Original Article

Adipose Tissue Hypoxia in Obesity and Its Impact on Adipocytokine Dysregulation

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Obesity is linked to a variety of metabolic disorders, such as insulin resistance and atherosclerosis. Dysregulated production of fat-derived secretory factors, adipocytokines, is partly responsible for obesity-linked metabolic disorders. However, the mechanistic role of obesity per se to adipocytokine dysregulation has not been fully elucidated. Here, we show that adipose tissue of obese mice is hypoxic and that local adipose tissue hypoxia dysregulates the production of adipocytokines. Tissue hypoxia was confirmed by an exogenous marker, pimonidazole, and by an elevated concentration of lactate, an endogenous marker. Moreover, local tissue hypoperfusion (measured by colored microspheres) was confirmed in adipose tissue of obese mice. Adiponectin mRNA expression was decreased, and mRNA of C/EBP homologous protein (CHOP), an endoplasmic reticulum (ER) stress–mediated protein, was significantly increased in adipose tissue of obese mice. In 3T3-L1 adipocytes, hypoxia dysregulated the expression of adipocytokines, such as adiponectin and plasminogen activator inhibitor type-1, and increased the mRNAs of ER stress marker genes, CHOP and GRP78 (glucose-regulated protein, 78 kD). Expression of CHOP attenuated adiponectin promoter activity, and RNA interference of CHOP partly reversed hypoxia-induced suppression of adiponectin mRNA expression in adipocytes. Hypoxia also increased instability of adiponectin mRNA. Our results suggest that hypoperfusion and hypoxia in adipose tissues underlie the dysregulated production of adipocytokines and metabolic syndrome in obesity. *Diabetes* 56:901–911, 2007

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CHOP, C/EBP homologous protein; eIF2α, eukaryotic translation initiation factor 2α; ER, endoplasmic reticulum; GRP78, glucose-regulated protein, 78 kD; HIF1, hypoxia-inducible factor-1; IRE1, inositol-requiring protein-1; MMP2, matrix metalloproteinase 2; PAI-1, plasminogen activator inhibitor type 1; PPAR, peroxisome proliferator–activated receptor; siRNA, small interfering RNA; UPR, unfolded protein response; WAT, white adipose tissue; XBP1, X-box binding protein-1.

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Recent studies have revealed that adipose tissue is not only a passive reservoir for energy storage but also produces and secretes a variety of bioactive molecules called adipocytokines, including tumor necrosis factor, leptin, resistin, and plasminogen activator inhibitor type 1 (PAI-1) (1–4). Dysregulated production of adipocytokines is associated with the pathophysiology of obesity-related metabolic diseases (5–9). We identified adiponectin as an adipocytokine in the human adipose tissue cDNA library (10). Plasma adiponectin levels are low in obesity and type 2 diabetes (11,12). The biological functions of adiponectin include improvement of glucose and lipid metabolism (4) and prevention of inflammation and atherosclerosis (13–15). Adiponectin is regarded as a link between obesity and metabolic disorders. However, the precise mechanisms responsible for the dysregulation of adiponectin have not been fully elucidated.

Obesity as excess of adipose tissue is attributed to hypertrophy and hyperplasia of adipocytes. Adipocytes become hypertrophic during the development of obesity, and their size increases up to 140–180 μm in diameter (16). Adipocytes have a limited capacity for hypertrophy; one reason for this is considered the diffusion limit of oxygen, which is at most 100 μm (17). Therefore, it is possible that hypertrophic adipocytes might endure less than adequate oxygen supply.

Hypoxia occurs when oxygen availability does not match the demand of the surrounding tissue, resulting in decreased oxygen tension. Hypoxic stress plays a pivotal role in normal human development and physiology, including embryogenesis and wound repair, and has been well studied for its importance in the pathogenesis of several human diseases, including heart disease, stroke, diabetes, and cancer (18). In the presence of hypoxia, cells must respond by coordinated expression of numerous genes to ensure adaptation. An important and well-characterized key regulator of the adaptive response to alterations in oxygen tension is hypoxia-inducible factor-1 (HIF1), a transcription factor that accumulates during hypoxia and increases the mRNA expression of a wide variety of genes that stimulate erythropoiesis, angiogenesis, and glycolysis (19). On the other hand, hypoxic cells also provoke HIF1-independent adaptive responses. Previous reports have shown that the unfolded protein response (UPR), an HIF1-independent signaling pathway, is activated in the presence of hypoxia and contributes to cellular adaptation of this stress (20). Newly synthesized secretory and membrane-associated proteins are correctly folded and assem-

bled by chaperones in the endoplasmic reticulum (ER) (21). Many disturbances including hypoxia cause accumulation of unfolded proteins in the ER, resulting in ER stress. To cope with the ER stress, cells trigger a set of pathways known as UPR, which is mediated by three types of ER-transmembrane proteins, inositol-requiring protein-1 (IRE1), RNA-dependent protein kinase–like ER eukaryotic translation initiation factor 2α kinase (PERK), and activating transcription factor 6 (ATF6) (22).

Recent studies have reported that ER stress is increased in liver and adipose tissue of obese mice (23,24). Various intracellular and extracellular stimuli including glucose or nutrient deprivation, hypoxia, viral infection, and increased synthesis of secretory proteins can trigger ER stress (21). However, the triggers that induce ER stress in obesity remained unclear.

In the present study, we provide evidence for hypoxia in adipose tissue of obese mice and that such hypoxia is partly due to inadequate blood supply. In addition, we found that exposure of adipocytes to hypoxia elicits dysregulated production of adipocytokines and that hypoxia-induced downregulation of adiponectin mRNA is mediated by ER stress–dependent transcriptional and –independent posttranscriptional mechanisms.

RESEARCH DESIGN AND METHODS

All animals were purchased from CLEA Japan, were housed in a room under controlled temperature (23 ± 1°C) and humidity (45–65%), and had free access to water and chow (Oriental Yeast Co.). We used male mice for the high-fat diet–feeding study and female mice for the KKAy mice study. For diet-induced obesity studies, male C57BL/6J mice were divided at random into two groups at 8 weeks of age. The first group was fed a high-fat diet containing 30% fat by weight (AIN93G), while the second group was fed normal chow containing 5.9% fat by weight (CRF-1; Oriental Yeast Co.) for 8 weeks. Normal chow-fed and high-fat–fed mice were killed at 16 weeks of age, and female C57BL/6J and female KKAy mice were killed at 10–11 weeks of age. Tissues were dissected and frozen in liquid nitrogen. Samples were stored at −80°C until use. For analysis of arterial blood gases, blood samples were collected from the left carotid artery and measured by a blood analyzer (i-STAT; Fuso Pharmaceutical Industries, Tokyo, Japan).

Detection of hypoxia. A Hypoxyprobe-1 Plus kit (Chemicon International, Temecula, CA) was used for the detection of tissue hypoxia. Mice were injected with 40 mg/kg pimonidazole intraperitoneally 1 h before they were killed. Organs were removed immediately, fixed in 10% neutral buffered formalin for 24–48 h, and then processed into paraffin blocks. The sections were prepared according to the instructions provided by the manufacturer, counterstained with hematoxylin, and analyzed in a standard fashion.

Quantification of perfusion capacity using microspheres. Mice were anesthetized by intraperitoneal injection of pentobarbital, and a polyethylene catheter was positioned in the aortic arch via the left carotid artery. Yellow dye microspheres (15.5 μm diameter, 4×10^4 beads; Triton Technology, San Diego, CA) were injected and then flushed with saline. Tissues were dissolved in 4 mol/l KOH and filtered with polyester membrane filters (Triton Technology). The fluorescent dye was extracted with acidified cellosolve acetate, and fluorescence was measured with a plate reader and normalized by the weight of each tissue.

