Role of hypoxia in obesity-induced disorders of glucose and lipid metabolism in adipose tissue

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Yin J, Gao Z, He Q, Zhou D, Guo Z, Ye J. Role of hypoxia in obesity-induced disorders of glucose and lipid metabolism in adipose tissue. Am J Physiol Endocrinol Metab 296: E333–E342, 2009.—Recent studies suggest that adipose tissue hypoxia (ATH) may contribute to endocrine dysfunction in adipose tissue of obese mice. In this study, we examined hypoxia’s effects on metabolism in adipocytes. We determined the dynamic relationship of ATH and adiposity in ob/ob mice. The interstitial oxygen pressure (PO2) was monitored in the epididymal fat pads for ATH. During weight gain from 39.5 to 55.5 g, PO2 declined from 34.8 to 20.1 mmHg, which are 40–60% lower than those in the lean mice. Insulin receptor-β (IRβ) and insulin receptor substrate-1 (IRS-1) were decreased in the adipose tissue of obese mice, and the alteration was observed in 3T3-L1 adipocytes after hypoxia (1% oxygen) treatment. Insulin-induced glucose uptake and Akt Ser473 phosphorylation was blocked by hypoxia in the adipocytes. This effect of hypoxia exhibited cell type specificity, as it was not observed in L6 myotubes and βTC6 cells. In response to hypoxia, free fatty acid (FFA) uptake was reduced and lipolysis was increased in 3T3-L1 adipocytes. The molecular mechanism of decreased fatty acid uptake may be related to inhibition of fatty acid transporters (FATP1 and CD36) and transcription factors (PPARγ and C/EBPα) by hypoxia. The hypoxia-induced lipolysis was observed in vivo after femoral arterial clamp. Necrosis and apoptosis were induced by hypoxia in 3T3-L1 adipocytes. These data suggest that ATH may promote FFA release and inhibit glucose uptake in adipocytes by inhibition of the insulin-signaling pathway and induction of cell death.

Abdominal obesity; insulin sensitivity; high-fat diet; hypoxia-inducible factor 1α; glucose transporter 1

IN OBESITY, METABOLIC DISORDERS in white adipose tissue contribute to pathogenesis of insulin resistance (1, 40). The decrease in triglycerides (TAG) biosynthesis and increase in lipolysis of adipose tissue lead to elevation in free fatty acids (FFA) in plasma and contribute to ectopic fat deposition in liver and skeletal muscle. The lipid disorder may promote systemic insulin resistance through several mechanisms (56), such as activation of PKCs/INK through intermediate products (diacylglyceride or ceramide), induction of oxidative stress through incomplete β-oxydation, and induction of inflammatory responses through activation of Toll-like receptor 4 (27, 30, 42). FFA may contribute to hyperinsulinemia in obesity by a direct effect in β-cells (54). Impairment of insulin action in adipocytes may contribute to metabolic disorders in adipose tissue, since insulin stimulates synthesis and storage of TAG and inhibits lipolysis. Given the role of insulin in adipose tissue, impairment of insulin action in adipose tissue may represent an early event in systemic insulin resistance in obesity. However, the cause of the adipocyte malfunction remains to be identified in obesity.

The role of hypoxia in chronic inflammation in adipose tissue was first proposed in a review article in 2004 (50). Recent studies from three different laboratories have provided consistent evidence that adipose tissue hypoxia (ATH) exists in obese mice and that it contributes to initiation of chronic inflammation and inhibition of adiponectin expression in the white adipose tissue (21, 37, 55). These studies have nicely addressed the impact of hypoxia in the endocrine functions, but not metabolism of adipose tissue. Hypoxia was shown to enhance insulin-independent glucose uptake in human adipocytes through induction of GLUT1 expression in mRNA and protein (53). However, the effect of hypoxia on insulin-dependent glucose uptake in adipocytes was not reported in the study. Additionally, the dynamic relationship of ATH and adiposity has not been characterized.

In the current study, we examined dynamic change in ATH in ob/ob mice and investigated the hypoxia effects on insulin-stimulated glucose uptake. The results suggest that ATH went up with body weight and that hypoxia powerfully inhibited insulin action in adipocytes. The inhibition led to an increase in lipolysis and cell death in adipocytes. The results suggest that hypoxia may be a cause of metabolic disorders in the adipose tissue of obese mice.

