Macrophage HIF-2α Ameliorates Adipose Tissue Inflammation and Insulin Resistance in Obesity

In obesity, adipose tissue macrophages (ATMs) play a key role in mediating proinflammatory responses in the adipose tissue, which are associated with obesity-related metabolic complications. Recently, adipose tissue hypoxia has been implicated in the regulation of ATMs in obesity. However, the role of hypoxia-inducible factor (HIF)-2α, one of the major transcription factors induced by hypoxia, has not been fully elucidated in ATMs. In this study, we demonstrate that elevation of macrophage HIF-2α would attenuate adipose tissue inflammation and improve insulin resistance in obesity. In macrophages, overexpression of HIF-2α decreased nitric oxide production and suppressed expression of proinflammatory cytokines through induction of arginase 1. HIF-2α-overexpressing macrophages alleviated proinflammatory responses and improved insulin resistance in adipocytes. In contrast, knockdown of macrophage HIF-2α augmented palmitate-induced proinflammatory gene expression in adipocytes. Furthermore, compared with wild-type mice, Hif-2α heterozygous-null mice aggravated insulin resistance and adipose tissue inflammation with more M1-like ATMs upon high-fat diet (HFD). Moreover, glucose intolerance in HFD-fed Hif-2α heterozygous-null mice was relieved by macrophage depletion with clodronate treatment, implying that increase of proinflammatory ATMs is responsible for insulin resistance by haplodeficiency of Hif-2α upon HFD. Taken together, these data suggest that macrophage HIF-2α would counteract the proinflammatory responses to relieve obesity-induced insulin resistance in adipose tissue.

Emerging evidence has suggested that obesity is characterized by chronic and low-grade inflammation accompanied by macrophage accumulation in the adipose tissue, which eventually leads to metabolic diseases, including insulin resistance and type 2 diabetes (1–3). Increased adipose tissue macrophages (ATMs) play crucial roles in the altered production of proinflammatory cytokines in the adipose tissue of obesity (1–3). In healthy lean mice, ATMs are mostly composed of alternatively activated (M2)-type macrophages, which express high levels of anti-inflammatory cytokines such as interleukin (IL)-10 and specific enzymes such as arginase (ARG) 1 in company with low levels of proinflammatory signals (4,5). Thus M2 ATMs contribute to metabolic homoeostasis by keeping down adipose tissue inflammation (4,5). In the progress of obesity, some ATMs are polarized from the M2 type to the classically activated (M1) type. In obese adipose tissue, M1 ATMs enhance nitric oxide (NO) production with stimulation of inducible NO synthase (iNOS) and the secretion of proinflammatory cytokines such as tumor necrosis factor α (TNFα), IL-6, and IL-1β. Proinflammatory cytokines secreted from M1 ATMs repress insulin action and potentiate proinflammatory transcription factors such as nuclear factor κB to further stimulate the expression of proinflammatory genes, which eventually leads to insulin resistance (1,6,7). Recently, it has been reported that M2 ATMs are also elevated in obesity, where they would function to repair and/or remodel dysregulated adipose tissue (4,5,8). Alternative activation of M2
ATMs by Th2 cytokines such as IL-4 and IL-13 contributes to relieve metabolic complication and to restore insulin sensitivity (9,10). Thereby, the balance shifting between M1 and M2 ATMs appears to be crucial for inflammatory responses in adipose tissue of obesity.

In obese adipose tissues, M1 polarization of ATMs is attributed to various changes of local microenvironments (2–5). Adipose tissue hypoxia is a tissue-specific phenomenon that occurs with rapid expansion of adipose tissue in obesity. Accordingly, it has been shown that ATMs isolated from obese adipose tissue exhibit elevated hypoxic state, implying that the pathophysiological role of ATMs is regulated by certain transcription factors induced by hypoxia in combination with metabolic stresses (11–14). Hypoxia-inducible factor (HIF)-1α and HIF-2α are key transcription factors to mediate hypoxic responses such as angiogenesis, glycolysis, adhesion, and infiltration. Most studies of adipose tissue HIFs have focused on HIF-1α (14–17). For instance, activation of HIF-1α has been implicated in adipose tissue inflammation, fibrosis, and adipocyte dysfunction. In addition, it has been suggested that HIF-1α is involved in the proinflammatory feature of ATMs in obesity (14). However, although HIF-1α has been extensively investigated in adipose tissue inflammation, the role of HIF-2α, also known as endothelial PAS domain protein 1, in obesity is largely unknown.

HIF-2α shares many features with HIF-1α in terms of its structure, reactivity to hypoxia, DNA-binding motif (so called the hypoxia-response element), and target genes such as GLUT1, vascular endothelial growth factor A (VEGF-A), and adrenomedullin (ADM) (18). However, despite the extensive homology it shares with HIF-1α, HIF-2α has its own physiological functions through induction of its unique target genes such as erythropoietin, octamer-binding transcription factor 4, and delta-like ligand 4 (18). Furthermore, studies of HIF-1α and HIF-2α in solid tumors have revealed their opposing roles in cell growth, energy metabolism, NO homeostasis, and other cell functions (18,19). These findings led us to test whether HIF-2α has functions that are distinct from those of HIF-1α in obese adipose tissue even though both HIF-1α and HIF-2α might be involved in angiogenesis in response to adipose tissue hypoxia. In particular, the functional roles of HIF-2α in the regulation of the inflammatory responses in ATMs to modulate adipose tissue inflammation remain to be elucidated.