Lactate concentration. Tissue lactate concentrations were determined with an assay kit using the instructions provided by the manufacturer (Roche, Mannheim, Germany) and normalized by protein concentration. Protein was quantified by the bicinchoninic acid method using the BCA Protein Assay Reagent obtained from Pierce (Rockford, IL) and BSA as the standard.

Cell culture. 3T3-L1 preadipocytes were grown to confluence and induced to differentiate into adipocytes, as described previously (25). The cells were then cultured for 12 h under hypoxia (1% O_2) or normoxia (21% O_2).

Quantitative real-time PCR. Total RNA from 3T3-L1 cells and tissues were prepared with an RNeasy Mini Kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized from total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time PCR was performed on the ABI7900 using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to the protocol provided by the manufacturer. Primer sets were the following: adiponectin, 5′-GTT CTA CTG CAA CAT TCC GG-3′ and 5′-TAC ACC TGG AGC CAG ACT TG-3′; PAI-1, 5′-TCA GCC CTT GCT TGC CTC AT-3′ and 5′-GCA TAG CCA GCA CCG AGG A-3′; peroxisome proliferator–activated receptor (PPAR)-γ, 5′-CCA GAG TCT GCT GAT CTG CG-3′ and 5′-GCC ACC TCT TTG CTC TGC TC-3′; GLUT1, 5′-CCA TCC ACC ACA CTC ACC AC-3′ and 5′-GCC CAG GAT CAG CAT CTC AA-3′; C/EBP homologous protein (CHOP), 5′-GTC CTG TCC TCA GAT GAA ATT GG-3′ and 5′-GCA GGG TCA AGA GTA GTG AAG GTT-3′; glucose-regulated protein, 78 kD (GRP78), 5′-ACC TAT TCC TGC GTC GGT GT-3′ and 5′-GCA TCG AAG ACC GTG TTC TC-3′; leptin, 5′-GAT GGA CCA GAC TCT GGC AG-3′ and 5′-AGA GTG AGG CTT CCA GGA CG-3′; matrix metalloprotease 2 (MMP2), 5′-GCA GGG AAT GAG TAC TGG GTC TAT-3′ and 5′-CAG TTA AAG GCA GCA TCT ACT TG-3′; adrenomedullin, 5′-AAG TGG AAT AAG TGG GCG CTA A-3′ and 5′-ACT GTC GTC TCA TCA GCG AGT C-3′; and 36B4, 5′-GCT CCA AGC AGA TGC AGC A-3′ and 5′- CCG GAT GTG AGG CAG CAG-3′. The levels of mRNA were normalized relative to the amount of 36B4 mRNA.

Analysis of hypoxia-induced X-box binding protein-1 mRNA splicing. Total RNA from 3T3-L1 cells cultured in hypoxia (1% O_2) was reverse transcribed and amplified using the sense primer (5′-AAACAGAGTAGCAGCGCAGACTGC-3′) and the antisense primer (5′-GGATCTCTAAAACTAGAGGCTTGGTG-3′). This fragment was further digested by *Pst*I as described previously (26). The cDNA fragments were resolved on a 2% agarose gel.

Plasmids. The luciferase reporter plasmids of human adiponectin promoter were generated by excising the promoter fragment from the genomic clone of adiponectin (generous gift from Dr. Junji Takeda) and inserting it into the *Kpn*I and *Sac*I sites of the pGL3 basic luciferase expression vector (Promega, Madison, WI). Expression plasmids encoding β-galactosidase (pCMX–β-gal) were generous gifts from Dr. David Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX). Mouse CHOP (accession number BC013718) was cloned and inserted into pCMV to generate the expression plasmid for mouse CHOP (pCMV-CHOP).

Determination of adiponectin transcriptional activity in 3T3-L1 cells. On day 5 after induction of differentiation, the media of 3T3-L1 cells in six-well plates were changed to OPTI-MEM (Invitrogen, San Diego, CA), and the cells were transfected with plasmids using LipofectAMINE 2000 reagent (Invitrogen) according to the instructions provided by the manufacturer. At 48 h after plasmid transfection, luciferase reporter assays were performed using Luciferase Assay System (Promega). Luciferase values were normalized by an internal β-galactosidase control and expressed as the relative luciferase activity.

Western blotting. Western blot analysis was carried out by using anti-eIF2α (eukaryotic translation initiation factor 2α) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Design and transfection of small interfering RNAs. Two pairs of small interfering RNAs (siRNAs) were synthesized chemically by Qiagen, annealed, and transfected into 3T3-L1 adipocytes using Lipofectamine 2000 (Invitrogen) as described previously (27). Twenty-four hours after transfection, cells were cultured for 12 h under hypoxia (1% O_2) and total RNA extracted as described above.

Statistical analysis and ethical considerations. All data are presented as means ± SEM and analyzed using unpaired Student's *t* test. For simultaneous multiple comparisons, differences between groups were analyzed by one-way ANOVA followed by Dunnet's multiple comparisons test. P values <0.05 were considered statistically significant. All study protocols described in this report were approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine or Astellas Pharma.

RESULTS

Tissue hypoxia in obese mice. Body weight of obese mice was significantly higher than that of the control group (male normal diet–fed C57BL/6J mice 29.07 ± 0.42 g ($n = 11$) and male high-fat diet–fed mice 36.32 ± 0.33 g ($n = 9$), $P < 0.001$; female C57BL/6J mice 21.12 ± 0.27 g ($n = 9$) and female KKAy mice 43.96 ± 0.4 g ($n = 9$), $P < 0.001$). Tissue hypoxia was evaluated by immunohistochemistry of pimonidazole, which is known to form adducts with thiol groups under hypoxia and has been used to quantify tissue oxygen concentration (28). To identify and quantitate oxygen concentrations of tissues, sections of liver and white adipose tissue (WAT) of control and obese mice were stained with pimonidazole and hematoxylin. Control experiments showed that pimonidazole binding in L6 cells was elevated under hypoxic conditions (supplementary Fig. 1*A* and *B* [available in an online appendix at http://dx.doi.org/10.2337/db06-0911]). More-

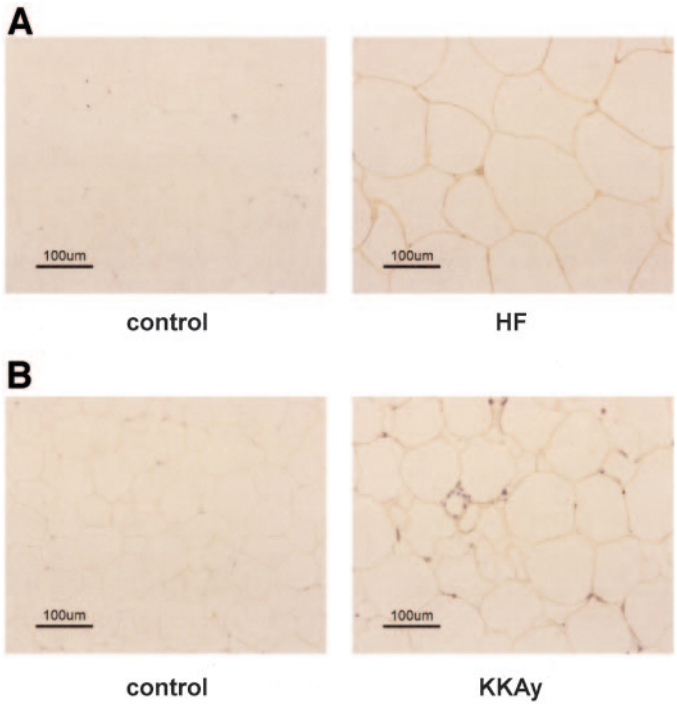

FIG. 1. Detection of epididymal or parametrial WAT hypoxia by immunohistochemistry for pimonidazole protein adducts. Sections of WAT were stained with a specific antibody for pimonidazole adducts (brown color) and hematoxylin and examined microscopically. *A*: WAT from mice fed normal diet (control) and high-fat diet (HF). *B*: WAT from C57BL6J (control) and KKAy mice (KKAy). Original magnification ×200.

over, in accordance with a previous report (29), pimonidazole staining predominated in pericentral regions of liver lobule in all mice, where oxygen supply is naturally low (data not shown). Fig. 1 shows the patterns of pimonidazole binding (brown) against a hematoxylin counter stain (blue) in epididymal or parametrial WAT of control and obese mice. Weak pimonidazole signals were identified in WAT of normal diet–fed mice and control C57BL/6J mice. On the other hand, pimonidazole staining (cytoplasm as well as cytoplasmic membrane) was markedly increased in WAT of high-fat diet–fed mice and KKAy mice compared with that of each group of control mice, indicating low oxygen concentrations in fat depots in obese mice.