RESEARCH DESIGN AND METHODS

Obese mice. Male ob/ob mice (B6.V-Lepob/Lepob, stock no. 000632) and C57BL/6 mice were purchased from the Jackson Laboratory at age of 4–5 wk and used at 6–12 wk in this study for genetic and dietary obesity. The ob/ob mice were fed normal chow diet (12.8% kcal in fat), and the sex-matched wild-type littermates of ob/ob mice were used as the lean control. In diet-induced obesity (DIO), male C57BL/6 mice at 5 wk of age were fed a high-fat diet (HFD, D12331; Research Diets, New Brunswick, NJ), which contained 58% calories in fat. In terms of weight, the total fat was 35.8% (wt/wt) in the diet (33.3% hydrogenated coconut oil and 2.5% soybean oil). There was 33.4% saturated, 0.86% monounsaturated, and 1.54% polyunsaturated fatty acids in the diet. In the control, mice (age- and sex-matched C57BL/6) were fed a normal chow diet. After 8 wk on HFD, the mice were used as dietary obese mice in this study. The fat composition was determined with nuclear magnetic resonance (model mq10; Brucker, Milton, ON, Canada). All of the mice were housed in the animal facility at the Pennington Biomedical Research Center on a 12:00 light-dark cycle and constant temperature (22–24°C). The

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mice had free access to water and diet. All procedures were performed in accordance with National Institute of Health guidelines for the care and use of animals and approved by the Institute’s Animal Care and Use Committee at the Pennington Biomedical Research Center.

**Cells and differentiation.** Mouse 3T3-L1 preadipocytes, rat skeletal L6 myoblasts, and mouse βTC6 insulinoma cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM culture medium supplemented with 10% fetal bovine serum (15% for βTC6), 4 mM glutamine, and 50 μg/ml gentamicin in an atmosphere of 5% CO2 at 37°C. The differentiations of 3T3-L1 into adipocytes and L6 myoblasts into myotubes were induced as described elsewhere (57).

**Hypoxia treatment.** Ambient hypoxia was generated by filling in a sealed metal chamber (self-designed) with low-oxygen air that contained 1% oxygen, 5% carbon dioxide, and 94% nitrogen. In hypoxia treatments, 3T3-L1 adipocytes were maintained in DMEM supplemented with 0.25% bovine serum albumin (BSA) and 25 μM HEPES. The hypoxia was monitored with an oxygen meter. To keep the humidity and temperature at 37°C in the chamber, 200 ml of water was kept in the chamber, and the chamber was maintained in a 37°C water bath with a constant temperature of 37°C.

**Interstitial PO2.** An oxygen meter with a needle type optic-fiber oxygen sensor (OXY-MICRO-AOT, World Precision Instruments) was used to determine interstitial PO2 in the epididymal fat pads, as described previously (55). The PO2 reading was taken from both sides of the fat pads, normalized with temperature and presented in millimeters of mercury. The mean value of three mice was used to represent PO2 in obese or lean mice.

**Quantitative RT-PCR.** Total RNA was extracted from homogenized epididymal fat or cultured cells using Tri reagent (T9424, Sigma) according to the manufacturer’s instructions. The epididymal fat pads were collected from mice and frozen in liquid nitrogen. Quantitative (q)RT-PCR was conducted using an ABI 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA). The primer and probe were ordered from Applied Biosystems: hif1a (Mm00468869_m1), vegf (Mm00437304_m1), glut1 (Mm00441473_m1), HO-1 (Mm00516004_m1), and pdk1 (Mm00554306_m1). The mRNA signal was normalized over the 18S ribosomal RNA (rRNA) signal. A mean value of triplicates was used for relative mRNA level or calculation of fold induction.

**Western blot.** Whole cell lysate was made with homogenization and sonication in a lysis buffer, and Western blotting was conducted as described elsewhere (14). Lipids were removed from the lysate before the protein assay was conducted. Antibodies to hypoxia-inducible factor-1α (HIF1α), glucose transporter 1 (GLUT1), glucose transporter 4 (GLUT4), actin, and tubulin were from Abcam (Cambridge, MA). Antibodies to IRβ, IRS-1, Akt1/2, CCAAT enhancer binding protein α (C/EBPα), and fatty acid transport protein-1 (FATP1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to Phospho-Akt (Ser473) was purchased from Calbiochem (Gibbstown, NJ). Rabbit antisera to peroxisome proliferator-activated receptor-γ (PPARγ) and CD36 were made in our laboratory. Horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG

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**Fig. 1.** Hypoxia in adipose tissue of ob/ob mice. A: PO2 in epididymal fat pads of lean and ob/ob mice was determined with an oxygen meter equipped with a fiber optic oxygen sensor. The assay was conducted in mice at 6, 9, and 12 wk in age. Fat composition and body weight of the mice were determined (n = 3). B: hypoxia response gene expression in epididymal fat of ob/ob mice was determined in quantitative RT-PCR. The assay was conducted in mice at 6 and 12 wk in age (n = 3). C: protein levels in epididymal fat of lean and ob/ob mice were examined in a Western blot (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. lean mice; #P < 0.05, ##P < 0.01 vs. 6-wk-old (6w) mice.
were purchased from GE Healthcare UK (Buckinghamshire, UK). ImageJ 1.37V was used to quantify the Western signals.

**Glucose uptake.** 3T3-L1 adipocytes, L6 myotubes, or βTC6 cells were cultured in 12-well plates and treated with hypoxia in serum-free medium (DMEM with 0.25% BSA). After hypoxia treatment, 2-deoxy-D-[3H]glucose uptake was performed immediately in normoxic condition in PBS buffer. The glucose uptake assay has been described elsewhere (57).

