Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice

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Ye J, Gao Z, Yin J, He Q. Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. Am J Physiol Endocrinol Metab 293: E1118–E1128, 2007. First published July 31, 2007; doi:10.1152/ajpendo.00435.2007.—Chronic inflammation and reduced adiponectin are widely observed in the white adipose tissue in obesity. However, the cause of the changes remains to be identified. In this study, we provide experimental evidence that hypoxia occurs in adipose tissue in obese mice and that adipose hypoxia may contribute to the endocrine alterations. The adipose hypoxia was demonstrated by a reduction in the interstitial partial oxygen pressure (Po2), an increase in the hypoxia probe signal, and an elevation in expression of the hypoxia response genes in ob/ob mice. The adipose hypoxia was confirmed in dietary obese mice by expression of hypoxia response genes. In the adipose tissue, hypoxia was associated with an increased expression of inflammatory genes and decreased expression of adiponectin. In dietary obese mice, reduction in body weight by calorie restriction was associated with an improvement of oxygenation and a reduction in inflammation. In cell culture, inflammatory cytokines were induced by hypoxia in primary adipocytes and primary macrophages of lean mice. The transcription factor NF-kB and the TNF-α gene promoter were activated by hypoxia in 3T3-L1 adipocytes and NIH3T3 fibroblasts. In addition, adiponectin expression was reduced by hypoxia, and the reduction was observed in the gene promoter in adipocytes. These data suggest a potential role of hypoxia in the induction of chronic inflammation and inhibition of adiponectin in the adipose tissue in obesity.

IN OBESITY, chronic inflammation and reduced adiponectin in the white adipose tissue (WAT) contribute to pathogenesis of insulin resistance, which links obesity to many complications, such as type 2 diabetes and cardiovascular diseases (23, 24, 30). The chronic inflammation is indicated by an increased expression of proinflammatory cytokines and elevated infiltration of macrophages into adipose tissue. Of the proinflammatory cytokines, TNF-α and IL-6 reduce insulin sensitivity and impair the homeostasis of lipid and glucose metabolism. Monocyte chemoattractant protein-1 (MCP-1) promotes macrophage infiltration into adipose tissue. Although expression of these cytokines is increased in adipose tissue of obese subjects, it is not clear what induces expression of these inflammatory cytokines in obesity. A decrease in adiponectin (ACRP30) production contributes to pathogenesis of insulin resistance (23). However, it remains to be investigated what obesity-associated factor leads to suppression of adiponectin.

Systemic hypoxia is associated with insulin resistance in human and animal. In patients with obstructive sleep apnea or sleep-disordered breathing, intermittent hypoxia is associated with a high risk for insulin resistance (20, 40, 50, 56). The inflammation markers (IL-6 and C-reactive protein) are increased in the patients’ plasma and reduced after improvement of breathing by surgery (28, 48, 52, 65). Several studies suggest that obese subjects are more susceptible to insulin resistance in hypoxic environment, such as high altitude (7, 12, 29, 39). The cellular and molecular mechanisms underlying the association of hypoxia and insulin resistance remain to be investigated in the obese subjects. It was hypothesized that hypoxia may exist in adipose tissue in obesity and lead to inflammation (54). However, there is no direct evidence in support of the possibility. In the current study, we demonstrated that hypoxia occurred in the adipose tissue of obese mice. The hypoxia was associated with chronic inflammation and reduced expression of adiponectin in vivo. In vitro, hypoxia is able to induce proinflammatory cytokines and decrease adiponectin expression in adipose tissue of lean mice. Our data provide experimental evidence that in obesity, hypoxia exists in adipose tissue, and adipose hypoxia may contribute to chronic inflammation and hypoadiponectinemia.

METHODS AND MATERIALS

Mice. Male ob/ob mice (B6.V-Lep+/-, stock no. 000632) were purchased from the Jackson Laboratory at the age of 4–5 wk and used at 10–12 wk in this study for genetic obesity. The sex-matched wild-type littermates were used as lean mice in the control. The ob/ob mice were fed on normal chow diet (12.8% kcal from fat). All of the mice were housed in the animal facility at the Pennington Biomedical Research Center with a 12:12-h light-dark cycle and constant temperature (22–24°C). The mice had free access to water and diet. All procedures were performed in accordance with the National Institutes of Health guidelines for the care and use of animals and were approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center.