We also quantified tissue lactate concentrations as another indicator of tissue hypoxia (Fig. 2). The lactate concentrations in WAT of high-fat diet–fed mice and KKAy mice were 1.7- and 1.5-fold higher than those of normal diet–fed mice and control C57BL/6J mice, respectively. In contrast, the lactate concentration in muscle of obese mice was similar to that of control mice (Fig. 2).

To exclude possible accumulation of pimonidazole and lactate by systemic hypoxia in obese mice, we analyzed arterial blood gases. As shown in Table 1, systemic arterial $PaCO_2$ and PaO_2 as well as hemoglobin concentration and oxygenation (arterial O_2 saturation: SaO_2) were not different between control and obese mice.

We next examined the possible role of tissue perfusion using colored microsphere analysis (Fig. 3). The numbers of microspheres were significantly decreased in WAT of both types of obese mice (i.e., high-fat diet–fed and KKAy mice) compared with each group of controls (Fig. 3*A* and *B*), indicating that the WAT of obese mice had less perfusion than that of control mice. There were no significant differences in the number of microspheres in the muscle, lung, heart, or kidney between the control and obese mice (Fig. 3). Similar results were obtained after correction for protein amounts (supplementary Fig. 2). These results indicate that tissue hypoxia in WAT of obese mice is, at least in part, due to decreased perfusion in WAT.

To assess the hypoxic condition in WAT, mRNA levels of hypoxia-inducible genes such as leptin, PAI-1, MMP2, and adrenomedullin were measured. Expression levels of these genes were significantly elevated in WAT of high-fat diet–fed and KKAy mice compared with each group of control mice (Fig. 4). We measured mRNA levels of these hypoxia-inducible genes in liver and muscle of high-fat diet–fed and control mice. In liver, mRNA expression levels of PAI-1, MMP2, and adrenomedullin were not elevated in high-fat diet–fed compared with the control mice. In muscle, MMP2 and adrenomedullin mRNA levels were not altered, while only PAI-1 mRNA level was increased (supplementary Fig. 3).

Adiponectin mRNA levels as well as PPARγ mRNA level were significantly reduced in WAT of high-fat diet–fed and KKAy mice compared with those of normal diet–fed and control C57BL/6J mice. The mRNA levels of ER stress marker genes, such as GRP78 and CHOP, were significantly elevated in WAT of high-fat diet–fed and KKAy mice compared with those of normal diet–fed and control C57BL/6J mice (Fig. 4) as previously reported (23,24).

Dysregulation of adipocytokine mRNA expression via hypoxia. To examine the effects of hypoxia on adipocytokine mRNA expression, 3T3-L1 adipocytes were cultured in 1% O_2 for 12 h. mRNA levels of PPARγ and adiponectin were decreased, whereas mRNA level of PAI-1 was increased in hypoxic cells compared with that in normoxic control cells (Fig. 5*A*). We further determined the effect of hypoxia on expression of adipogenic marker genes. Exposure of differentiated 3T3-L1 adipocytes to hypoxic condition did not affect stearoyl-CoA desaturase 1 (SCD1) and preadipocyte factor-1 (pref1) mRNA expression levels (Fig. 5*A*). These results suggested that hypoxia did not alter the adipocyte differentiation stage itself. To elucidate whether hypoxia inactivates the adiponectin promoter, luciferase activity driven by −3.6 kb of the human adiponectin promoter was tested in 1 and 21% O_2. As shown in Fig. 5*B*, luciferase activity under hypoxia was significantly lower than under normoxia.

Induction of ER stress in adipocytes after hypoxic incubation. To determine whether hypoxia induces ER stress in 3T3-L1 adipocytes, we investigated the expression patterns of several molecular indicators of ER stress in these cells. The mRNA expression levels of CHOP and GRP78 in the 3T3-L1 adipocytes, incubated under hypoxia, were significantly higher than those under normoxia (Fig. 6*A*). PERK is an ER-resident type I transmembrane Ser/Thr protein kinase and phosphorylates eIF2α in response to ER stress. Immunoblot analysis demonstrated marked accumulation of phosphorylated eIF2α protein in cell lysates after 2–6 h hypoxia compared with the normoxic cells (Fig. 6*B*). ER stress also activates the IRE1-dependent pathway (26). Activated IRE1 cuts out 26 nucleotides of the unspliced X-box binding protein-1 (XBP1) mRNA to generate the spliced XBP1 mRNA. The RT-PCR products produced from the spliced and unspliced XBP1 mRNAs can be easily detected following *Pst*I digestion of PCR products generated using primers flanking the XBP1 intron, since the unspliced product contains a *Pst*I site that