**Glycerol and TAG assays.** 3T3-L1 adipocytes were cultured in 12-well plates and treated with hypoxia in serum-free medium (DMEM with 0.25% BSA). After hypoxia treatment, 2-deoxy-D-[3H]glucose uptake was performed immediately in normoxic condition in PBS buffer. The glucose uptake assay has been described elsewhere (57).

Glycerol and TAG assays. 3T3-L1 adipocytes were cultured in 12-well plates and treated with hypoxia in serum-free medium (DMEM with 0.25% BSA). After hypoxia treatment for 24 h, the medium was collected and tested for glycerol and TAG with the Serum TAG Determination Kit (Sigma, St. Louis, MO). In the preparation of conditioned medium, the adipocytes were treated with normoxia or hypoxia for 24 h, and the supernatant was collected immediately after the treatment. The conditioned medium was used to treat cells for 24 h, and glycerol concentration was determined in the culture. The net change in glycerol was obtained by comparing glycerol before and after the treatment to determine lipolysis.

**Necrosis and apoptosis assay.** 3T3-L1 adipocytes were cultured in six-well plates. After hypoxia treatment for 24 h, both detached and attached cells were collected and used in analysis of necrosis and apoptosis with Vybrant Apoptosis Assay Kit #2 (Molecular Probes, Eugene, OR). Flow cytometric analysis was performed using the FACScalibur cytometer (BD Biosciences, San Jose, CA). A total of 20,000 cells were examined in each sample, and the data were analyzed using Cellquest Pro software (BD Biosciences) and a Macintosh G5 computer (Apple, Cupertino, CA).

**Fatty acid uptake.** 3T3-L1 adipocytes were cultured in a 12-well plate. After hypoxia treatment in serum-free DMEM with 0.25% BSA for 24 h, [14C]palmitic acid uptake was examined as described elsewhere (34).

**Nonviable cell assay.** 3T3-L1 adipocytes were cultured in normal DMEM supplemented with serum and 25 μM HEPES in a six-well plate. After hypoxia treatment for 8 or 16 h, the medium was collected for counting the detached dead cells, which were collected after centrifugation and stained with trypan blue. The attached cells were trypsinized, diluted in serum-free DMEM, and stained with trypan blue for dead cells. The stained cells in the supernatant and on the plate were combined together to represent the nonviable cells in each well.

**Plasma FFA after femoral artery clamp.** The effect of hypoxia on lipolysis was assessed in rats by determining the changes in plasma FFA level in femoral vein after occlusion of the femoral artery for 15 min in lean rats (400 g body wt; Harlan Sprague Dawley, Indianapolis, IN). The rats fed on regular rat chow were anesthetized by pentobarbital sodium (50 mg/kg iv), and the abdominal cavity was surgically opened to expose the femoral artery and vein. A basal blood sample was taken first from the femoral vein. Then, a small clamp was placed at the corresponding femoral artery to block the blood flow. Fifteen minutes later, the clamp was removed, and two blood samples were taken at 3-min intervals from the same vein. Plasma FFA concentration was determined enzymatically (19).

**Statistical analysis.** In this study, all of the in vitro experiments were conducted three times with consistent results. The data of representative experiments are presented. In the bar figures, a mean value and standard error of multiple data points or samples were used to represent the final result. Student’s t-test or two-way ANOVA was used in statistical analysis of the data with significance P < 0.05.

**RESULTS**

**Hypoxia in adipose tissue of ob/ob mice.** If hypoxia is a risk factor for adipocyte insulin resistance in vivo, it should increase with adiposity in the obese condition. To test this possibility, we conducted three experiments in ob/ob mice. In

**Nonviable cell assay.** 3T3-L1 adipocytes were cultured in normal DMEM supplemented with serum and 25 μM HEPES in a six-well plate. After hypoxia treatment for 8 or 16 h, the medium was collected for counting the detached dead cells, which were collected after centrifugation and stained with trypan blue. The attached cells were trypsinized, diluted in serum-free DMEM, and stained with trypan blue for dead cells. The stained cells in the supernatant and on the plate were combined together to represent the nonviable cells in each well.

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**RESULTS**

**Hypoxia in adipose tissue of ob/ob mice.** If hypoxia is a risk factor for adipocyte insulin resistance in vivo, it should increase with adiposity in the obese condition. To test this possibility, we conducted three experiments in ob/ob mice. In
the first, interstitial PO2 was measured in the epididymal fat pads of \textit{ob/ob} mice during weight gain between 6 and 12 wk in age. In the lean controls, PO2 was in the range of 56.8 to 60.4 mmHg at the same age range (Fig. 1A). Although their body fat content was increased from 10 to 18\%, PO2 did not decrease in the fat tissue when the body fat content reached 18\% at 12 wk of age. In the \textit{ob/ob} mice, PO2 was 34.8 mmHg (40.4\% lower than that in lean controls) at 6 wk of age. It further dropped to 20.1 mmHg (64.8\% lower than that in lean mice) at 12 wk of age with a gain in adiposity (Fig. 1A). At 6 wk, the low PO2 was associated with 50\% of body fat content in the obese mice. This adiposity was 400\% higher than that of lean mice. The data suggest that ATH may occur when the body fat content is over 20\% (Fig. 1A) and becomes more severe with gain in fat content thereafter.