Dietary obesity and calorie restriction. Male C57BL/6 mice (4 wk of age) were purchased from the Jackson Laboratory and fed a high-fat diet (HFD; no. D12331, 58 kcal% from fat). All of the mice were housed in the animal facility at the Pennington Biomedical Research Center with a 12:12-h light-dark cycle and constant temperature (22–24°C). The mice had free access to water and diet. All procedures were performed in accordance with the National Institutes of Health guidelines for the care and use of animals and were approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center. In the control, mice (age- and sex-matched C57BL/6) were fed on normal chow diet. After 3 and 5 mo on HFD, the ob/ob mice were compared for the association of body weight and adipose hypoxia. At 4 mo on HFD, a group of mice was subjected to weight reduction by caloric restriction to confirm the association of body weight and adipose hypoxia. In the caloric restriction, one-third of the daily requirement of HFD was supplied to the obese mice for 4 wk. The control mice were supplied with sufficient HFD in the caloric restriction study.

Cells and reagents. The cell line 3T3-L1 (CL-173) was purchased from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.
becco’s modified Eagle’s culture medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 4 mM glutamine. Differentiation of 3T3-L1 was induced as described elsewhere (16). Antibodies to hypoxia-inducible factor (HIF)-1α (ab1), actin (ab6276), and tubulin (ab7291) were obtained from Abcam (Cambridge, UK). Human insulin (I9278) was obtained from Sigma. Adiponectin antibody (MAB3608) was obtained from Chemicon International (Temecula, CA).

Tissue collection and Western blot analysis. Epididymal fat pads and skeletal muscles were collected immediately after cervical dislocation in mice and frozen in liquid nitrogen. The tissue collection was done within 1–2 min after cervical dislocation. The tissues were kept in a −80°C freezer until preparation of whole lysate or extraction of RNA. The whole cell lysate protein was made with homogenization and sonication in a lysis buffer (1% Triton X-100, 50 mM KCl, 25 mM HEPES, pH 7.8, 10 μg/ml leupeptin, 20 μg/ml apro tin, 125 μM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate). Triglycerides were removed from the lysate before protein assay. Extraction of nuclear protein and Western blot analysis were conducted as described elsewhere (16).

Nuclear protein extraction. The nuclear extract was prepared according to a protocol described elsewhere (64). The epididymal fat tissues were collected and snap-frozen in liquid nitrogen within 2 min of cervical dislocation of mice. Tissue samples were stored at −80°C until further processing. The fat sample of ~200–300 mg was cut into small pieces on dry ice and homogenized in 1 ml of lysis buffer. After centrifugation at 10,000 rpm for 10 min at 4°C, the nucleus was pelletted and collected. The top-layer lipid was removed with a cotton stick before collection of the nucleus. After washing, the nucleus pellet was treated with extraction buffer. The supernatant was collected for nuclear protein after centrifugation at 14,000 rpm for 5 min at 4°C.

Interstitial partial pressure of oxygen. A fiber-optic oxygen meter with a needle-type optic-fiber oxygen sensor (OXY-MICRO-AOT; World Precision Instruments) was used to determine the interstitial partial pressure of oxygen (P\textsubscript{o2}) in the epididymal fat pads. A surgical operation was conducted with an abdominal incision to expose the epididymal and retroperitoneal fat pads. During the surgery, mice were anesthetized with a rodent cocktail (100 mg/kg ketamine-5 mg/kg xylazine-2 mg/kg acepromazine) under ambient air. The measurements were made in epididymal and retroperitoneal fat pads. A mean value was obtained for each mouse from the value of both left and right fat pads. Each reading was taken after 3 s of probe insertion. As a control, oxygen pressure in the blood of the abdominal inferior vena cava (vein) was measured. The reading was converted (into mmHg) using the formula \(P_{\text{o2}} = 159.6 \times \text{mmHg} \times \text{reading.} \) The mean value of 10 mice was used to represent \(P_{\text{o2}} \) in obese or lean mice.

Hypoxia probe. Western blotting and immunohistostaining for hypoxia probe were done using the Hoxypoxyprobe-1 kit (HP2-100; Chemicon International). The kit contains three key reagents: pimonidazole hydrochloride (Hoxypoxyprobe-1; no. 90203), Hoxypoxyprobe-1 antibody (no. 90531) conjugated with FITC, and anti-FITC secondary antibody (no. 90532) conjugated with horseradish peroxidase (HRP). Hoxypoxyprobe-1 was injected (intraperitoneally) at 60 mg/kg body wt at 30 min before tissue collection. The epididymal fat pads were collected immediately after cervical dislocation. For Western blotting, the fresh tissue was frozen immediately in liquid nitrogen after isolation and used for preparation of the whole cell lysate. The gel (7% SDS-PAGE) running time was 30 min for analysis of the hypoxia probe. The Hoxypoxyprobe-1 antibody was used as the primary antibody in the Western blot. For immunohistostaining, the fresh adipose tissue was fixed in 10% formalin, dehydrated, and embedded in paraffin. Immunohistostaining was conducted with the primary Hoxypoxyprobe-1 antibody conjugated with FITC and a secondary antibody of anti-FITC antibody conjugated with HRP. Since the secondary antibody does not cross-react with antigen in rodent tissues, there is no nonspecific staining in the adipose tissue.