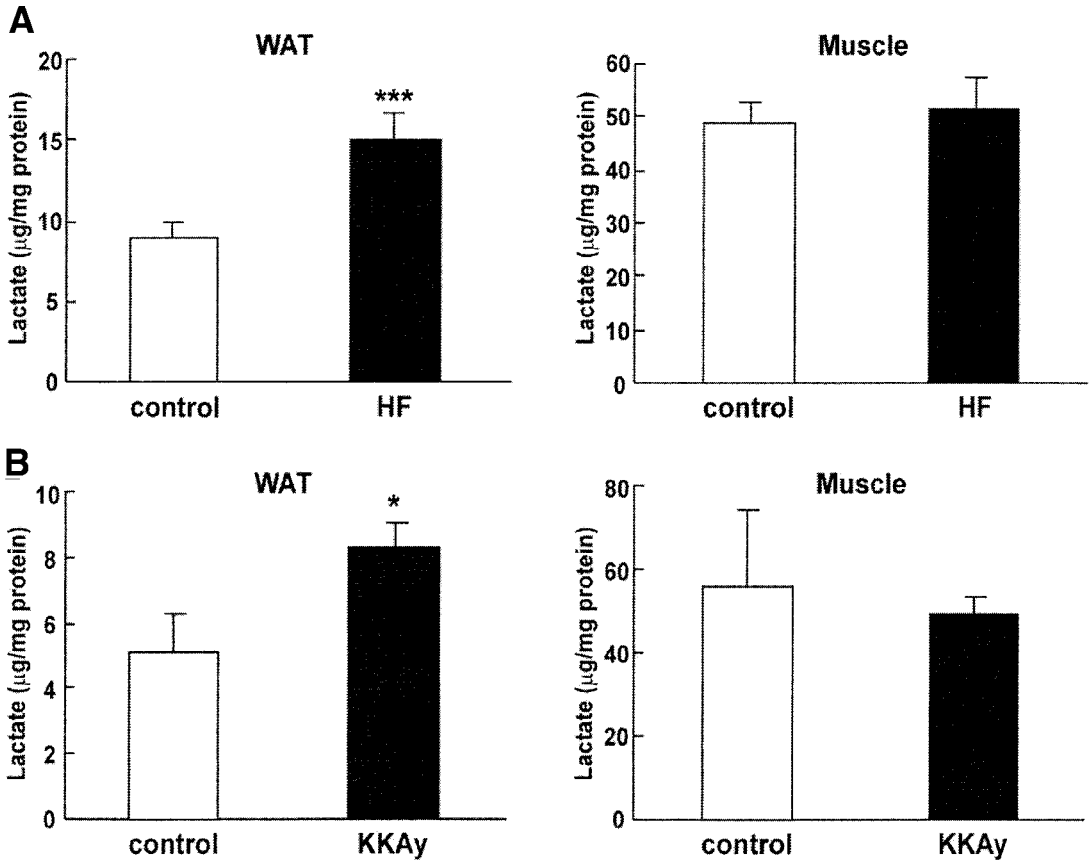

FIG. 2. Increased lactate concentration in WAT of obese mice. *A*: Tissue levels of lactate in skeletal muscles and WAT of mice fed normal diet (control, $n = 11$) and high-fat diet (HF, $n = 9$). *B*: Tissue levels of lactate in skeletal muscles and epididymal or parametrial WAT of C57BL6J (control, $n = 9$) and KKAy mice (KKAy, $n = 8$). Values are expressed as means ± SEM. *$P < 0.05$, ***$P < 0.001$ compared with the control group.

is lost in the spliced product. As shown in Fig. 6*C*, the *Pst*I-undigested fragment increased after 6 h hypoxia and was still noted at 24 h. Taken together, these results indicate that hypoxia induces ER stress in 3T3-L1 adipocytes.

Effect of ER stress on PPARγ and adiponectin mRNA expression. Treatment with tunicamycin, an ER stress inducer, decreased the mRNA expression levels of PPARγ and adiponectin in 3T3-L1 adipocytes with the induction of CHOP mRNA (Fig. 7*A*), suggesting that ER stress results in downregulation of PPARγ and adiponectin mRNA.

Effect of CHOP on adiponectin promoter activity. The CHOP protein is known to heterodimerize with other C/EBP proteins to transcriptionally inactive complexes (30). Previous reports showed that C/EBP is critical for the regulation of adiponectin expression (31). To confirm the negative regulatory effect of CHOP on the transcriptional activity of adiponectin, we measured luciferase activity driven by −3.6 kb adiponectin promoter in 3T3-L1 adipocytes. Simultaneous transfection of the CHOP vector significantly inhibited the transcriptional activity of adiponectin promoter in a dose-dependent manner (Fig. 7*B*).

Gene silencing of CHOP and HIF1α. To further confirm the role played by CHOP in adiponectin expression, we knocked down CHOP expression using a specific siRNA. Transfection of CHOP siRNA in 3T3-L1 adipocytes reduced the endogenous CHOP mRNA and protein levels (Supplementary Fig. 4*A* and *B*) and reduced mRNA levels of tribble 3 (TRB3), a target gene of CHOP (supplementary Fig. 4*C*). CHOP siRNA partially reversed the downregulation of adiponectin mRNA in 3T3-L1 adipocytes incubated under hypoxia (Fig. 7*C*). Under the same condition, interference with CHOP had no effect on downregulation of PPARγ by hypoxia (Fig. 7*C*). These findings suggest that hypoxia-induced reduction of adiponectin mRNA is mediated in part through induction of CHOP.

Because it is well established that HIF1α is a major regulator of cellular-adaptive responses to hypoxia (32), we next determined the role of HIF1α in hypoxia-induced downregulation of adiponectin mRNA. Transfection of 3T3-L1 adipocyte cells with HIF1α siRNA significantly reduced endogenous HIF1α mRNA and protein levels (supplementary Fig. 4*A* and *B*). The same treatment partially inhibited hypoxia-induced upregulation of GLUT1 mRNA, a known target of HIF1α (Fig. 7*D*). However, interference with HIF1α did not alter the gene expression levels of adiponectin under hypoxia (Fig. 7*D*) and reduced mRNA levels of PPARγ under normoxia and hypoxia (Fig. 7*D*).

Stability of adiponectin mRNA under hypoxia and ER stress. Finally, we examined the effect of hypoxia on the stability of adiponectin mRNA. The remaining amounts of adiponectin mRNA under normoxia and hypoxia were determined at various time points after addition of the transcriptional inhibitor actinomycin D. The mRNA degradation of adiponectin was accelerated under hypoxia compared with normoxia (Fig. 8*A*). To examine the effect of ER stress on adiponectin mRNA stability, 3T3-L1 adipocytes cells were incubated for 12 h with medium alone or medium supplemented with tunicamycin under normoxia, and the rate of adiponectin mRNA decay was determined as above. In contrast to the effect of hypoxia on adiponec-

TABLE 1
Systemic arterial $PaCO_2$, PaO_2, and SaO_2 of control and high-fat diet–fed mice

Mice	*n*	$PaCO_2$ (mmHg)	PaO_2 (mmHg)	SaO_2 (%)	Hb (g/dl)
Normal diet fed	9	29.1 ± 1.3	120.8 ± 6.3	98.6 ± 0.3	15.4 ± 0.2
High-fat diet fed	9	30.8 ± 4.9	108.0 ± 5.0	97.8±-0.4	15.0 ± 0.5
Control	8	30.7 ± 1.4	110.0 ± 4.6	98.1 ± 0.2	13.6 ± 0.6
KKAy	7	27.4 ± 1.7	99.0 ± 6.2	97.3±-0.3	14.1 ± 0.7

Data are means ± SEM.

tin mRNA decay, tunicamycin did not alter adiponectin mRNA stability, suggesting that hypoxia-induced degradation of adiponectin mRNA is not dependent on increased ER stress (Fig. 8*B*).

DISCUSSION

There is increasing evidence that obesity impairs adipocyte function and secretion of adipocytokines and that dysregulated release of adipocytokines contributes to insulin resistance and metabolic syndrome. One possible mechanism for the dysregulation is inflammatory reaction (33). In addition, recent studies revealed that obesity induces ER stress and that the latter in turn activates inflammatory response, thus contributing to insulin resistance in the liver and adipose tissue (23,24). However, it is still not clear how inflammation and ER stress begin in obesity. To answer this key question in the present study, we focused on oxygen availability in WAT of obese mice and investigated the effect of oxygen tension on the regulation of adipocytokines in adipocytes.

Although previous studies demonstrated changes in WAT blood flow and vasculature network in obesity (34–38), there is no evidence that the changes actually affect intracellular ambient oxygen tension. In the current study, we demonstrated that WAT of obese mice is under hypoxia as confirmed using pimonidazole hydrochloride adduction (physical evidence) as well as lactate concentration (physiological evidence). In this study, tissue hypoxia was detected in WAT of obese mice using pimonidazole staining, a 2-nitroimidazole, which is reductively activated at low oxygen concentrations (39–41) and adducts to cellular proteins. Hypoxia induces energy metabolism alteration and increases lactate concentration in tissue (42,43). We demonstrated that lactate concentration was markedly increased specifically in WAT of obese mice, providing physiological evidence of tissue hypoxia.