In the second test, expression of hypoxia-responsive genes was examined by qRT-PCR to determine the hypoxia response. The genes include HIF1\(\alpha\), GLUT1, Heme oxygenase-1 (HO-1), pyruvate dehydrogenase kinase-1 (PDK1), and vascular endothelial growth factor (VEGF). Among the five genes, HIF1\(\alpha\) is a transcription factor that controls expression of the other four genes (41). In the \textit{ob/ob} mice, four of the five genes (except VEGF) increased in the epididymal fat pads compared with the control mice (Fig. 1B). The fold induction in these genes was positively associated with the degree in ATH and fat content in the \textit{ob/ob} mice. Expression of these genes suggests a hypoxia response in the adipose tissue of obese mice.

In the third test, protein levels of hypoxia-responsive molecules including HIF1\(\alpha\) and GLUT1 were examined in a Western blot. These proteins increased significantly in the adipose tissue of the \textit{ob/ob} mice (Fig. 1C). In contrast, proteins of IR\(\beta\) and IRS-1 decreased significantly in the obese mice (Fig. 1C). These data suggest that hypoxia exists in the visceral fat tissue of obese mice and that adipose tissue hypoxia may lead to impairment in the insulin signaling pathway.

**Gene expression in diet-induced obesity.** We hypothesized that the hypoxia response is present in adipose tissue in obesity regardless of model system. The above data suggest the hypoxia response in \textit{ob/ob} mouse, a genetic model of obesity. To test the hypothesis, we examined the hypoxia response in dietary obese mice, which also suffer adipose tissue hypoxia (37). The dietary obese mice were used at 8 wk on HFD. Their body weight was increased by 27.7\% and fat content by 71.6\% at that time (Fig. 2A). mRNA of hypoxia-responsive genes, including HIF1\(\alpha\), GLUT1, HO-1, PDK-1, and VEGF, was increased significantly in epididymal fat pads (Fig. 2B). Proteins for HIF1\(\alpha\) and GLUT1 were upregulated (Fig. 2C). Proteins for IR\(\beta\) and IRS-1 were downregulated (Fig. 2C). These data suggest that hypoxia reaction is present in diet-induced obesity. The hypoxia may impair the insulin-signaling pathway in adipocytes or adipose tissue similar to what was observed in the \textit{ob/ob} mice.

**Inhibition of insulin-induced glucose uptake by hypoxia.** To understand the effect of hypoxia on insulin-dependent glucose uptake, 3T3-L1 adipocytes were exposed to hypoxia (1\% oxygen), and proteins were determined by Western blot in a time course study (\(n = 3\) or 6). 2-deoxy-D-\([3\text{H}]\)glucose uptake was examined in 3T3-L1 adipocytes after hypoxia treatment for different times. The assay was conducted in normoxic condition immediately after hypoxia treatment (\(n = 3\)). C: 15 min prior to cell collection, 100 nM insulin was added to normoxia- or hypoxia-treated 3T3-L1 adipocytes. Western blot was conducted to detect the phosphorylation of Akt in whole cell lysate. 0 represents normoxic condition. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) vs. normoxic control (CON or 0 h).

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**Fig. 3. Inhibition of insulin-induced glucose uptake by hypoxia.**

A: 3T3-L1 adipocytes were exposed to hypoxia (1\% oxygen), and proteins were determined by Western blot in a time course study (\(n = 3\) or 6). B: 2-deoxy-D-\([3\text{H}]\)glucose uptake. Uptake was examined in 3T3-L1 adipocytes after hypoxia treatment for different times. The assay was conducted in normoxic condition immediately after hypoxia treatment (\(n = 3\)). C: 15 min prior to cell collection, 100 nM insulin was added to normoxia- or hypoxia-treated 3T3-L1 adipocytes. Western blot was conducted to detect the phosphorylation of Akt in whole cell lysate. 0 represents normoxic condition. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) vs. normoxic control (CON or 0 h).
metabolism, we examined hypoxia regulation of glucose uptake in 3T3-L1 adipocytes. Insulin-independent glucose uptake is mediated by GLUT1, and insulin-induced glucose uptake is mediated by both GLUT1 and GLUT4 (31, 32). In the study, expression of GLUT1 and GLUT4 proteins was determined in adipocytes after hypoxia treatment. In a time course study, a 4.3-fold elevation in GLUT1 protein level was observed at 30 min under hypoxia (Fig. 3A). The proteins for GLUT1 and HIF1α were both upregulated at high levels throughout the time of 24 h in the study. GLUT1 expression peaked at 24 h with an 18.5-fold increase, and HIF1α peaked at 8 h with an 8.6 fold increase. In this system, GLUT4 expression was not significantly changed. In this time course study, a reduction in IRβ (50%) and IRS-1 (64%) was observed at 24 h (Fig. 3A).