In this study, we demonstrated that macrophage HIF-2α attenuates the proinflammatory property via induction of ARG1, which prevents proinflammatory responses and insulin resistance in adipocytes. Consistently, haplo-deficiency of Hif-2α in mice exacerbated adipose tissue inflammation and insulin resistance in high-fat diet (HFD)-fed obese mice. In addition, depletion of ATMs by clodronate injection restored insulin sensitivity in HFD-fed Hif-2α heterozygote (Hif-2α+/−) mice, indicating that the increase of proinflammatory ATMs observed in severe obesity is one of major criminals for insulin resistance in HFD-fed Hif-2α+/− mice. Collectively, these data suggest that macrophage HIF-2α would alleviate insulin resistance in obese adipose tissue by suppressing the proinflammatory responses of ATMs induced by metabolic stresses.

**RESEARCH DESIGN AND METHODS**

**Animals and Treatment**

*db/db* and *db/+* mice were purchased from Central Laboratory Animal Inc., South Korea, and were killed at 12 weeks of age to detect HIF-2α in the adipose tissue. *Hif-2α−/−* mice were generously provided by Jang-Soo Chun. All mice were maintained under pathogen-free conditions and were housed in solid-bottom cages with wood shavings for bedding in a room maintained at 25°C with a 12:12 h light:dark cycle (lights on at 0700 h). Heterozygous mice were bred to generate *Hif-2α−/−* and wild-type (WT) littersmates. *Hif-2α−/−* and WT mice were maintained on normal diet (ND) until 10 weeks of age and were then fed HFD (60% of calories derived from fat; Research Diets Inc., New Brunswick, NJ) for 12–16 weeks. For the oral glucose tolerance test (OGTT), mice were fasted for 6 h, and then they were administered glucose (3 g/kg body weight; Sigma-Aldrich, MO). Blood glucose levels were measured at indicated time points with a Freestyle blood glucose meter (TheraSense, Uppsala, Sweden). For the insulin tolerance test (ITT), mice were fasted for 3 h and then administered insulin (1 unit/kg body weight; Lilly, IN), and then blood glucose levels were measured at the indicated time points. For macrophage depletion, clodronate liposome (FormuMax Scientific Inc., CA) was intraperitoneally injected two times at an interval of 3 days, as recommended by the manufacturer. All mice were killed and dissected, and tissue specimens were immediately stored at −80°C until analysis. All animal procedures were conducted in accordance with the research guidelines of the Seoul National University Animal Experiment Ethics Committee.

**Adipose Tissue Fractionation**

Adipose tissue was fractionated as previously described, with minor modifications (20). Briefly, epididymal adipose tissue were digested with type I collagenase buffer and filtered through nylon mesh. After centrifugation, the floating adipocytes and pelleted stromal vascular cellular (SVC) fractions were washed several times and then collected for RNA extraction. The SVC fraction was also used for flow cytometry.

**Flow Cytometric Analysis**

Flow cytometric analysis was performed as previously described (21). Erythrocytes were removed from the SVC fraction by adding red blood cell lysis buffer. After incubation with a blocking antibody, we stained the SVCs with monoclonal antibodies against CD11b (BD Bioscience, CA), F4/80, CD11c, and CD206 (eBioscience) for macrophage analysis. SVCs were analyzed using FACS Canto II.
Adenovirus Infection
HIF-2α adenovirus was generously provided by Jang-Soo Chun (22). As a negative control, a green fluorescent protein (GFP) adenovirus (Neurogenex, South Korea) was used. Primary cultured peritoneal macrophages were incubated with primary antibodies against HIF-2α (1:1,000; Novus, CO), perilipin (1:1,000; Fitzgerald, MA), and CD11b (1:1,000; ebioscience) overnight at 4°C. After washing for 1 h, the samples were incubated with fluorescence-labeled secondary antibody for 4 h at room temperature and washed again. Following staining with DAPI (Vector Laboratory, CA), samples were observed using a Zeiss LSM510NLO confocal microscope.

Transfection with Small Interfering RNA
Small interfering RNA (siRNA) was delivered into and peritoneal macrophage by electroporation. Peritoneal macrophages were electroporated as soon as the primary culture was prepared without attachment. The sequences of the siRNA targeting HIF-2α were sense, 5′-CUCA GUUACGCCCAACUGUGACUG-3′, and antisense, 5′-CAGU GACGAUGUGUCGUAGAG-3′ (22). As a negative control, a scrambled siRNA (siCTL) was used.

Isolation of Peritoneal Macrophage
Mice were injected intraperitoneally with sterile thiglycollate solution (2 mL per mouse). After 3 days, peritoneal cells were harvested by washing the peritoneal cavity with PBS containing 5 mmol/L EDTA. Primary peritoneal macrophages were cultured with DMEM (HyClone, Logan, UT) with 10% FBS (HyClone) to allow cell adherence. Nonadherent cells were removed by washing.

3T3-L1 Adipocyte Differentiation and Coculture Experiment
3T3-L1 preadipocytes were grown to confluence in DMEM supplemented with 10% bovine calf serum (HyClone). Two days after confluence, differentiation was stimulated with DMEM containing 10% FBS, 500 μmol/L methylisobutylxanthine, 1 μmol/L dexamethasone, and 5 μg/mL insulin for 2 days. Then the culture medium was replaced with DMEM containing 10% FBS and 5 μg/mL insulin. For the indirect coculture experiment, 3T3-L1 adipocytes were differentiated in the lower chamber of a Transwell apparatus and cocultured with macrophages in which the expression of HIF-2α was modified and were then prepared to grow in 0.4-μm pore Transwell. After 48 h of incubation, the Transwell-attached macrophages were removed and the 3T3-L1 cells were used for RNA extraction, an insulin-stimulated glucose uptake assay, and Western blotting to detect insulin signaling after insulin treatment. For the macrophage coculture experiments, epididymal adipose tissue was chopped into ~1-mm pieces, and then 50 mg of adipose tissue was placed in the upper chamber of each well of the Transwell apparatus and cocultured with 5 × 10^5 macrophages in the lower chamber separated by 0.4-μm pore membrane.