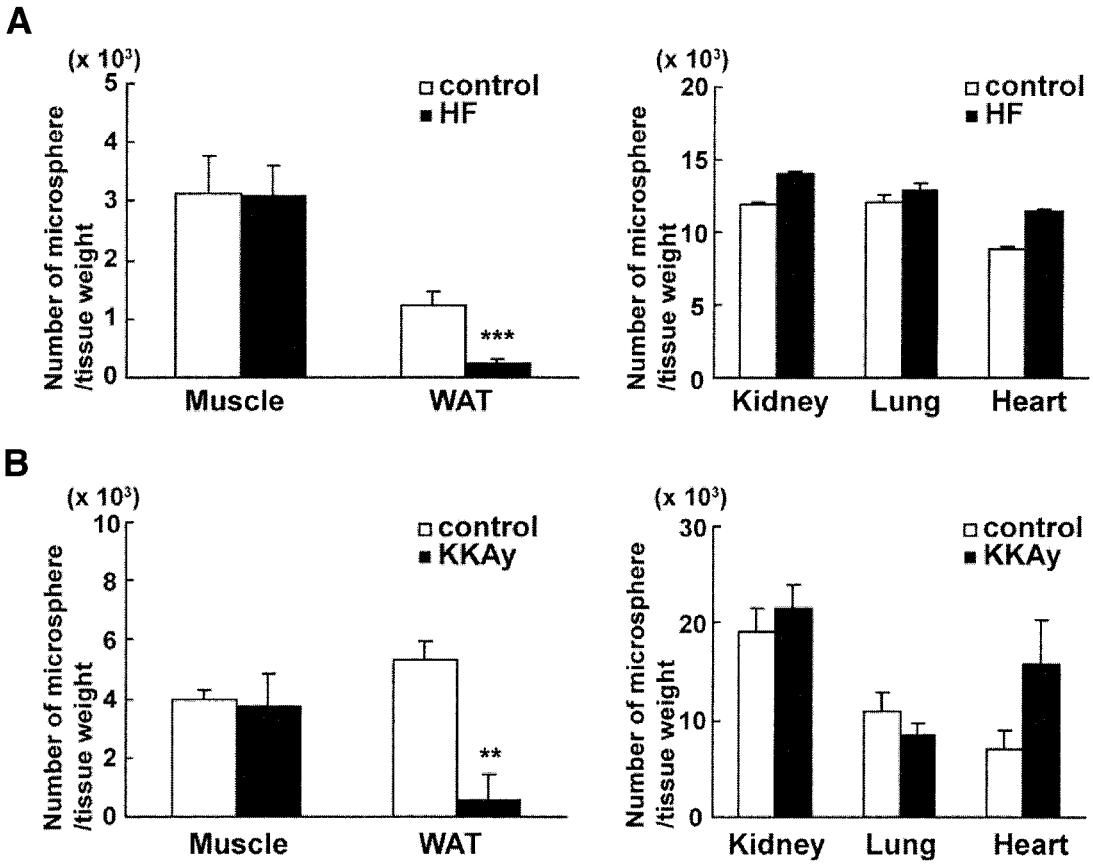

FIG. 3. Reduced perfusion in WAT of obese mice. Number of microspheres in skeletal muscles, WAT, kidney, lung, and heart of normal diet–fed mice (control, $n = 11$) and high-fat diet–fed mice (HF, $n = 9$) (*A*) and C57BL/6J (control, $n = 9$) and KKAy mice ($n = 8$) (*B*). Values are expressed as means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group.

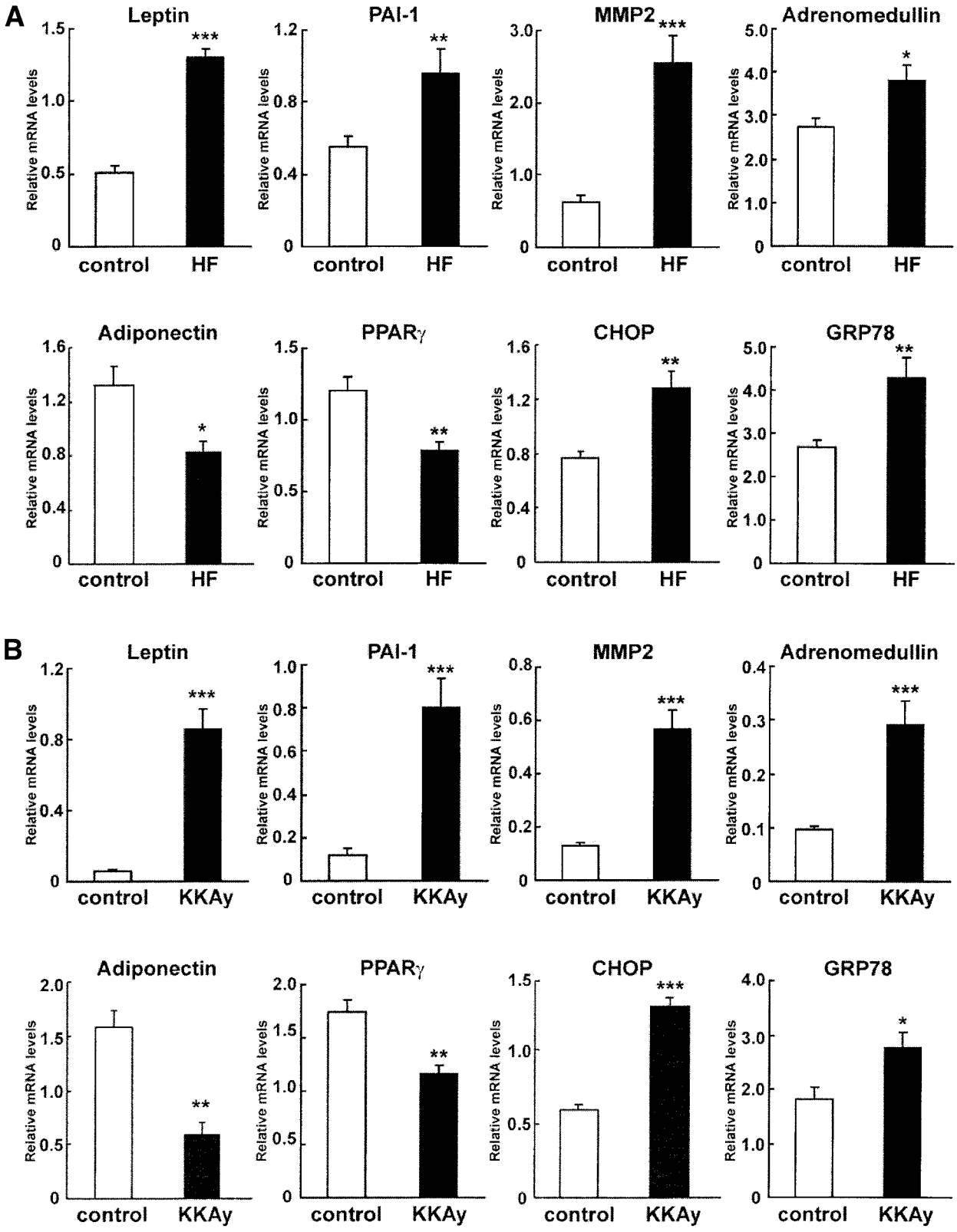

FIG. 4. Changes of mRNA levels in WAT of obese mice. The mRNA levels of leptin, PAI-1, MMP2, adrenomedullin, adiponectin, PPARγ, CHOP, and GRP78 in WAT of normal diet–fed (control) and high-fat diet–fed (HF) mice (*A*) and C57BL6J (control) and KKAy mice (KKAy) (*B*) were quantified by real-time PCR. Values are normalized to the level of 36B4 mRNA and expressed as means ± SEM (n = 8–11). *P < 0.05, **P < 0.01, ***P < 0.001 compared with the control group.