Preparation of primary cells from adipocyte tissue. Primary cells were prepared from epididymal fat pads of lean C57BL/6 mice. The cell isolation was conducted in sterile conditions using a protocol modified from that described by Malide et al. (33). Briefly, the fat pads were collected immediately from the mice after cervical dislocation, minced, and digested with collagenase (1 mg/ml; Sigma C6885) at 37°C for 60 min. The cell suspension was centrifuged at 400 g for 2 min, and the cells in the top fraction of medium were collected as primary cells of adipose tissue. After being washed two times in DMEM, the cells were incubated in 35-mm dishes in DMEM with 10% FBS. Forty-eight hours later, the cells were used in the hypoxia study.

Hypoxia treatment. Primary cells of adipose tissue, fully differentiated 3T3-L1 cells, and primary macrophages were maintained in fresh medium for 6 h before hypoxia treatment. HEPES was used at 15 mM in the cell culture medium to maintain pH in the hypoxia condition. The cells were exposed to hypoxia in a sealed chamber (metal chamber, self-designed). The hypoxia was generated by filling the chamber with low-oxygen air that was made to contain 1% oxygen, 5% CO\(_2\), and 94% nitrogen. The ambient air was removed from the chamber by vacuum before the chamber was filled with customized air. To keep the humidity in the chamber, 200 ml of water were kept in the chamber. The chamber was maintained in a 37°C water bath for a constant temperature.

Real-time quantitative RT-PCR. Total RNA was extracted from homogenized fat pads or cells using the Tri reagent (Sigma T9424) according to the manufacturer’s instructions. Real-time quantitative RT-PCR (qRT-PCR) was conducted using the ABI 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA). The following primers and probes were ordered from Applied Biosystems: HIF-1α (Mm00468869_m1), vascular endothelial growth factor (VEGF; Mm00437304_m1), glucose transporter 1 (GLUT1; Mm00441473_m1), heme oxygenase 1 (Hmox; Mm00516004_m1), pyruvate dehydrogenase kinase 1 (PDK1; Mm00554306_m1), macrophage migration inhibition factor (MIF; Mm01611157_gH), IL-1β (Mm00434228_m1), IL-6 (Mm004446190_m1), TNF-α (Mm00443258_m1), transforming growth factor-β (TGF-β; Mm00441724_m1), matrix metalloproteinase 9 (MMP9; Mm00442991_m1), and adiponectin (Acrd; Mm00456425_m1). The mRNA signal was normalized over 18S rRNA signal. A mean value of triplicates was used for relative mRNA level or calculation of the degree of mRNA induction.

Peritoneal macrophages. Primary peritoneal macrophages were isolated from C57BL/6J mice. The macrophages were induced intraperitoneally by injection of 2 ml of sterilized solution of 2% starch (Sigma 85643). The macrophages were harvested 3 days later with 20 ml of cold PBS in lavage and then cultured in RPMI 1640 (supplemented with 10% FBS and 50 μg/ml gentamicin) in a 100-mm culture dish. Three days later, the cells were transferred to 35-mm tissue culture dishes and treated with hypoxia in serum-free RPMI 1640 medium.

Transfection and reporter assay. The luciferase reporter driven by adiponectin gene promoter (~1,300/+18) was kindly provided by Dr. Jianhua Shao (Graduate Center for Nutritional Sciences, University of Kentucky, Lexington, KY) (41). The NF-κB luciferase reporter and TNF-α luciferase reporter (~1,200 bp) are described elsewhere (66). The reporter plasmids were transfected into 3T3-L1 adipocytes by electroporation using the Nucleofector II electroporator (Amaza Biosystems, Cologne, Germany). Program T030 was used in electroporation. For transfection into NIH3T3 fibroblasts, Lipopectamine was used. The details of luciferase assay and reporter normalization are described elsewhere (15).