Insulin-Stimulated Glucose Uptake Assay
Insulin-stimulated glucose uptake into 3T3-L1 adipocytes was determined by measuring [14C]-2-deoxy-D-glucose uptake as previously described (23). Briefly, 3T3-L1 adipocytes cocultured with macrophages were incubated in low-glucose DMEM containing 0.1% BSA for 8 h. Cells were stimulated with or without 50 nmol/L insulin for 15 min. Glucose uptake was initiated by the addition of [14C]-2-deoxy-D-glucose (Perkin-Elmer, MA) at a final concentration of 3 μmol/L for 15 min in HEPES-buffered saline. The reaction was terminated by several washes with cold PBS. After the cells were lysed with 0.1% SDS, 14C was measured using a scintillation counter. 14C was normalized to total protein in the whole cell lysates.

Quantitative RT-PCR
Total RNA was isolated from peritoneal macrophages, 3T3-L1 adipocytes, and epididymal adipose tissues as described previously (24). cDNA was synthesized using the M-MuLV reverse transcriptase kit according to the manufacturers’ instructions (Thermo Fisher Scientific, MA). The primers used for quantitative real-time PCR were obtained from Bioneer (South Korea), and their sequences are provided in Supplementary Table 1.

Western Blot Analysis
Western blot analysis was performed as described previously (24). Peritoneal macrophages, 3T3-L1 adipocytes, and epididymal adipose tissues were lysed with NETN buffer. The proteins were separated on SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Millipore). The blots were blocked with 5% nonfat milk and probed with the following primary antibodies: HIF-2α (Novus), HIF-1α, ARG1 (Abcam, MA), iNOS (Santa Cruz Biotechnology, CA), and β-actin (Sigma-Aldrich). The blots were visualized with horseradish peroxidase–conjugated secondary antibodies (Sigma-Aldrich) developed with chemiluminescence. For the detection of insulin signaling in 3T3-L1 adipocytes cocultured with macrophages, 3T3-L1 cells were incubated in low-glucose DMEM containing 0.1% BSA for 8 h, and then they were lysed after 30 min of 50 nmol/L insulin stimulation. To detect insulin signaling, antibodies against pAKT (Ser473), AKT,
pGSK (Ser9; Cell Signaling, MA), and GSK (BD biosciences) were used.

Measurement of NO
Nitrite was measured using the Griess reaction as described previously (20). Culture medium (100 μL) was collected and incubated with an equal volume of Griess reagent (Sigma-Aldrich) for 10 min at room temperature. The nitrite concentration was measured by the absorbance at 550 nm, using sodium nitrite as a standard. S-(2-boronoethyl)-l-cysteine (BEC), an ARG inhibitor, was purchased from Cayman Chemical, MI.

Statistical Analysis
Results represent data from multiple (three or more) independent experiments. Error bars represent SD, and P values were calculated using Student t test or two-way ANOVA.

RESULTS
HIF-2α Is Increased in ATMs of Obese Mice
It has been shown that the expression levels of HIF-1α mRNA and protein are increased in obese adipose tissue (12,13). However, even though HIF-2α is another major transcription factor to mediate hypoxia, it is unclear whether the level of HIF-2α is altered in the adipose tissue of obese animals. To address this, we quantitatively analyzed the level of Hif-2α mRNA in several adipose tissues of db/db mice. Similar to Hif-1α, the level of Hif-2α mRNA was significantly increased in many fat depots, including epididymal and subcutaneous adipose tissue of db/db mice (Supplementary Fig. 1A and B). Compared with lean mice, Hif-2α mRNA was elevated in both the SVCs and adipocytes from the epididymal adipose tissue of obese db/db mice (Supplementary Fig. 1C). Especially, the level of HIF-2α protein was markedly elevated in CD11b+ ATMs from obese db/db mice (Fig. 1), implying that accumulated macrophage HIF-2α might be involved in the regulation of adipose tissue inflammation in obesity. To determine whether HIF-2α abundantly expresses in either M1 or M2 ATMs of obese adipose tissue, M1 ATMs and M2 ATMs were isolated by using flow cytometry, and then we examined the level of Hif-2α mRNA in them. As shown in Fig. 1C, Hif-2α mRNA was more highly expressed in M2 ATMs along with M2 markers such as macrophage galactose N-acetyl-galactosamine–specific lectin 1 (Mgl1), mannose receptor C type 1 (Mrc1), and chitinase-like 3 (Chil3). In contrast, Hif-1α mRNA was abundantly expressed in the M1-like ATMs of obese animals. These results suggest that M2 ATMs would highly express HIF-2α protein in response to hypoxia compared with M1 ATMs.