Assessment of mRNA levels of hypoxia-inducible genes such as leptin, PAI-1, MMP2, and adrenomedullin was consistent with the above data, showing that WAT of obese mice was hypoxic and liver was not. In muscle, MMP2 and adrenomedullin mRNA levels were not altered, while only PAI-1 mRNA level was increased in high-fat

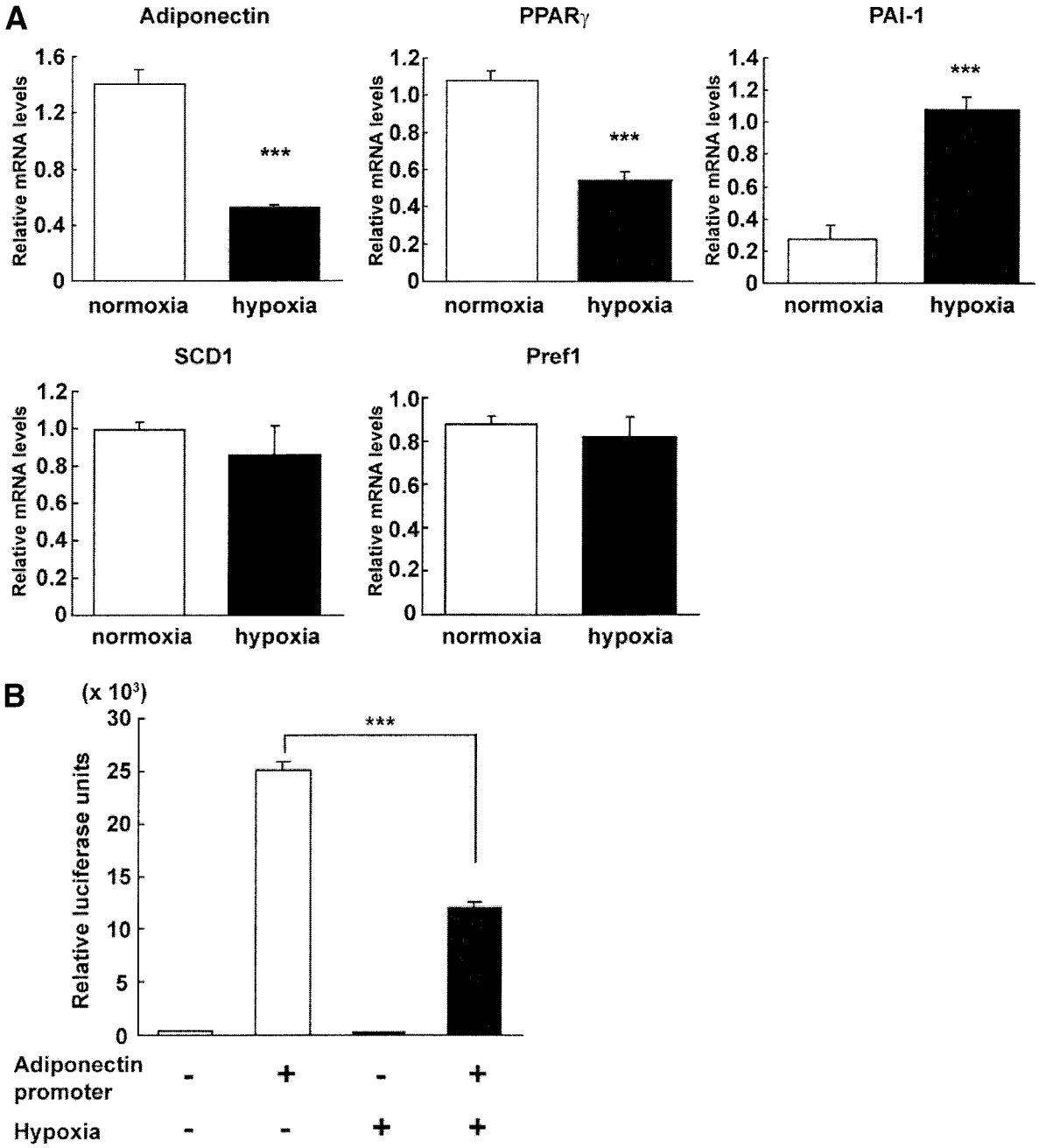

FIG. 5. Dysregulation of adipose genes in hypoxic 3T3-L1 adipocytes. *A*: The mRNA expression of adiponectin, PPARγ, PAI-1, SCD1, and Pref1 in fully differentiated 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 12 h under normoxia or hypoxia (1% O_2). The mRNA levels were quantified by real-time PCR. Values are normalized to the level of 36B4 mRNA and expressed as means ± SEM (n = 6). ***P < 0.001 compared with the normoxia group. *B*: Effects of hypoxia on adiponectin promoter activity. 3T3-L1 adipocytes were cotransfected with expression plasmids for pGL3-adipo-luc and pCMV-β-gal. After 48 h, the transfected cells were further incubated for 12 h under hypoxia (1% O_2) or maintained under normoxia for 12 h. Data are means ± SEM (n = 6). ***P < 0.001, compared with normoxia group.

diet–fed mice compared with normal diet–fed mice (supplementary Fig. 3). PAI-1 may be regulated by other mechanisms in muscle.

There are two possible explanations for the tissue hypoxia in the WAT of obese mice: *1*) reduced oxygen pressure and oxygen content of the blood and *2*) tissue hypoperfusion. The first possibility was ruled out by measurement of arterial blood gases, showing no difference in arterial PaO_2, hemoglobin concentration, or oxygenation. Moreover, pimonidazole staining and lactate concentration showed no significant differences in tissues other than WAT. Thus, local tissue hypoxia in WAT in obese mice is not due to reduced systemic oxygen supply.

The second possibility, i.e., tissue hypoperfusion in WAT of obese mice, was confirmed using colored microspheres (Fig. 3). West et al. (36) demonstrated that blood flow to adipose tissue, measured with radiolabeled microsphere, was reduced in an adipose-specific manner in Zucker obese rats. In humans, the levels of adipose tissue blood flow were measured with positron emission tomography using [^{15}O]-labeled water (44) and the ^{133}Xe washout method (45) and were lower in obese compared with

