interactions among the bHLH-PAS proteins (6). The cis-acting elements for various dimers such as Arnt/Arnt, Arnt/AhR, Arnt/HIF-1α, Arnt/Sim, and Arnt/Sim are summarized in Fig. 7. The DNA recognition sequences for various heterodimers or the Arnt homodimer revealed that the 3’ recognition sequence contains a CGTG motif. Crosslinking experiments demonstrated that the typical basic region of Arnt contacts this CGTG sequence, which is part of a dioxin-responsive element and which is identical to half of the typical basic region of Arnt that binds the perfect palindromic E box sequence (CACGTG). Swanson et al. (42) used a DNA selection and amplification method, the consensus sequence for Sim/Arnt heterodimer binding was deduced (42). C, using an oligonucleotide amplification method, the consensus sequence for Sim/Arnt heterodimer binding was deduced (42). D, the consensus sequence for HIF-1α/Arnt binding is shown. Several HRE sequences. in hypoxia-induced genes are shown (43). Epo, erythropoietin; HO-1, heme oxygenase-1.

### Table: Comparisons of cis-acting elements for Arnt-containing heterodimeric transactivators

| Heterodimers       | cis-elements |
|--------------------|--------------|
| (A) Arnt+Arnt      | 5′-T T G C G T G N A A A G G N N-3′ |
| (B) Arnt+Arnt      | 5′-C A C C C T G 5 N N,-3′ |
| (C) Sim+Arnt       | 5′-G T T C G T G A N N T C C-3′ |
| (D) HIF-1α/Arnt    | 5′-A A C C C T G C N N N-3′ |

1. PKG1: CGCCTGTCAGAGAGAGAGAGA
2. PKG1: CAAGAAGCTAGAA
3. PKG1: TGGAGAGCTGTCTAAGT
4. Glut1: TGGAGAGCTGTCTAAGT
5. Glut1: TGGAGAGCTGTCTAAGT
6. Glut1: TGGAGAGCTGTCTAAGT
7. VEGF: CATACGTTGGCTC
8. HO-1: CGGCGTGCAGGC

Arnt-containing heterodimers. By analogy, in *Drosophila* bHLH-PAS proteins Trh (tracheaeless) and Sim (Single-minded) bind to the same DNA recognition sequences, which contain the CGTG motif even though they activate different sets of target genes. Studies using various Trh-Sim chimeras revealed that replacement of the basic DNA binding domains does not alter target specificity, whereas replacement of the Trh PAS domain with the PAS domain of Sim dramatically changes the ability of the chimera to recognize target genes of Sim (44).

In this study, our results demonstrate that insulin and hypoxia induce a common set of target genes, including VEGF, PGK-1, and Glut-1, through an HRE- and Arnt-dependent pathway. Our work demonstrates that (i) the anti-HIF-1α antibody that we used cannot detect a protein in insulin-treated cells, (ii) hypoxia but not insulin can repress HIF-1α-VHL interaction, and (iii) PI 3-kinase and H2O2 are responsible for insulin-induced gene expression but not for hypoxia-induced gene expression. Together these results suggest that insulin activates other putative Arnt partner proteins that can interact with the HRE of genes that are a part of PI 3-kinase- and H2O2-dependent pathways.

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