Statistical analysis. In this study, all of the experiments were conducted at least three times with consistent results. The data from representative experiments are presented. Values are means ± SE of multiple data points or samples to represent the final result. Student’s t-test or one-way ANOVA was used in statistical analysis of the data with a significance of P ≤ 0.05.
RESULTS

Hypoxia in adipose tissue of ob/ob mice. In an effort to identify risk factors for insulin resistance, we observed that adipocytes developed insulin resistance in response to vacuum in a sealed chamber. This result led us to investigate the hypoxia effect on adipose tissue, since hypoxia was generated in the sealed chamber under vacuum. To investigate hypoxia in the adipose tissue, we employed three approaches. In the first approach, the interstitial PO2 was measured in epididymal and retroperitoneal fat pads of obese mice. A surgical operation was conducted to expose the fat pads in mice under anesthesia. An oxygen meter that was equipped with a needle-type oxygen probe was used to detect PO2 in the fat pads. In the control lean mice, PO2 was 47.9 mmHg in the epididymal fat pad. In the obese mice, PO2 dropped to 15.2 mmHg (Fig. 1A). The 70% reduction in PO2 suggests hypoxia in the adipose tissue of obese mice. In the retroperitoneal fat pads, a similar reduction was observed in PO2. In the inferior vena cava (venous blood), PO2 was not reduced in the obese mice, suggesting that the adipose hypoxia is not likely a result of systemic hypoxia. The temperature of adipose tissue was 35.5–36.5°C when PO2 was measured.

In the second approach, a chemical probe (pimonidazole hydrochloride) for hypoxia was used to detect hypoxia in the adipose tissue of obese mice. The chemical probe reacts with proteins under hypoxia, leading to generation of new protein adducts, which can be detected with a monoclonal antibody. The probe was injected into the peritoneal cavity and detected in the adipose tissue 30 min after injection. To detect the probe, the epididymal fat pads were collected and used in Western blotting and immunohistostaining. The probe signal was quantified in the whole cell lysate with Western blotting. The probe signal was observed in both lean and obese mice. However, it was increased eightfold in the ob/ob mice as determined by the
signal of total bands (Fig. 1B). The distribution of hypoxia probe was examined in the tissue with immunohistostaining (Fig. 1C). Consistently, the obese mice exhibited more hypoxia in the immunohistostaining. The probe represented by the brown color in the microscopic image was mainly detected in the intercellular space. The staining was increased in the obese mice.

In the third approach, a group of widely accepted genes for hypoxia response was used to examine the hypoxia (44). The gene expression was determined in qRT-PCR. The genes included HIF-1α, VEGF, GLUT1, Hemox, and PDK1 (38, 44). Among the five genes, HIF-1α is a transcription factor that controls expression of the other four genes (44). In the adipose tissue, four of the five genes were increased in ob/ob mice, with the exception being VEGF (Fig. 1D). The increase was only observed in the adipose tissue, not in the skeletal muscle, of obese mice (Fig. 1E), suggesting that hypoxia is limited to the fat tissue in the obese mice. The data do not support a role of insulin in the upregulation of these genes in the obese mice. Insulin was reported to induce HIF-1α expression in cell cultures (55). This activity of insulin suggests that hyperinsulinemia may contribute to the expression of hypoxia response genes in obesity. However, the fat-specific expression of hypoxia response genes does not support such a role of insulin in our model. Data for P22 and the hypoxia probe suggest that hypoxia is likely the primary cause of hypoxia gene expression. It is interesting that an increase in VEGF was not observed in the adipose tissue of ob/ob mice. The unresponsiveness of VEGF may be related to hyperglycemia in the obese mice. It was reported that VEGF transcription was inhibited by a high level of glucose (25).

Association of adipose hypoxia with body weight in dietary obese mice. The ob/ob mice are deficient in leptin. It is not clear whether the hypoxia is a result of leptin deficiency or obesity. To answer this question, the hypoxia was examined in dietary obese mice. The obese mice were generated by feeding the wild-type C57BL/6J mice with a HFD (58% calories from fat). The hypoxia was determined using the five hypoxia response genes. HIF-1α protein was examined in the nucleus of adipocytes, and a significant increase was observed in the WAT of the obese mice (Fig. 2A). The HIF-1α increase was also observed at the mRNA level (Fig. 2B). All of the five hypoxia response genes were upregulated. Although an increase in VEGF mRNA was not detected in ob/ob mice, the increase was detected in the adipose tissue of dietary obese mice. Similar to that observed in ob/ob mice, the hypoxia response genes were not changed in the skeletal muscle of HFD mice (Fig. 2C). This group of data suggests that the adipose hypoxia is a result of obesity, not a direct consequence of leptin deficiency.