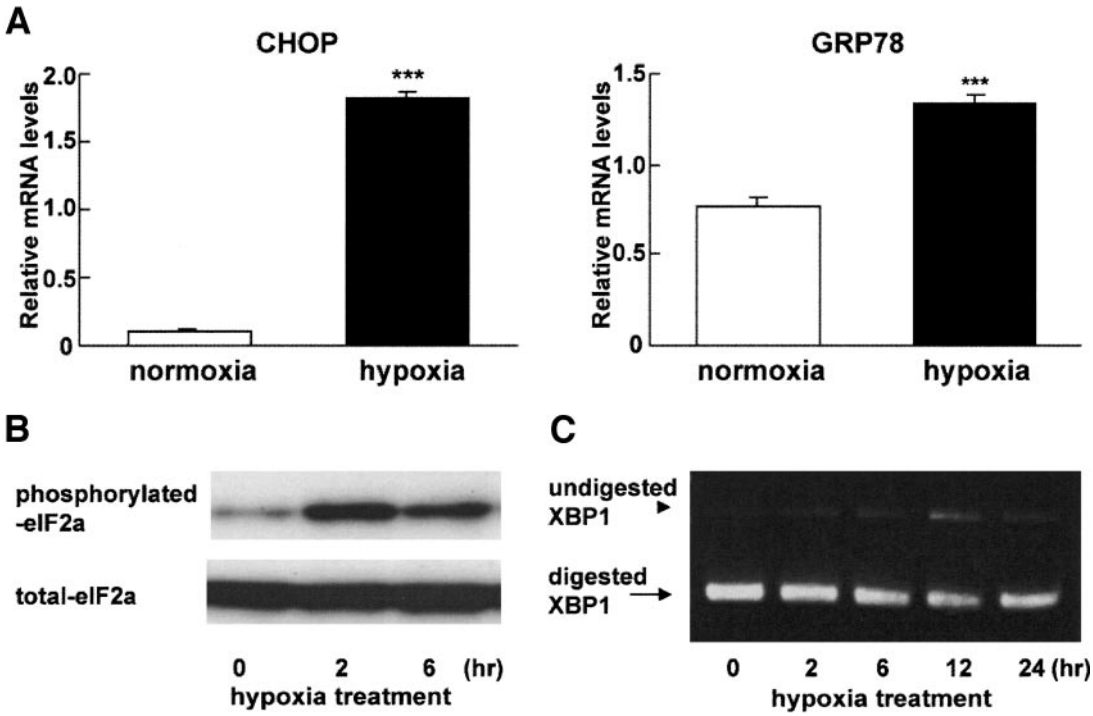

FIG. 6. Induction of ER stress in hypoxic 3T3-L1 adipocytes. *A*: The mRNA expression levels of CHOP and GRP78 in fully differentiated 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 12 h under normoxia or hypoxia (1% O_2). The mRNA levels were quantified by real-time PCR. Values are normalized to the level of 36B4 mRNA and expressed as means ± SEM ($n = 6$). ***$P < 0.001$ compared with normoxia. *B*: Effects of hypoxia on phosphorylated eIF2α protein during differentiation of 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 2 or 6 h under normoxia or hypoxia (1% O_2). *C*: RT-PCR analysis of XBP1 mRNA splicing. Total mRNA from 3T3-L1 adipocytes incubated under normoxia or hypoxia (1% O_2) for 12 h were subjected to real-time PCR analysis as described in RESEARCH DESIGN AND METHODS. Arrowhead, undigested bands; arrow, *Pst*I-digested bands.

nonobese subjects. These reports are consistent with the current data, suggesting that a decrease of adipose tissue perfusion is a common feature in obesity. We measured the numbers of endothelial cell nuclei and adipocytes per sections of WAT in control, high-fat diet–fed, and KKAy mice. The ratio of endothelial cell number per adipocyte increased significantly in obese compared with control mice (data not shown). Considering these results, blood flow per vessels may decrease severely in WAT of obese mice.

Considering that oxygen diffusion is limited at most to 100 μm, hypertrophied adipocytes up to 140–180 μm in diameter are assumed to be in a relatively hypoxic state. However, in our study, pimonidazole staining was detected in small as well as large adipocytes of obese animals. These results suggest that reduced perfusion capacity rather than cell size seems the main culprit for tissue hypoxia.

We evaluated the effect of hypoxia on adipocytokine expression. Adiponectin and PPARγ mRNA expression levels were reduced while PAI-1 level was increased in hypoxic 3T3L1 adipocytes. In addition, hypoxia decreased adiponectin promoter activity, suggesting a suppressive effect of hypoxia on adiponectin transcription.

It has been reported that severe hypoxia (46,47) or anoxia (48) induces ER stress, and UPR plays a role in adaptation to hypoxic stress in an HIF1α-independent manner in other organs and cells (49). Our results establish the role of ER stress in altering adiponectin mRNA level in hypoxic adipocytes. This conclusion was based on the following findings: *1*) hypoxia induced ER stress in adipocytes, concurrent with previous reports (20); *2*) tunicamycin, an ER stress inducer, suppressed adiponectin mRNA expression; *3*) CHOP dose-dependently inhibited adiponectin promoter activity; and *4*) hypoxia-induced downregulation of adiponectin mRNA was inhibited partially by CHOP siRNA. CHOP is a member of the C/EBP family of bZIP transcription factors, and its expression is induced to high levels by ER stress (30) and mitochondrial reactive oxygen species (ROS) (47). Adiponectin transcription is regulated by C/EBP (31), thus CHOP should inhibit adiponectin transcription as a suppressor of C/EBP as the CHOP-C/EBP heterodimer cannot bind to the C/EBP binding site. Cycloheximide, a potent inhibitor of protein synthesis, blocked hypoxia-induced suppression of adiponectin mRNA (supplementary Fig. 5), indicating that de novo production of protein mediators was required. Production of CHOP via ER stress might be partly responsible for downregulation of adiponectin mRNA. These results suggest that tissue hypoxia in the WAT of obesity leads to ER stress and suppression of adiponectin transcription.

Posttranscriptional regulation of mRNA stability is another control of gene expression by hypoxia, as shown in several hypoxia-regulated genes including vascular endothelial growth factor, tyrosine hydroxylase, GLUT1, erythropoietin, and endothelial NO synthase (50,51). In our study, hypoxia increased adiponectin mRNA degradation in adipocytes, and this effect was independent of ER stress. Adiponectin mRNA, however, does not contain classical AU-rich motifs for mRNA stability (52), suggesting unknown sequence motifs involved in its stability. These data indicate that adiponectin mRNA transcription was regulated via ER stress and that mRNA stability was regulated posttranscriptionally via hypoxia.