Glucose uptake was examined in 3T3-L1 adipocytes by use of 2-deoxy-[3H]glucose. After 4 h of hypoxia treatment, insulin-independent (basal) and insulin-stimulated glucose uptake was enhanced by hypoxia for 142 and 72%, respectively (Fig. 3B). Subsequently, the glucose uptake decreased in a time-dependent manner. At 24 h, only 50.8% of the increase was observed in the basal glucose uptake. Under hypoxia for 24 h, the insulin-stimulated glucose uptake was completely blocked by hypoxia (Fig. 3B). The loss of insulin action was associated with a reduction in IRβ and IRS-1 proteins at the 24-h time point, suggesting that a long-term hypoxia response may lead to insulin resistance in adipocytes.

Insulin signaling was investigated in 3T3-L1 adipocytes after hypoxia treatment for 24 h. Insulin treatment for 15 min induced Akt phosphorylation (Ser473) in the control cells. However, the Akt phosphorylation was absent in the hypoxia-treated cells (Fig. 3C), suggesting that the insulin signaling pathway was impaired by hypoxia in 3T3-L1 adipocytes.

**Regulation of IRβ and IRS-1 mRNA expression.** To understand the mechanism of protein change in hypoxia, we examined mRNA for IRβ, IRS-1, and GLUT1 and insulin and adipose tissue and 3T3-L1 adipocytes. In the epididymal fat of ob/ob mice, mRNA was increased significantly for IRβ and GLUT1 (Fig. 4A). In contrast, mRNA was decreased markedly for IRS-1 (Fig. 4A). In 3T3-L1 adipocytes, IRβ mRNA was induced and IRS-1 mRNA was reduced by hypoxia in a time-dependent manner (Fig. 4B). In the positive control, GLUT1 mRNA was increased as much as 10-fold in the hypoxia-treated 3T3-L1 adipocytes (Fig. 4B). These data suggest that the mRNA changes in IRβ, IRS-1, and GLUT1 in the adipose tissue of ob/ob mice may be a result of hypoxia response in obesity.

**Hypoxia effects on L6 myotubes and TC6 cells.** To test hypoxia effects on skeletal muscle cells and pancreatic β-cells, we repeated the experiments with L6 myotubes and TC6 cells. In these two cell lines, GLUT1 protein was induced markedly by hypoxia (Fig. 5, A and B). However, no significant reduction was observed in IRβ and IRS-1 proteins. In the glucose uptake assay, a 50% increase was observed under insulin treatment in the L6 myotubes in the normoxia condition. In contrast, a fivefold induction was observed under hypoxia in the absence of insulin (Fig. 5C). This activity of hypoxia was so strong that insulin-induced glucose uptake was not detectable in the presence of hypoxia. In the TC6 cells, insulin-independent glucose uptake increased 45% under hypoxia (Fig. 5D). Insulin-induced glucose uptake was not examined, as the TC6 cells do not express GLUT4. These data suggest that hypoxia is able to stimulate GLUT1-mediated glucose uptake in muscle cells and β-cells. In these two types of cells, hypoxia did not inhibit the IRβ and IRS-1 proteins.

**Hypoxia regulation of lipid metabolism.** Plasma FFA is increased in obesity and is involved in pathogenesis of systemic insulin resistance. An imbalance in biosynthesis and lipolysis of TAG in adipocytes may contribute to the elevation of FFA in the circulation. To test hypoxia regulation of TAG metabolism, we examined TAG lipolysis by analysis of glycerol concentration in the culture medium of 3T3-L1 adipocytes. In response to hypoxia, glycerol level was doubled in the culture medium after the treatment. The net glycerol concentration in the normoxic condition. Glycerol concentration was determined with real-time quantitative RT-PCR. A: mRNA in epididymal fat pads of lean and ob/ob mice were determined with real-time quantitative RT-PCR. B: mRNA levels in 3T3-L1 adipocytes were determined with real-time quantitative RT-PCR after hypoxia treatment. *P < 0.05, **P < 0.01 vs. lean mice or untreated cells (0 h).

**Hypoxia response in 3T3-L1 adipocytes.** Insulin-stimulated glucose uptake was completely blocked by hypoxia (Fig. 3B). The loss of insulin action was associated with a reduction in IRβ and IRS-1 proteins at the 24-h time point, suggesting that a long-term hypoxia response may lead to insulin resistance in adipocytes.