Overexpression of Macrophage HIF-2α Diminishes Proinflammatory Responses via ARG1
It has been recently demonstrated that HIF-1α and HIF-2α counteract each other in the regulation of NO synthesis, which partly affects macrophage polarity (25). To examine the effect of HIF-2α elevation on the proinflammatory activity of macrophages, we analyzed the expression of genes associated with NO metabolism and several inflammatory cytokines. In peritoneal macrophages, adenoviral overexpression of HIF-2α stimulated the mRNA expression of HIF-2α target genes such as VEGF-A and ADM without the change of HIF-1α mRNA (Fig. 24). In addition, macrophage HIF-2α overexpression significantly elevated the levels of ARG1 mRNA and protein, while mRNA levels of ARG2, an isozyme encoded by a different gene, and iNOS were not altered (Fig. 2B and C). ARG1 competitively uses L-arginine, which is also a common substrate of iNOS for NO synthesis, and actively prevents
NO production in macrophages (26). In accordance with the gene expression profiles, NO production was significantly suppressed by HIF-2α overexpression in macrophages (Fig. 2D). In contrast, treatment with the ARG inhibitor, BEC, reversed the HIF-2α overexpression-mediated reduction of NO generation, which was more evident in the presence of L-arginine, (Fig. 2E). Moreover, macrophage HIF-2α overexpression decreased the levels of expression and secretion of proinflammatory cytokines such as TNFα, IL-6, and IL-1β, whereas BEC reversed the inhibitory effect of HIF-2α on the expression and secretion of proinflammatory cytokine genes in macrophages (Fig. 2F and G).

Because macrophages sensitively alter their functional phenotypes in response to the local microenvironmental inputs, GFP adenovirus (Ad-GFP)– and HIF-2α adenovirus (Ad-HIF-2α)–infected macrophages were indirectly cocultured with freshly isolated adipose tissues of lean WT or obese db/db mice. As shown in Fig. 2H, certain signaling molecules released from adipose tissues potently stimulated the expression of TNFα in cocultured macrophages, whereas the induction of TNFα was largely abolished by HIF-2α overexpression in macrophages. Interestingly, ARG1 mRNA was strongly induced in macrophages cocultured with lean adipose tissue, and macrophage HIF-2α overexpression further augmented ARG1

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**Figure 2**—Macrophage HIF-2α overexpression decreases NO production and suppresses expression of proinflammatory cytokine genes via induction of ARG1. Peritoneal macrophages infected with an adenovirus containing HIF-2α (Ad-HIF-2α) or GFP (Ad-GFP). Total RNA was isolated and analyzed for HIF-2α, HIF-1α, and proangiogenic genes (VEGF-A and ADM) (A) and NO metabolic genes (ARG1, ARG2, and iNOS) (B). **P < 0.01 vs. Ad-GFP control by Student t test. Total lysates were subjected to Western blot analysis using specific antibodies (HIF-2α, ARG1, iNOS, and β-actin). C: β-Actin was used as the loading control. Ad-GFP– or Ad-HIF-2α–infected macrophages were treated with or without L-arginine (0.1–2 mmol/L) (D) or BEC (0.1 mmol/L) (E). After 48 h, the culture media from these cells were harvested and the NO concentration was measured. F: mRNA expression levels of TNFα, IL-6, and IL-1β were analyzed by quantitative RT-PCR. G: Secretion of TNFα and IL-6 were measured in culture media from macrophages cultured in the same condition. §§§P < 0.005 vs. Ad-GFP control by two-way ANOVA; *P < 0.05 and **P < 0.01 vs. Ad-GFP control; #P < 0.05 and ##P < 0.01 vs. Ad-HIF-2α control by Student t test. H: Ad-GFP– or Ad-HIF-2α–infected macrophages were cocultured with or without chopped epididymal adipose tissue of WT or db/db mice in Transwell chambers for 48 h. Total RNA was isolated from the cocultured macrophages, and then HIF-2α, ARG1, iNOS, and TNFα levels were analyzed. *P < 0.05 and **P < 0.01 vs. noncocultured control by Student t test. #P < 0.05; ##P < 0.01. All data represent mean ± SD for n = 3 in each group. All quantitative RT-PCR data were normalized to the level of Cyclophilin mRNA.
mRNA levels (Fig. 2H). Meanwhile, coculturing with obese adipose tissue relatively attenuated induction of ARG1 mRNA but further increased iNOS mRNA, which may lead to reduce the effect of macrophage HIF-2α on expression of TNFα mRNA. Collectively, these results suggest that macrophage HIF-2α could affect the inflammatory response of ATMs through induction of ARG1, which might be influenced by signal molecules from adipose tissues.

**Macrophage HIF-2α Relieves Proinflammatory Responses and Insulin Resistance in Adipocytes**

To explore the role of macrophage HIF-2α during the interaction with adipocytes, macrophages overexpressing HIF-2α or GFP were cocultured with differentiated 3T3-L1 adipocytes (Fig. 3A). Adipocytes cocultured with macrophages increased the mRNA levels of proinflammatory genes such as iNOS, IL-6, and serum amyloid A (SAA), which were significantly reduced by coculture with HIF-2α–overexpressing macrophages (Fig. 3B). Compared with control adipocytes, adipocytes cocultured with macrophages inhibited the phosphorylation levels of AKT and GSK3β in the presence of insulin, whereas adipocytes cocultured with HIF-2α–overexpressing macrophages reversed the inhibitory effect of macrophages on the insulin signaling in adipocytes (Fig. 3C). Accordingly, macrophages overexpressing HIF-2α restored the insulin-stimulated glucose uptake ability of adipocytes, which was disrupted by coculturing with macrophages (Fig. 3D).