To further investigate the association of adipose hypoxia and adiposity, an influence of body weight on adipose hypoxia was investigated in the dietary obese mice. When the body weight was increased by HFD from 3 to 5 mo, the hypoxia was significantly increased as indicated by the increase in expression of hypoxia response genes (Table 1). Under food restriction, when the body weight was reduced, the hypoxia genes were reduced together with the body weight (Table 1), sug-

Table 1. Association of adipose hypoxia with body weight

<table>
<thead>
<tr>
<th>Assay</th>
<th>HFD 3 mo</th>
<th>HFD 5 mo</th>
<th>Control</th>
<th>CR</th>
</tr>
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<tr>
<td>Metabolism</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW, g</td>
<td>40±2.2</td>
<td>53±3.2*</td>
<td>50±3.2</td>
<td>33±5.2*</td>
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<tr>
<td>Insulin, ng/ml</td>
<td>416±25</td>
<td>652±43*</td>
<td>590±32</td>
<td>196±12*</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>100±9</td>
<td>141±7*</td>
<td>139±8</td>
<td>105±6*</td>
</tr>
<tr>
<td>Hypoxia genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIF-1α</td>
<td>3.7±0.5</td>
<td>4.9±0.7*</td>
<td>4.7±0.6</td>
<td>3.4±0.1*</td>
</tr>
<tr>
<td>VEGF</td>
<td>2.0±0.2</td>
<td>5.5±0.4*</td>
<td>4.2±0.4</td>
<td>1.6±0.2*</td>
</tr>
<tr>
<td>GLUT1</td>
<td>0.4±0.1</td>
<td>1.3±0.2*</td>
<td>0.8±0.1</td>
<td>0.3±0.02*</td>
</tr>
<tr>
<td>Hemox</td>
<td>2.7±0.3</td>
<td>4.9±0.9*</td>
<td>5.5±0.2</td>
<td>3.1±0.3*</td>
</tr>
<tr>
<td>PDK1</td>
<td>1.5±0.41</td>
<td>3.1±0.5*</td>
<td>3.3±0.2</td>
<td>2.0±0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 6 mice). HFD, high-fat diet; CR, calorie restriction; BW, body weight; HIF-1α, hypoxia-inducible factor-1α; GLUT1, glucose transporter 1; Hemox, heme oxygenase 1; PDK1, pyruvate dehydrogenase kinase 1. Expression of hypoxia gene (relative mRNA) was used to indicate hypoxia in epididymal fat of dietary obese mice. Mice on HFD for 3 and 5 mo were compared for the association of BW gain and degree of hypoxia. CR for 4 wk was used to study the association of weight loss with improvement of oxygenation. Mice on HFD for 4 mo were used in the CR study. *P < 0.05.

Fig. 2. Hypoxia in adipose tissue of dietary obese mice. The obesity was induced in male C57BL/6J mice with a high-fat diet (HFD; 58% kcal from fat, D12331; Research Diets) at 5 wk of age. The epididymal fat pads were collected at 12 wk on HFD. Tissues from sex- and age-matched lean mice (fed on chow diet) were used in the control. A: Western blot of HIF-1α. The nuclear protein was extracted from epididymal fat tissue and examined in a Western blot. Seven mice were examined in each group. An increase in HIF-1α protein was observed consistently in the obese mice. A representative Western blot is shown. Each lane represents 1 mouse. SP3 is a loading control for the nuclear protein. B: mRNA of hypoxia response genes determined by qRT-PCR. Total RNA was extracted from the epididymal fat and used in TaqMan qRT-PCR. Values are means ± SE (n = 7 mice). C: mRNA in the skeletal muscle determined by qRT-PCR. Values are means ± SE (n = 6 mice).
suggesting an improvement of oxygenation in the adipose tissue after reduction of obesity. Fasting plasma insulin and glucose were positively associated with the body weight and adipose hypoxia, indicating a link between the adipose hypoxia and insulin resistance.

**Association of adipose hypoxia and inflammatory response in obese mice.** Hypoxia induces expression of inflammatory cytokines in cells (36). In the adipose tissue, the chronic inflammation might be a result of hypoxia response. To test this possibility, we used a group of inflammation-related genes to evaluate the inflammation response. These genes include cytokines (TNF-α, IL-1, IL-6, and TGF-β), chemokine (MIF), extracellular enzyme (MMP9), and macrophage markers (CD11 and F4/80). In the adipose tissue of ob/ob mice, all of these genes were increased over the control (Fig. 3A), suggesting an elevation in inflammation and macrophage infiltration in obesity. The increase was observed together with hypoxia response genes. In the study, mRNA expression of resistin and sterol regulatory element-binding protein (SREBP) was examined, and no significant change was observed (data not shown). The association of inflammation and hypoxia was also observed in the dietary obese mice (Fig. 3B). These results suggest that in adipose tissue of obese mice, chronic inflammation is associated with hypoxia.