**Hypoxia regulation of lipid metabolism.** Plasma FFA is increased in obesity and is involved in pathogenesis of systemic insulin resistance. An imbalance in biosynthesis and lipolysis of TAG in adipocytes may contribute to the elevation of FFA in the circulation. To test hypoxia regulation of TAG metabolism, we examined TAG lipolysis by analysis of glycerol concentration in the culture medium of 3T3-L1 adipocytes. In response to hypoxia, glycerol level was doubled in the cell culture (Fig. 6A). This effect of hypoxia is comparable to that of TNF-α (20 ng/ml), which was used as a positive control in the stimulation of lipolysis. Both hypoxia and TNF-α generated the maximal level of lipolysis, as no additive effects were observed in combination of the two factors (Fig. 6A). To determine the role of autocrine in the stimulation of lipolysis by hypoxia, conditioned medium was prepared from 3T3-L1 adipocytes treated with hypoxia for 24 h. The supernatant was used to treat the fresh 3T3-L1 adipocytes for 24 h in the normoxic condition. Glycerol concentration was determined in the culture medium after the treatment. The net increase in the glycerol level was obtained by comparing glycerol concentrations before and after 24-h culture in the conditioned medium. The glycerol concentration was increased

Fig. 4. IRβ, IRS-1 and GLUT1 gene expression in adipose tissue and hypoxia-treated 3T3-L1 adipocytes. A: mRNA in epididymal fat pads of lean and ob/ob mice were determined with real-time quantitative RT-PCR. B: mRNA levels in 3T3-L1 adipocytes were determined with real-time quantitative RT-PCR after hypoxia treatment. *P < 0.05, **P < 0.01 vs. lean mice or untreated cells (0 h).
significantly by the treatment. In addition to lipolysis, fatty acid uptake was investigated in 3T3-L1 adipocytes with radiolabeled palmitic acid. After 24 h in hypoxia, the uptake was decreased by 21% (Fig. 6B).

To test whether hypoxia is able to induce lipolysis in vivo, we generated hypoxia in the hindleg in rats by clamping the femoral artery for 15 min. After reestablishment of the circulation by removal of the clamp, blood samples were collected from the femoral vein and used in the test for plasma FFA. The FFA level was significantly increased by clamp-induced hypoxia. Compared with the FFA level in the vein blood before the clamp, FFA was increased 110% (P<0.001) at 3 min and 150% (P<0.0001) at 6 min after clamp (Fig. 6C). Adipocytes are the source of FFA. These data suggest that hypoxia is able to induce strong lipolysis in adipose tissue in vivo. These data support the idea that hypoxia may contribute to elevation of plasma FFA in obesity.

**Hypoxia regulation of lipid metabolism-related protein levels.** Since lipolysis was induced and fatty acid uptake was reduced by hypoxia, we wanted to investigate the mechanism of these reactions. We hypothesized that these effects might be related to changes in lipid metabolism-related protein levels. To test this possibility, expression of transcription factors (such as PPARγ and C/EBPα) and fatty acid transporters (such as FATP1 and CD36) were examined. In the adipocytes, all of these proteins were decreased significantly by 24-h hypoxia treatment (Fig. 7A). In adipose tissue, these proteins were reduced in the ob/ob mice as indicated by the Western blot, proteins for PPARγ, C/EBPα, FATP1, and CD36 (Fig. 7B). These data suggest that the reduction may be a result of chronic hypoxia response.

**Hypoxia induction of necrosis and apoptosis.** It was reported that adipocyte necrosis increased in adipose tissue of human and mice with obesity (9). The cell death may contribute to macrophage infiltration into adipose tissue. However, the cause of adipocyte death remains to be identified (46). In our study, we observed that total protein was reduced in the whole cell lysate of hypoxia-treated cells. This observation suggests that hypoxia may induce cell death, which in turn leads to protein reduction by reducing viable cell number. To test this possibility, cell death was determined by measuring TAG in the culture medium. TAG is released into the cell culture supernatant after adipocyte death. In 24-h hypoxia exposure, the TAG level was increased by 35% in the culture medium. TAG release was not significantly induced by TNF-α (Fig. 8A). These data suggest that hypoxia is stronger than TNF-α in the induction of TAG release.

The cell death was examined with trypan blue staining and flow cytometry. The results showed that adipocyte death was increased by hypoxia in a time-dependent manner under hypoxia (Fig. 8B). With 16-h hypoxia treatment, the number of nonviable cells was increased by 75% (Fig. 8B). To determine necrosis and apoptosis for cell death, we used flow cytometry after the cells were stained with fluorescent dye. The results showed that necrosis and apoptosis of the adipocytes were increased by 40% and 35%, respectively (Fig. 8C). The data suggest that hypoxia was able to induce adipocyte death through necrosis and apoptosis.

**DISCUSSION**

Results from the current study suggest that hypoxia may lead to insulin resistance in adipocytes in obesity. It has been known for more than 30 years that large adipocytes isolated from
The reduction in PO2 continued in the body fat content was 50% at 6 wk of age, and PO2 was at 39 mmHg. The net increase in glycerol level was obtained by comparing glycerol concentrations before and after 24-h culture in CM. B: 1[14C]palmitic acid uptake was performed in 3T3-L1 adipocytes after hypoxia treatment for 24 h (n = 3). C: FFA in femoral artery of lean rats. The femoral artery was clamped to block blood flow for 15 min to generate a hypoxia response in vivo. Blood samples were collected from the femoral vein before surgery and after clamp to determine the change in FFA (n = 11). *P < 0.05, **P < 0.01, ***P < 0.001, ****p < 0.0001.