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**Figure 3**—Macrophage HIF-2α overexpression relieves proinflammatory gene expression and insulin resistance in cocultured adipocytes. **A:** Illustration of the indirect co-culture system for adipocytes and macrophages. Peritoneal macrophages were seeded in the upper chamber of a Transwell system (0.4-μm pores) and infected with or without Ad-GFP or Ad-HIF-2α. 3T3-L1 adipocytes were differentiated in the lower chamber and then cocultured with the prepared macrophages. After 48 h, the adipocytes were harvested and total RNA was extracted. **B:** The relative mRNA levels of the inflammatory genes iNOS, IL-6, and SAA were analyzed by quantitative RT-PCR and were normalized to the level of Cyclophilin mRNA. Data represent mean ± SD for n = 3 in each group. *P < 0.05 and **P < 0.05 vs. noncoculture control; #P < 0.05 vs. Ad-GFP control by Student t test. **C and D:** Insulin signaling cascade and insulin-dependent glucose uptake activity were analyzed in adipocytes after coculturing with or without Ad-GFP- or Ad-HIF-2α–infected macrophages as described in the RESEARCH DESIGN AND METHODS. C: Adipocytes were harvested, and total lysates were blotted and probed with various antibodies to detect iNOS and the insulin signaling cascade (pAKT, total AKT, pGSK3β, total GSK3β), β-Actin was used as the loading control. Quantitative data represent the mean ± SD of three separate experiments. *P < 0.05 and **P < 0.01 vs. no insulin control. D: Relative glucose uptake is shown in the graph. Data represent mean ± SD for n = 5 in each group. *P < 0.05 and **P < 0.01 vs. no insulin control; #P < 0.05 vs. Ad-GFP–infected macrophage cocultured control adipocytes by Student t test. Media only, adipocytes cultured without macrophages; Ad-GFP MAC, adipocytes cocultured with Ad-GFP–infected macrophages; Ad-HIF-2α MAC, adipocytes cocultured with Ad-HIF-2α–infected macrophages.
These data indicate that activation of macrophage HIF-2α would suppress proinflammatory responses and insulin resistance in adipocytes.

**Macrophage HIF-2α Knockdown Accelerates Palmitate-Induced Proinflammatory Responses in Adipocytes**

To confirm the effects of macrophage HIF-2α on proinflammatory gene expression in adipocytes, we suppressed macrophage HIF-2α expression using siRNA. In macrophages, suppression of HIF-2α via siRNA decreased the expression of ARG1 mRNA, while mRNA levels of HIF-1α, ARG2, and iNOS were not affected by HIF-2α suppression (Fig. 4A). Because NO generation in macrophages is restrained in the absence of stimuli, including inflammatory signals, downregulation of ARG1 by HIF-2α siRNA did not affect basal NO production (Fig. 4B). However, HIF-2α suppression significantly increased NO production upon palmitate challenge to mimic the condition of metabolic stress in macrophages (Fig. 4B), indicating that HIF-2α-suppressed macrophages could not maintain their ability to repress palmitate-induced NO production. In macrophages, HIF-2α suppression further promoted the mRNA expression of TNFα and IL-6, which are key mediators of proinflammatory responses in obese adipose tissue (Fig. 4C). Furthermore, when differentiated 3T3-L1 adipocytes were indirectly cocultured with macrophages

![Figure 4](https://example.com/figure4.png)

**Figure 4**—Knockdown of macrophage HIF-2α stimulates proinflammatory responses in adipocytes. A–C: Macrophages were transfected with control siRNA (siCTL) or HIF-2α–specific siRNA (siHIF-2α) and then treated with 0.5 mmol/L palmitate in 0.1% BSA or with 0.1% BSA only. After 48 h, total RNA was extracted and HIF-2α, HIF-1α, ARG1, ARG2, iNOS (A), TNFα, and IL-6 (C) mRNA levels were analyzed by quantitative RT-PCR. B: The concentration of NO in cultured media was measured. D: HIF-2α knocked-down macrophages were indirectly cocultured with adipocytes (as illustrated in Fig. 3A). During coculture (48 h), both adipocytes and macrophages were treated with or without 0.5 mmol/L palmitate. The relative mRNA levels of iNOS, IL-6, and SAA were analyzed by quantitative RT-PCR. Data represent mean ± SD for n = 3 in each group. *P < 0.05 and **P < 0.01 vs. BSA control; #P < 0.05 and ##P < 0.01 vs. control siRNA by Student t test. mRNA expression levels were normalized to the level of Cyclophilin mRNA. Media only, adipocytes cultured without macrophages; siCTL MAC, adipocytes cocultured with control siRNA-transfected macrophages; siHIF-2α MAC, adipocytes cocultured with HIF-2α siRNA-transfected macrophages. PA, palmitate.
in the presence or absence of HIF-2α siRNA, macrophage HIF-2α suppression increased the mRNA levels of proinflammatory genes such as iNOS, IL-6, and SAA in adipocytes upon palmitate stimulation (Fig. 4D). These results clearly suggest that macrophage HIF-2α may attenuate proinflammatory responses, which would be aggravated by cross talk between adipocytes and recruited macrophages upon metabolic stresses such as hyperlipidemic condition in obesity.