Other mechanisms might also be involved in the hy-

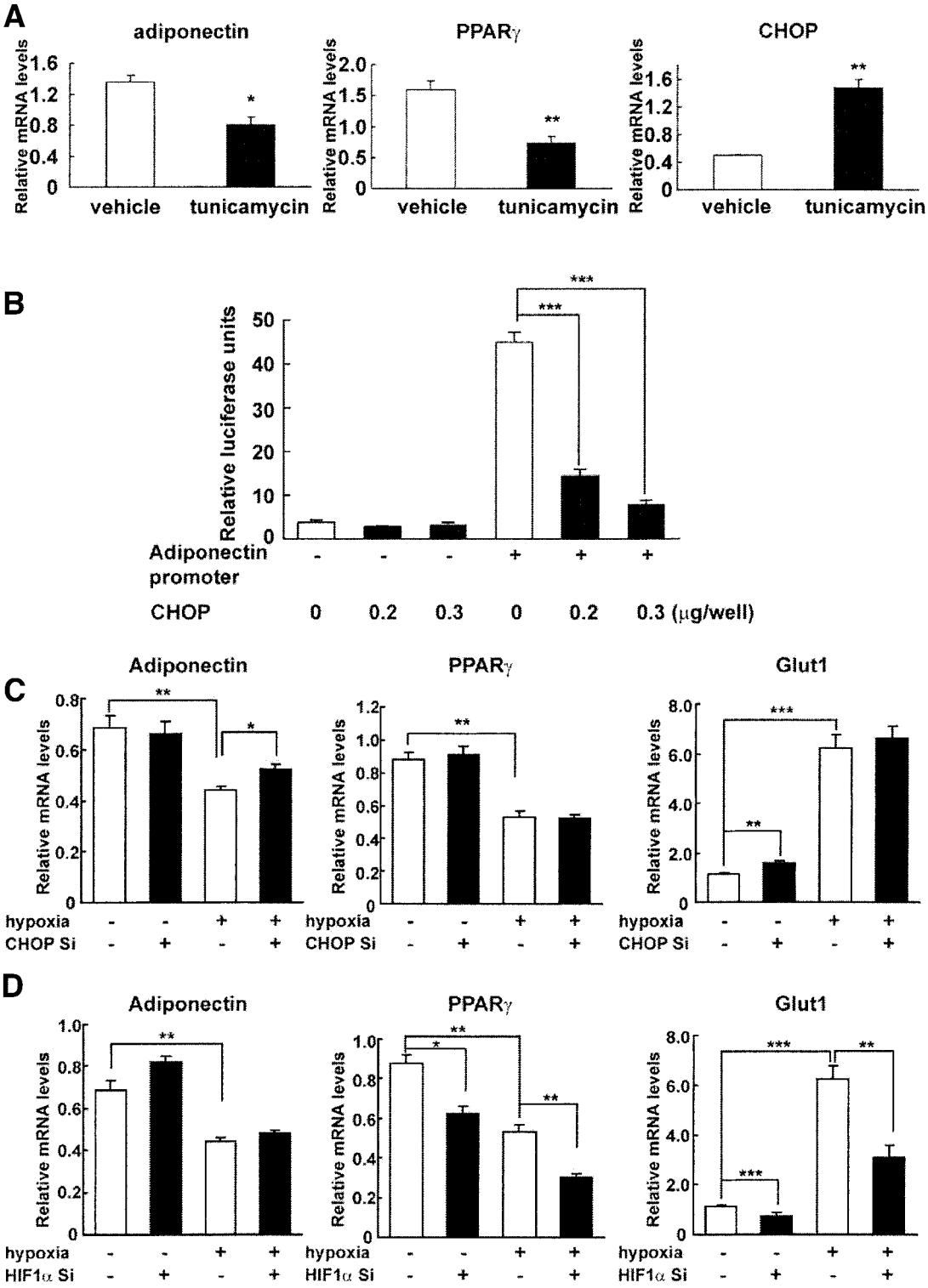

FIG. 7. Dysregulation of adipose genes by an ER stress inducer in 3T3-L1 adipocytes. *A*: The mRNA expression levels of PPARγ, adiponectin, and CHOP in fully differentiated 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 12 h with 2 μg/ml tunicamycin. The mRNA levels were quantified by real-time PCR. Values are normalized to the level of 36B4 mRNA and expressed as means ± SEM (n = 3). *P < 0.05, **P < 0.01 compared with the vehicle group. *B*: Adiponectin promoter activity in 3T3-L1 adipocytes. 3T3-L1 adipocytes were transfected with expression plasmids for pGL3-adipo-luc, pCMV-CHOP, and pCMV-β-gal. After 48 h, luciferase activity in cell lysates was measured and normalized with β-galactosidase activity and expressed as means ± SEM (n = 6), ***P < 0.001 compared with the control group. *C*: Effect of knockdown of CHOP on mRNA expression of adiponectin, PPARγ, and GLUT1 in fully differentiated 3T3-L1 adipocytes. 3T3-L1 adipocytes were transfected with control siRNA or CHOP siRNA. After 24 h, cells were incubated under normoxia or hypoxia (1% O_2) for 12 h. The mRNA levels were quantified by real-time PCR. Values are normalized to the level of 36B4 mRNA and expressed as means ± SEM (n = 4). *D*: Effects of knockdown of HIF1α on mRNA expression of Adiponectin, PPARγ, and GLUT1 in differentiated 3T3-L1 adipocytes. 3T3-L1 adipocytes were transfected with control siRNA or CHOP siRNA. After 24 h, cells were incubated for 12 h under normoxia or hypoxia (1% O_2). The mRNA levels were quantified by real-time PCR. Values are normalized to the level of 36B4 mRNA and expressed as means ± SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared with the control group.

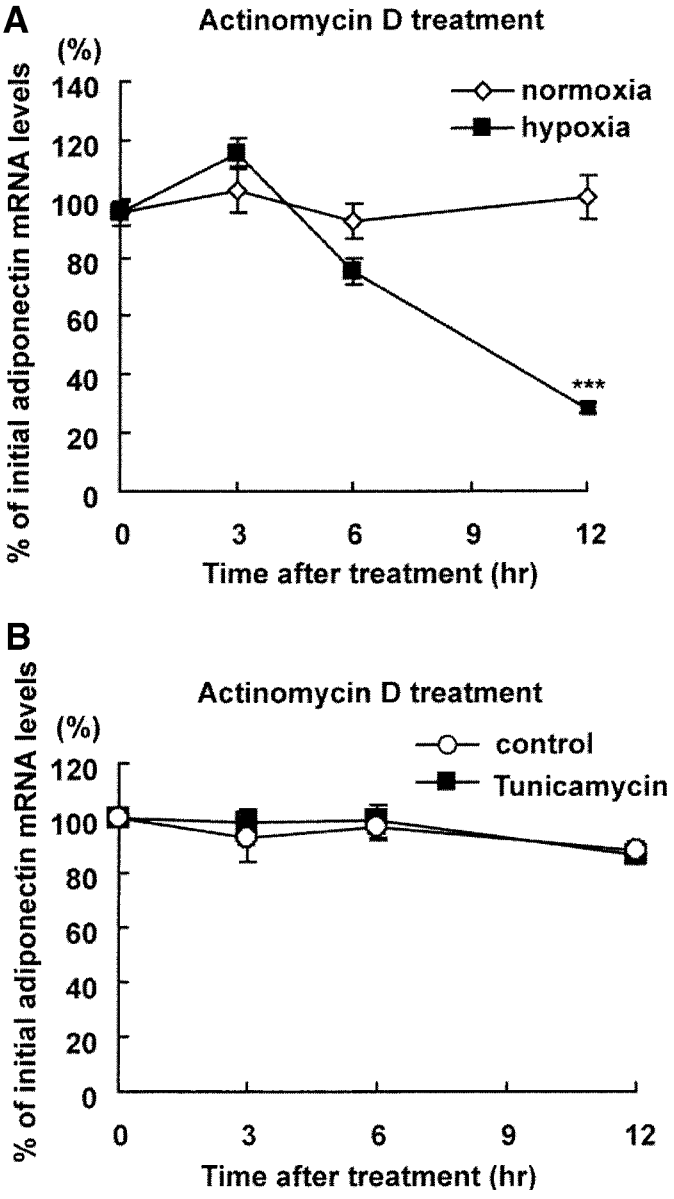

FIG. 8. Influence of hypoxia and ER stress on adiponectin mRNA stability. *A*: De novo mRNA transcription was inhibited by the addition of actinomycin D, and 3T3-L1 adipocytes were incubated under normoxia or hypoxia (1% O_2) for the indicated time intervals. *B*: 3T3-L1 adipocytes were pretreated with or without tunicamycin for 12 h. The cells were further incubated for various time periods in the presence of AcD. The residual mRNA was quantified by real-time PCR. Values represent percentage of residual mRNA versus mRNA level at time 0. Results are means ± SEM (n = 3). ***P < 0.001 compared with the normoxia group.

poxia-induced downregulation of adiponectin mRNA such as HIF1α, ROS, redox, and inflammation. Hypoxia-induced downregulation of adiponectin mRNA was not affected by HIF1α siRNA, hence hypoxia-induced suppression of adiponectin expression should not be dependent on HIF1α. We also tested the effects of several antioxidants such as *N*-acetyl-cysteine, butylated hydroxyanisole, apocynin, catalase, and agents that alter the cellular redox status by affecting NAD(P)H-to-NAD(P) ratio, i.e., rotenone (an inhibitor of complex I [NADH-dehydrogenase] of the respiratory chain) and lactate (a product of the anaerobic energy metabolism, which induces an increase in NAD(P)H/NAD(P) ratio while oxidized to pyruvate), and protein kinase inhibitors such as SB203580 (a p38MAPK inhibitor), LY294002 (a PI3K inhibitor), PD98059 (a MEK inhibitor), and SP600125 (a JNK inhibitor). None of them attenuated the hypoxia-induced downregulation of adiponectin mRNA expression. This evidence may rule out roles of ROS, redox, and inflammation in reduced adiponectin production in hypoxic adipocytes.

In summary, we demonstrated that WAT of obese mice is hypoxic and that hypoxia dysregulates the production of adipocytokines in adipocytes. This effect is mediated by ER stress and posttranscriptional regulation. Collectively, our results suggest that local adipose tissue hypoxia is partly responsible for dysregulated adipocytokines production and metabolic syndrome in obesity. Hypoxia, per se, as well as signals mediated by hypoxia, could become a therapeutic target for the treatment of obesity-linked metabolic syndrome.

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