Our data suggest that reduction of IRβ and IRS-1 by hypoxia may account for insulin resistance in adipose tissue of obese subjects. In this study, we examined many signaling molecules (IR, IRS-1, Akt, GSK-3, mTOR, p70S6K, and GLUT4) in the insulin-signaling pathway. IRβ and IRS-1 were decreased by hypoxia in 3T3-L1 adipocytes. The reduction was observed at 24 h in hypoxia. This effect of hypoxia suggests that IR function, loss of insulin sensitivity may lead to adipocyte dysfunction in obesity, as the average size of adipocytes is increased in obesity (39). We tested the effects of mechanical pressure and vacuum on insulin-signaling activity in adipocytes. An increase in mechanical pressure to 20 psi for 72 h did not lead to any insulin-signaling activity in adipocytes. An increase in mechanical pressure to 20 psi for 72 h did not lead to any stimulation of IRS-1 reduction in the adipose tissue of ob/ob mice remains to be identified. We were unable to identify the threshold in this study, as the body fat content was 50% in ob/ob at the start of this study. The reason(s) for ATH remains to be investigated. An increase in adipocyte size may be a factor. In obesity, adipocyte size increases up to 140–180 µm in diameter. This size is bigger than the diffusion distance of oxygen, which is 100–120 µm at most. A large adipocyte may block oxygen diffusion. Additionally, a reduction in adipose tissue blood flow may be another factor.

In this study, we observed that all of the hypoxia response genes were increased in the two obesity models except for VEGF. VEGF expression was increased in the dietary obese mice but not in the ob/ob mice. In the cell culture, VEGF expression was upregulated by hypoxia in all of the cells tested in this study. These data suggest that the nonresponsiveness of VEGF in ob/ob mice might be related to a factor that is unique in ob/ob mice. In the two obese models, a major difference is the absence of leptin in ob/ob mice. Leptin was reported to upregulate VEGF transcription through activation of the JAK/STAT3-signaling pathway (47). The leptin signal may be required for the VEGF response to hypoxia in vivo. The lack of VEGF response may lead to relatively poor vascularization, which has been reported in muscular arterioles of ob/ob mice (33).

Our data suggest that reduction of IRβ and IRS-1 by hypoxia may account for insulin resistance in adipose tissue of obese subjects. In this study, we examined many signaling molecules (IR, IRS-1, Akt, GSK-3, mTOR, p70S6K, and GLUT4) in the insulin-signaling pathway. IRβ and IRS-1 were decreased by hypoxia in 3T3-L1 adipocytes. The reduction was observed at 24 h in hypoxia. This effect of hypoxia suggests that IRβ and IRS-1 reduction in the adipose tissue of ob/ob mice may be a result of chronic hypoxia response. This study provides a new explanation for the reduction of IR protein in the adipose tissue of obese subject that has been known for 30 years (2, 13, 35). It was believed that the reduction was a consequence of hyperinsulinemia, which reduces IRβ protein level through ligand-mediated receptor degradation in adipocytes. Although TNF-α is able to reduce IRS-1 protein (22–24, 45), it does not reduce IRβ up to 24 h (45). Inflammation seems not to be able to explain the reduction in both IRβ and IRS-1.

The current study provides evidence that hypoxia regulates glucose metabolism in adipocytes by modifying both insulin-dependent and -independent pathways. Hypoxia is known to enhance insulin-independent glucose uptake in muscle cells (3, 11, 25). However, the effects of hypoxia on adipocytes and β-cells have been less studied. Our data suggest that hypoxia may increase glucose uptake through both insulin-dependent and -independent pathways in a short-term (~4 h) exposure.

Glucose uptake declined in both pathways after the long-term (~24 h) hypoxia treatment. The insulin-induced glucose uptake was completely blocked after 24-h hypoxia exposure. The inhibition was associated with loss of IRβ and IRS-1 proteins. Since insulin action is critical in the maintenance of adipose tissue function, loss of insulin sensitivity may lead to adipocyte dysfunction in the storage of TAG as observed in this study. This effect of hypoxia seems specific to adipocytes, as the insulin-signaling pathway was not impaired by hypoxia in β-cells and muscle cells.
In this study, we observed that the basal glucose uptake was increased by hypoxia treatment in 3T3-L1 adipocytes. This result is likely a consequence of upregulation of GLUT1 expression by hypoxia. In response to hypoxia, the basal glucose uptake was increased by 142% at 4 h and then by 73 and 50.8% at 8 and 24 h, respectively. The reduction in glucose uptake after 8-h hypoxia treatment may be a result of reduced demand for energy in response to persistent hypoxia. ATP production in mitochondria is reduced by hypoxia. The loss of ATP supply may be responsible for cell necrosis and apoptosis in the current study. The cell death may contribute to the reduction in glucose uptake as well. Another possibility is inhibition of glucose uptake by FFA that is derived from the hypoxia-induced lipolysis. The current study indicates that a 24h hypoxia treatment was able to induce lipolysis in 3T3-L1 adipocytes. A high level of FFA in the medium may interfere with the glucose uptake via lipotoxicity (15). However, in these conditions, the basal glucose uptake remains higher than that in normoxia condition. The GLUT4 protein in 3T3-L1 adipocytes was unchanged by hypoxia treatment in this study. It was reported that, in matured 3T3-L1 adipocytes, the half-life of GLUT4 protein was longer than 16 h (43). In the current study, the test was conducted for 24 h. The literature suggests that hypoxia may reduce GLUT4 mRNA through downregulation of C/EBP activity (26). A study with a longer exposure time may be required to test the hypoxia effect on GLUT4 expression.