**Hif-2α/+** Mice Exhibit Insulin Resistance Upon HFD

To further investigate the in vivo role of HIF-2α in adipose tissue inflammation and insulin resistance, we decided to examine an Hif-2α knockout mouse model. Since Hif-2α whole-body knockout mice are embryonic lethal (27), Hif-2α haploinsufficient mice (Hif-2α+/−) were fed with either a normal chow diet (ND) or an HFD for 16 weeks and compared with their WT (Hif-2α+/+) littermates as a control group. As shown in **Fig. 5A**–**F**, body weight, adipose tissue weight, adipocytes size, serum triglycerides, and cholesterol did not differ between WT and Hif-2α+/− mice fed with either an ND or an HFD. In addition, there were no significant differences in the lipid metabolism gene expression profiles between WT and Hif-2α+/− mice (Supplementary Fig. 2). However, HFD-fed Hif-2α+/− mice showed higher fasting insulin levels and tended to have elevated fasting glucose levels than HFD-fed WT mice (Fig. 5G and H). To determine whether Hif-2α haploinsufficiency would affect systemic insulin sensitivity, OGTT and ITT were performed. Similar to fasting insulin and glucose levels, there were no significant difference in OGTT and ITT between WT and Hif-2α+/− mice under ND feeding. However, it is of interest to note that Hif-2α+/− mice were more glucose and insulin intolerant than WT mice upon HFD (Fig. 5I–K). These observations evidently indicate that Hif-2α haploinsufficiency is associated with systemic insulin resistance in diet-induced obesity (DIO).

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**Figure 5**—Hif-2α+/− mice are insulin resistant upon HFD. Ten-week-old Hif-2α+/− mice and their WT littermates were fed a ND or HFD for 16 weeks. A: Body weight was measured every 2 weeks. Body weight gain (B) and the weights of various tissues (C) from Hif-2α+/− and WT mice were measured after 16 weeks of feeding either a ND or HFD. D: Average adipocyte size was quantified in section images of epididymal white adipose tissue. Levels of triglycerides (E), total cholesterol (F), and insulin (G) were measured in serum samples. H: Levels of fasting glucose were measured in blood sample. I and J: OGTT and area under the curve analysis of Hif-2α+/− mice and WT mice. K: ITT in Hif-2α+/− mice and WT mice. Data represent mean ± SD for n = 7–9 in each group. *P < 0.05 and **P < 0.01 vs. WT group; ###P < 0.01 vs. ND-fed WT group by Student t test. N, normal chow diet; H, HFD; WAT, white adipose tissue; Sub, subcutaneous fat; Epi, epididymal fat; Peri, perirenal fat; BAT, brown adipose tissue; AUC, area under the curve; GTT, glucose tolerance test.
In DIO, HIF-2α/− Mice Aggravate Adipose Tissue Inflammation With Increased ATM Accumulation

To determine whether insulin resistance is linked with adipose tissue inflammation in HFD-fed Hif-2α−/− mice, we examined gene expression profiles such as inflammatory response, fibrosis, and macrophage infiltration. In adipose tissues from HFD-fed mice, Hif-2α haplo deficiency decreased Arg1 expression and slightly but substantially increased iNOS mRNA and their protein expression (Fig. 6A–C), which appeared to be consistent with data from various in vitro cell culture and ex vivo experiments (Figs. 2–4). In ND- or HFD-fed Hif-2α−/− mice, the levels of Hif-1α mRNA were not altered (Fig. 6B). However, the expression of proinflammatory genes, including Tnfa, Il-6, and Saa was further augmented in the adipose tissue of HFD-fed Hif-2α−/− mice (Fig. 6D). In addition, the mRNA levels of fibrotic collagen genes such as Col1a1, Col3a1, and Col6a1 and trichrome-positive area—indicating fibrotic collagens were slightly but substantially increased in HFD-fed Hif-2α−/− mice (Supplementary Fig. 3). Furthermore, we observed that HFD-fed Hif-2α−/− mice had greater numbers of crown-like structures (CLSs) than HFD-fed WT mice (Fig. 6E). In accordance with these data, the number of F4/80 and CD11b double-positive macrophages as well as F4/80, CD11b, and CD11c triple-positive ATMs (M1-like) per gram of fat mass was significantly higher in the adipose tissue of HFD-fed Hif-2α−/− mice than in HFD-fed WT mice (Fig. 6F). The percentage of M1-like ATMs in SVCs was also elevated in the adipose tissue of Hif-2α−/− mice, whereas the proportion of F4/80, CD11b+, CD11c− ATMs (containing M2-like macrophages) in Hif-2α−/− mice was not significantly different from that of WT mice (Fig. 6G and H). To explore the effect of HIF-2α on macrophage polarity, the expression levels of M1 and M2 marker genes were examined in adipose tissue of HFD-fed mice. As shown in Fig. 6I, in DIO, mRNA expression of M1 marker genes such as F4/80 and Cd11c were higher in Hif-2α−/− mice than in WT mice, whereas that of M2 marker genes such as Mgl1, Mrcl, and Chil3 was not changed or were slightly decreased. Taken together, these results strongly suggest that Hif-2α−/− mice are susceptible to adipose tissue inflammation, accompanied with increased M1 ATMs in DIO.

In HIF-2α/− Mice, Macrophage Depletion Improves Insulin Resistance and Adipose Tissue Inflammation

To test whether increased numbers of M1 ATMs might be essential for insulin resistance in HFD-fed Hif-2α−/− mice, we depleted phagocytic macrophages by using clodronate liposomes. Expectedly, clodronate treatment significantly decreased the numbers of CLSs in adipose tissues from both HFD-fed WT and HFD-fed Hif-2α−/− mice without changing adipocyte size or morphology (Fig. 7A and Supplementary Fig. 4). Consistently, the mRNA levels of F4/80 and Cd11c were greatly decreased by clodronate (Fig. 7B). Therefore, the difference in ATM contents between WT and Hif-2α−/− mice was insignificant. Interestingly, macrophage depletion also decreased the level of Arg1 mRNA, while the expression level of Hif-2α was not altered (Fig. 7C). These observations suggest that expression of ARG1 might be more abundant in the ATMs of adipose tissue, whereas HIF-2α seems to be broadly expressed in various cell types, including adipocytes, macrophages, and endothelial cells. Despite of the decrease in ARG1 expression, which mediates anti-inflammatory effects of macrophage HIF-2α, clodronate-mediated depletion of macrophages clearly improved the glucose intolerance of HFD-fed Hif-2α−/− mice to the levels comparable to that of HFD-fed WT mice (Fig. 7D). Therefore, these data suggest that enhanced recruitment and proinflammatory polarization of ATMs would be closely associated with systemic insulin resistance in HFD-fed Hif-2α−/− mice.