**Induction of inflammatory response in primary adipocytes of lean mice by hypoxia in vitro.** To test the cause-and-effect relationship between hypoxia and inflammation, the primary adipocytes were prepared from adipose tissue of lean mice and tested in a hypoxia study in vitro. The primary adipocytes were prepared from epididymal fats and exposed to 1% oxygen to examine the hypoxia response. One percent oxygen is often used in the study of cellular response to hypoxia in vitro (45). Its Po2 is 7.6 mmHg, which is comparable to 15.2 mmHg in the peritoneal cavity of lean C57BL/6 mice and treated with hypoxia in vitro. Before the hypoxia treatment, all of the inflammatory genes were detected at the basal levels. After hypoxia treatment, these inflammatory genes were all significantly upregulated (Fig. 4B). The induction was observed together with hypoxia response genes. All of the hypoxia genes were increased in mRNA except HIF-1α. The result suggests that expression of inflammatory genes was induced by hypoxia in macrophages. The cause-and-effect study was also conducted in adipocytes. In 3T3-L1 adipocytes, four of the inflammatory genes (IL-6, MIF, TGF-β, and MMP9) were detected, and three of them (IL-6, MIF, and MMP9) were induced by hypoxia (Fig. 4C). The hypoxia response genes are all induced by hypoxia in 3T3-L1 adipocytes except HIF-1α.

The time course of gene expression was analyzed in this study. The result suggests that in response to hypoxia, expression of inflammatory genes happens earlier than that of hypoxia response genes in the cell culture. In the primary adipocytes and macrophages, inflammatory genes (TNF-α, IL-1, IL-6, and TGF-β) was significantly induced between 0.5 and 2 h, and hypoxia genes were significantly increased between 4 and 8 h during the hypoxia treatment (Fig. 4B legend). In primary macrophages, inflammation genes were significantly increased at 2 h. At this time point, hypoxia response genes were not significantly increased. An increase in hypoxia genes were observed after 4 h in hypoxia. In the 3T3-L1 adipocytes, an increase in IL-6 mRNA was observed at 0.5 h during hypoxia treatment. At this time point, the hypoxia response genes were not changed. An increase in the hypoxia genes was

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Fig. 3. Association of hypoxia and inflammation in adipose tissue: mRNA was examined in the total RNA extract of epididymal fat. To present all of the genes together, a mean value for the degree of (fold) induction was used to indicate the change in each gene. These genes have a big difference in signal strength, and it is hard to read the results if the relative mRNA signal is used. The inflammatory genes include TNF-α, IL-1, IL-6, macrophage migration inhibition factor (MIF), transforming growth factor-β (TGF-β), matrix metalloproteinase 9 (MMP9), CD11, and F4/80. The hypoxia response genes were included as a control for hypoxia response. The experiments were repeated 3 times with consistent results. A: gene expression in ob/ob mice at 12 wk of age. B: gene expression in dietary obese mice after 12 wk on HFD. Values are means ± SE (n = 6 mice). *P < 0.05; **P < 0.001.
observed at 8 h. This is consistent with the nature of inflammatory genes that is quick in response to stimulation.

**Activation of NF-κB and TNF-α gene promoter by hypoxia.** Expression of inflammatory cytokines (such as TNF-α, IL-1, and IL-6) is precisely controlled at the gene transcription level. The transcription factor NF-κB is a master transcriptional regulator of these inflammatory genes. The above data suggest that adipose hypoxia may lead to the gene expression by activation of NF-κB. To test this possibility, the NF-κB activation of NF-κB/H9260 protein was examined using the luciferase reporter and nuclear extract in the positive control. To test transcription of the NF-κB gene promoter was used in this transfection system. In response to hypoxia, the TNF promoter was activated in both 3T3-L1 adipocytes and NIH3T3 fibroblasts (Fig. 5C). These data suggest that activation of NF-κB by hypoxia may be involved in the inflammatory gene expression.

**Inhibition of adiponectin expression by hypoxia.** A reduction in plasma adiponectin is widely observed in obesity and type 2 diabetes (22). The reduction was observed at mRNA and protein levels in the obese mice (Fig. 6, A and B). To explore the role of hypoxia in the regulation of adiponectin, mRNA was examined in the primary adipocytes after hypoxia treatment. A significant reduction of adiponectin was observed in a time-dependent manner in 3T3-L1 adipocytes (Fig. 6C). Adiponectin mRNA was reduced significantly at 8 and 24 h. To understand the mechanism of adiponectin inhibition by hypoxia, we examined the gene promoter activity for adiponectin in 3T3-L1 adipocytes. The assay was conducted with transient transfection of the adipocytes with a luciferase reporter that is driven by the adiponectin gene promoter (-1,300/+18). The reporter activity was inhibited by hypoxia as well as CoCl2,
which mimics the hypoxia effect (Fig. 6D). Since gene transcription is controlled by the promoter activity, the inhibition of promoter activity suggests that the suppression of adiponectin by hypoxia may happen at the transcriptional level.