The current study provides direct evidence that hypoxia is able to induce lipolysis and inhibit fatty acid uptake at the same time. Increased release of FFA from adipose tissue is a risk factor for FFA elevation in the circulation that contributes to systemic insulin resistance in obesity (5, 10, 54). Although the
FFA release is related to insulin resistance in adipocytes that reduces insulin activity in the inhibition of lipolysis (12), other causes of lipolysis remain to be identified. The current study demonstrated that ATH may be a potential signal to induce FFA release from adipocytes through induction of lipolysis. This possibility is supported by FFA data from the supernatant of hypoxia-treated adipocytes and FFA in the vein blood samples of clamped leg in rats. In vivo, inhibition of adipogenesis by hypoxia may also contribute to the FFA elevation. Generation of new adipocytes from adipogenesis can make more space for storage of FFA in the form of TAG. Hypoxia was reported to inhibit adipocyte differentiation and adipogenesis (8, 16, 28, 58, 59). Downregulation of PPARγ gene expression was proposed as a mechanism of hypoxia action (28, 58, 59). However, the effects of hypoxia on fatty acid uptake and lipolysis in adipocytes were not reported in those earlier studies. In the current study, we have demonstrated that FFA uptake by adipocytes is decreased by hypoxia. The inhibition is associated with a decrease in FATP and CD36 proteins. It is likely that hypoxia reduces FFA uptake by suppression of FFA transporter expression.

Our previous study (55) suggests a potential role of hypoxia in the induction of chronic inflammation and inhibition of adiponectin in adipose tissue in obesity. Hypoxia was able to stimulate the gene expression of proinflammatory cytokines like TNF-α directly in primary adipocytes and macrophages (55). In this study, we observed that hypoxia led to insulin resistance and lipolysis in the adipocytes. However, it was not certain whether the insulin resistance was due to increased proinflammatory cytokines and/or decreased adiponectin secretion. Our conditioned medium experiments revealed that the autocrine effect of adipocytes plays a role in the hypoxia inhibition of insulin signaling.

Induction of adipocyte death may be another mechanism by which hypoxia contributes to chronic inflammation in obesity. Hypoxia was reported to induce an inflammatory response in adipocytes and macrophages (51, 55). Activation of transcription factors such as NF-kB and HIF1α plays an important role in the induction of inflammatory cytokines and chemokines by hypoxia. These cytokines are likely involved in macrophage infiltration into adipose tissue (4, 6, 17). The present study suggests that adipocyte death may be another mechanism by which hypoxia contributes to chronic inflammation. Macrophage infiltration was proposed to remove dead cells in adipose tissue in obesity (9). However, the cause of cell death is not clear in adipose tissue in obesity. We show here that hypoxia induces necrosis and apoptosis in 3T3-L1 adipocytes. In adipose tissue, macrophages were predominantly found in the hypoxic areas in adipose tissue of obese mice (37). The results provide a new mechanism for obesity-associated cell death in adipose tissue. Necrosis and apoptosis are two different types of cell death that are dependent on ATP supply. A complete loss of ATP leads to cell necrosis and a partial loss induces apoptosis. In response to hypoxia, ATP production is reduced as a result of inhibition of mitochondrial respiration. In some cells, this change makes the ATP supply unable to meet the minimal demand for cell survival; then, necrosis occurs. If the cells are able to maintain the minimal ATP supply, they will either survive or become apoptotic. Apoptosis is a programmed cell death that requires ATP. Inhibition of protein synthesis by hypoxia may be involved in the mechanism of mitochondrial inhibition and ATP reduction. An increase in permeability in the inner mitochondrial membrane is observed in hypoxia response (18). In this condition, cell apoptosis is associated with release of cytochrome c. In ischemia research, hypoxia-induced cell death has been well established in cardiac injury and brain damage (44, 49).

In summary, insulin resistance in large adipocytes in adipose tissue is likely a result of hypoxia in obesity. Hypoxia is found in adipose tissue of obese mice and is a potential risk factor for metabolic disorders in the adipose tissue. Hypoxia may inhibit insulin-induced glucose uptake by reducing insulin-signaling molecules IRβ and IRS-1. Hypoxia may stimulate lipolysis and inhibit uptake of FFA in adipocytes leading to FFA elevation in the plasma of obese subjects. Induction of adipocyte necrosis and apoptosis provides a mechanism for cell death observed in adipose tissue of obesity and may serve as a new mechanism for hypoxia-induced macrophage infiltration into adipose tissue. These data suggest that adipose tissue hypoxia may play an important role in adipose dysfunction and the development of insulin resistance in obesity.

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