DISCUSSION

In obese adipose tissue, decreases in the vasculature network and blood flow reduce local oxygen tension, which leads to adipose tissue hypoxia (11). Emerging evidences have suggested that activation of HIF-1α in obesity mediates adipose tissue inflammation and macrophage infiltration as well as classical hypoxic responses such as angiogenesis and glycolysis (11,14–16). In obesity, adipose tissue hypoxia induces M1-like polarization of ATMs, where HIF-1α contributes to increase the proinflammatory responses in ATMs (14). Despite these findings, it is largely unknown which pathways are involved in the anti-inflammatory responses or resolution mechanisms against excessive proinflammatory responses in ATMs of the obese. It has been reported that both M1- and M2-like ATMs are increased in obese adipose tissue (4,5,8). The recent finding that HIF-2α, another key player in the hypoxic responses, has its own target genes and is induced by Th2 cytokines associated with M2 macrophage polarization (25) led us to explore the functional role of HIF-2α in obese adipose tissue. Our data suggest that HIF-2α activation in ATMs, in opposite to HIF-1α, is involved in the amelioration of adipose tissue inflammation and insulin resistance in obesity.

NO is a signaling molecule to mediate various physiological roles, including vasodilator, neurotransmitter, and muscle relaxer (28). However, excessive NO generation by iNOS is well-known to interfere with insulin signaling in obesity (1,2,29). For example, iNOS knockout mice are protected from HFD-induced insulin resistance, whereas treatment with NO donors deteriorates insulin signaling and adipocytokines (30,31). Recently, ARG1 has been suggested to negatively regulate iNOS activity by competitive utilization of a common precursor, l-arginine, in lean adipose tissue (4,5). It is not yet completely understood whether HIF-2α activation might drive M2 polarization in ATMs. In this study, our data suggest that activation of macrophage HIF-2α stimulates ARG1 expression, which helps macrophages to maintain M2 type characters by reducing NO production and proinflammatory cytokine expression (Fig. 2). Furthermore, HIF-2α–
Figure 6—Hif-2α+/− mice are susceptible to adipose tissue inflammation upon HFD. A: HIF-2α protein levels in adipose tissue of Hif-2α+/− and WT mice fed a ND or HFD. Whole-mount immunofluorescence analyses were performed to detect the nucleus (blue), perilipin (green), and HIF-2α (red). Relative mRNA levels of Hif-2α, Hif-1α, Arg1, and iNOS transcripts in epididymal adipose tissue were measured by quantitative RT-PCR (B), and total lysates were subjected to Western blot analysis with antibodies specific for HIF-2α, ARG1, iNOS, and β-actin (C). β-Actin was used as the loading control. D: Relative mRNA levels of inflammatory cytokine genes (Tnfα, Il-6, and Saa) in epididymal adipose tissue were measured by quantitative RT-PCR. E: Histological analysis was performed with epididymal adipose tissue from Hif-2α+/− and WT mice, and the numbers of CLSs were quantified in DIO mice. F: Macrophage infiltration and M1 polarization were measured in epididymal adipose tissue using flow cytometric analysis. The total number of macrophages (double positive; CD11b+ and F4/80+) and M1-like macrophages (triple positive; CD11b+, F4/80+, and CD11c+) among the SVCs per gram of fat mass; (G) percentages of CD11c+ and (H) CD11c− macrophages among the SVCs in adipose tissue. I: Macrophage polarization marker gene expression was analyzed by quantitative RT-PCR in total RNA extracted from SVCs fractionated from the epididymal adipose tissues of HFD-fed mice. Data represent mean ± SD for n = 7 in each group. *P < 0.05 and **P < 0.01 vs. WT group; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. ND group by Student t test. mRNA expression levels were normalized to the level of Cyclophilin mRNA.
overexpressing macrophages were able to suppress the proinflammatory responses induced by adipocyte-derived inflammatory mediators, including free fatty acids (Figs. 2 and 4). Given that ARG1 would be a key mediator of the anti-inflammatory function of macrophage HIF-2α (Fig. 2), it is likely that the anti-inflammatory features of macrophage HIF-2α would be mediated, at least partly, through the reduction of reactive nitrogen species that could readily react with reactive oxygen species to boost proinflammatory responses and insulin resistance. Accordingly, macrophage HIF-2α overexpression protected against inflammatory signals and insulin resistance driven by cross talk between adipocytes and macrophage (Figs. 3 and 4). Therefore, our data imply that macrophage HIF-2α activation would be part of the resolving pathways to induce M2 activation in obese adipose tissue.