DISCUSSION

We demonstrated hypoxia in adipose tissue in obese mice. The data suggest that hypoxia may be a risk factor for chronic inflammation and adiponectin inhibition in white adipose tissue in obesity. In vivo, the adipose hypoxia was associated with inflammation. The cause-and-effect relationship was demonstrated for hypoxia and inflammation by in vitro assay using primary adipocytes and primary macrophages. The 1% oxygen (7.6 mmHg) was used in cell culture experiment to mimic hypoxia in vivo. This condition is widely used in hypoxia research. The 1% oxygen (7.6 mmHg) is close to the adipose tissue oxygen level (2%, 15.2 mmHg) that was detected in the obese mice. The tissue oxygen pressure was a mean value of multiple measurements. In some measurements, oxygen pressure was 1% in the adipose tissue. This suggests that 1% oxygen exists in the adipose tissue of obese mice.

The animal data in Table 1 suggest that improvement of oxygenation in adipose tissue led to attenuation in inflammation. As indicated by the reduced oxygen pressure and increased gene expression, the adipose hypoxia and inflammation were induced by obesity. In dietary obesity, the cause-and-effect relationship was examined for hypoxia and inflammation through calorie restriction in the dietary obese mice. The adipose hypoxia was attenuated by weight loss in the calorie restriction, suggesting improvement of oxygenation in the adipose tissue. In this condition, expression of inflammatory genes was reduced. This is consistent with the positive association of adipose hypoxia and inflammation during weight gain in the dietary obese model. The hypoxia response in adipose tissue may explain insulin resistance induced by a hypoxia environment or systemic hypoxia (39, 48, 50). We expect that low oxygenation (Po2) occurs in adipose tissue of obese patients. This possibility may be used to explain susceptibility of obese patients to insulin resistance under systemic hypoxia, such as obstructive sleep apnea (20, 40, 50, 56).

The high level of plasma blood glucose may be responsible for the nonresponsiveness of VEGF to hypoxia in the adipose tissue.
tissue of ob/ob mice. 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 fasting glucose, which was 197 mg/dl in ob/ob mice (12 wk of age) and 100 mg/dl in dietary obese mice (12 wk on HFD) when the gene expression was examined. In the dietary obese mice, the blood glucose level was ~140 mg/dl at most in this study, 40% lower than that of ob/ob mice. The difference in glucose may account for the difference in VEGF response to hypoxia. The high glucose in ob/ob mice may be responsible for the unchanged VEGF mRNA in ob/ob mice. It has been reported that VEGF transcription was inhibited by a high level of glucose (25).

Hypoxia-induced expression of inflammatory genes may be a result of transcriptional activation. The mechanism might be related to activation of transcriptional activities of several nuclear factors, such as HIF-1, NF-κB, and C/EBP (35, 53, 63). HIF-1 is a dominant transcription factor for gene expression in response to hypoxia (44). NF-κB is a major transcription factor for expression of inflammatory genes, and its activation by hypoxia is independent of IKK (1, 35). In this study, we observed NF-κB activation and transcriptional activation of TNF-α gene promoter in response to hypoxia. This suggests the role of NF-κB in hypoxia-induced inflammatory response. Although it is generally believed that NF-κB is directly activated by hypoxia, it also is possible that the activation is an indirect effect of hypoxia, such as secretion of the cytokine TNF-α. C/EBP is required for adipogenesis as well as gene expressions for lipid metabolism and inflammation (2, 59).

C/EBP is also activated by hypoxia (53, 63). Of the six (TNF-α, IL-1, IL-6, MIF, TGF-β, and MMP9) proinflammatory genes examined in this study, two (MIF and TGF-β) are HIF-1 target genes (26, 46), four (TNF-α, IL-1, IL-6, and MMP9) are NF-κB target genes (34), and two (IL-1 and IL-6) are C/EBP target genes (59). The role of each transcription factor remains to be confirmed in the hypoxia-induced expression of inflammatory genes and hypoxia genes. Our data suggest that in response to hypoxia, expression of inflammatory genes happened at 2 h and expression of hypoxia response genes occurred at 8 h. Thus inflammatory genes may change earlier than hypoxia response genes in the response to hypoxia.

In response to hypoxia, c-Jun NH2-terminal kinase (JNK) and endoplasmic reticulum (ER) stress are activated in many experimental systems. JNK is activated by hypoxia in several studies of apoptosis (9, 11, 31). Activation of transcription factor AP-1 by JNK may also contribute to the expression of inflammatory genes in response to hypoxia. ER stress is induced by hypoxia as indicated by PERK (protein kinase-like ER stress kinase) expression (3, 27). The ER stress may contribute to JNK activation.

MIF may contribute to macrophage infiltration into the adipose tissue in obesity. MIF is a 114-amino acid protein that circulates in homotrimeric, dimeric, and monomeric forms (37). MIF expression is increased by hypoxia and glucocorticoids (6, 26). Study of MIF knockout mice suggests that MIF is required for normal function of macrophage (43). MIF is produced by many types of cells, including adipocytes, monocytes/macrophages, and lymphocytes (19, 49). In human adipocytes, MIF expression is positively associated with patient body mass index and negatively correlated with insulin sensitivity (49, 58). Although these studies suggest a pathological connection of MIF to insulin resistance, it remains to be understood how MIF contributes to insulin resistance. Our
data suggest that the increased expression of MIF in obesity may be a result of adipose hypoxia. It is possible that MIF contributes to the macrophage infiltration into WAT by inhibiting macrophages from moving away from the tissue in obesity. In macrophage infiltration, MIF may synergize with MCP-1.

Hypoxia may inhibit adiponectin expression by targeting peroxisome proliferator-activated receptor-γ (PPARγ). In this study, we observed that adiponectin expression was reduced in mRNA and protein in adipose tissue of obese mice. In vitro, adiponectin mRNA was reduced by hypoxia in 3T3-L1 adipocytes. During preparation of this report, a similar observation was reported in 3T3-L1 adipocytes (8). In our study, we demonstrate that the adiponectin promoter activity was inhibited by hypoxia. In this study, the Adc gene promoter activity was induced by troglitazone. The inhibition of Adc transcription might be a result of PPARγ suppression by hypoxia (67). The promoter DNA of adiponectin contains response elements for several transcription factors, such as PPARγ, FOXO1, C/EBP, and SREBP (21, 42, 47). It was reported that PPARγ activity was inhibited by hypoxia and that the inhibition was dependent on HIF-1α (67). This effect of HIF-1 may contribute to the adiponectin inhibition by hypoxia. It is not clear whether there is a direct binding site for HIF-1 in the adiponectin gene promoter. The transcriptional activity of C/EBP was induced by hypoxia (53, 63). It is unlikely that C/EBP activation by hypoxia contribute to the adiponectin inhibition. C/EBP was reported to enhance adiponectin gene transcription (42). SREBP may not be involved in the inhibition, since it is not directly regulated by hypoxia (14). The relationship of FOXO1 and hypoxia is not clear. The data suggest that hypoxia is a new factor for inhibition of adiponectin expression. In vivo, hypoxia and inflammation may both contribute to the inhibition of adiponectin expression.

The mechanism by which hypoxia is generated in the adipose tissue remains to be identified in obesity. A reduction in adipose tissue blood flow (ATBF; ml/min × 100 g tissue−1) may be involved in the development of hypoxia. ATBF is reduced in both human and animal obesity (51, 61). The ATBF reduction may lead to inflammation through hypoxia. Hypoxia-induced inflammation may further reduce the blood flow through the endothelial dysfunction. TNF-α is reported to reduce insulin sensitivity in endothelial cells (32). This endothelial alteration may block vasodilation and capillary recruitment effects of insulin (10, 57), leading to more reduction in ATBF. ATBF is increased by physical exercise and fasting status, which are associated with improvement of insulin sensitivity (5, 13). In addition, an increase in adipocyte size may also contribute to the intrstitial hypoxia in adipose tissue. Diffusion distance of oxygen is ~120 μm in tissues (18). When the diameter of adipocytes becomes ≥120 μm, the large adipocytes will form a barrier to block oxygen diffusion to the distal area in adipose tissue. The diameter of a large adipocyte can reach 150 μm in mice (4). This possibility may explain the severity of hypoxia in the adipose tissue. The blood flow was reduced by ~50% in the adipose tissue in obesity (51). Our data suggest that oxygen tension can reduce by 75%. The changes in blood flow and oxygen tension are not in proportion in adipose tissue. The barrier effect of large adipocytes may contribute to the difference in the two parameters.

In summary, hypoxia is induced in the white adipose tissue by obesity. Hypoxia may serve as an initial signal for chronic inflammation and adiponectin inhibition in obesity. It may also be responsible for plasma elevation of leptin, since hypoxia induces leptin expression in adipocytes (17). The adipose hypoxia may be a potential risk factor for chronic inflammation and impairment of adipose tissue function in obesity. These possibilities remain to be tested in vivo.

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