Without any stress, Hif-2α−/− mice appeared to be normal, with hematocrit levels similar to WT mice (Supplementary Fig. 5). However, several phenotypes have been reported upon various prolonged hypoxia-inducing stimuli (32). In addition, it has been demonstrated that Hif-2α haploinsufficiency suppresses osteoarthritis developments independent of hypoxia (22). In the current study, we provide another characteristic of Hif-2α−/− mice: susceptibility to adipose tissue inflammation and insulin resistance in DIO. In the adipose tissue of Hif-2α−/− mice, the CD11c+ M1-like macrophage population was greatly elevated upon HFD (Figs. 5 and 6). In DIO, both WT and Hif-2α−/− mice similarly increased body weight, adiposity...
and serum lipid metabolites except serum glucose and insulin. Furthermore, HFD-fed Hif-2α−/− mice were more insulin resistant than HFD-fed WT mice. Given that macrophage depletion using clodronate improved the insulin resistance in HFD-fed Hif-2α−/− mice, it is very likely that the polarity change of ATMs induced by Hif-2α haploinsufficiency would be critical for systemic insulin intolerance in Hif-2α−/− mice (Fig. 7). Therefore, these results led us to consider macrophage HIF-2α as a key mediator of anti-inflammatory responses in adipose tissue in response to metabolic stresses. Nevertheless, we cannot exclude the possibility that haploinsufficiency of Hif-2α might suffer from systemic insulin resistance through alternative pathways. For example, it has been reported that HIF-2α is able to regulate GLUT1, GLUT4, insulin receptor substrate (IRS) 2, and IRS3 to promote insulin signaling in adipocytes and hepatocytes (33,34). In addition, HIF-2α is able to regulate the expression of several matrix metalloproteinases to digest collagens (35), which would protect against fibrosis, probably by HIF-1α, in obese adipose tissue. Therefore, it remains to be elucidated whether deteriorated insulin resistance of HFD-fed Hif-2α−/− mice might result from the increased inflammatory response in HIF-2α–insufficient ATMs.

In accordance with the finding that macrophage HIF-2α overexpression would decrease proinflammatory cross talk between adipocytes and macrophages by the induction of ARG1, we observed that proinflammatory responses in the adipose tissue of HFD-fed Hif-2α−/− mice was augmented. However, our in vivo data showed that HIF-2α accumulation in the ATMs of obese mice would not be sufficient to induce ARG1 to resolve the proinflammatory responses (Fig. 6), implying that the expression level of ARG1 in obese adipose tissue might be uncoupled from the increase of HIF-2α in vivo. Although it is unclear why or how elevated HIF-2α fails to stimulate ARG1 expression in obese adipose tissue, there are several possible explanations for the uncoupled expression profiles of HIF-2α and ARG1 in vivo. One of the possible mechanisms for incapability of macrophage HIF-2α to elevate ARG1 in obesity might be related to the control of HIF-2α activity by protein modification. HIF-2α is primarily regulated by oxygen-dependent posttranslational stabilization. However, its transcriptional activity is influenced by various oxygen-independent modifications such as acetylation and phosphorylation (18). In particular, mammalian silent information regulator 2 homolog (SIRT1), which is a key energy sensor involved in the regulation of adipose tissue inflammation, deacetylates HIF-2α. It has been previously reported that SIRT1-dependent HIF-2α deacetylation stimulates its activity (36). However, in DIO, the reduced SIRT1 activity in the adipose tissue could provoke to downregulate HIF-2α activity owing to the high energy status (37,38). Another possibility for the inability of HIF-2α to induce ARG1 in obese adipose tissue might result from reduction of Th2 cytokines. It has been reported that alternative activation of macrophages by Th2 cytokines enhances ARG1 expression in both HIF-2α–dependent and HIF-2α–independent manners (25,39). It has been shown that the level of Th2 cytokines is decreased in obese adipose tissue due to decreased numbers of Th2 cytokine-expressing cells such as eosinophils (35), which may suppress ARG1 expression. In contrast, the relative increase in Th1 cytokines such as interferon-γ in obese adipose tissue potentiates proinflammatory responses (40) and might prevail over the effect of HIF-2 on ARG1. Moreover, we cannot rule out the possibility that the expression of ARG1 in vivo may require accessory cofactors that appear to be suppressed in obese adipose tissue. Therefore, it is feasible that elevated macrophage HIF-2α activity would be incompetent to overcome the augmented proinflammatory microenvironment of obese adipose tissue, where adipocytes actively cross talk with ATMs to amplify inflammatory responses.

Under hypoxia, HIF isoforms, HIF-1α and HIF-2α, directly or indirectly regulate various target genes. Depending on stresses and cellular contexts, HIF-1α and HIF-2α have unique and sometimes opposite functions as well as overlapping functions for physiological adaptations to hypoxia (18). For example, HIF-2α activation promotes cell proliferation by enhancing activity of the c-Myc oncoprotein in hypoxic tumors, whereas HIF-1α activation induces cell cycle arrest by inhibiting c-Myc activity (41,42). Interestingly, in response to hypoxia, the inflammatory status of adipose tissue also seems to be controlled through competitive interplay between HIF-1α and HIF-2α. It has been reported that activation of HIF-1α in obesity could be a crucial factor in stimulating adipose tissue inflammation and fibrosis (14–16). On the other hand, our data have indicated that HIF-2α could resolve adipose tissue inflammation against metabolic stress. However, in obesity, the imbalance between two HIF isoforms might amplify adipose tissue inflammation in response to hypoxia.

In conclusion, our data suggest that macrophage HIF-2α would act as a resolving power against adipose tissue inflammation in response to metabolic changes, which leads to keeping the homeostatic function of ATMs for healthy adipose tissue remodeling. In this regard, maintaining proper HIF-2α activity would be crucial for preventing adipose tissue dysregulation in obesity, implying that enhancing HIF-2α activity in ATMs might be an attractive approach to treat obesity-induced metabolic disorders.

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References


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