Molecular Bases of Disease:
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Increased Angiogenesis Protects against Adipose Hypoxia and Fibrosis in Metabolic Disease-resistant 11β-Hydroxysteroid Dehydrogenase Type 1 (HSD1)-deficient Mice*

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In obesity, rapidly expanding adipose tissue becomes hypoxic, precipitating inflammation, fibrosis, and insulin resistance. Compensatory angiogenesis may prevent these events. Mice lacking the intracellular glucocorticoid-amplifying enzyme 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1−/−) have “healthier” adipose tissue distribution and resist metabolic disease with diet-induced obesity. Here we show that adipose tissues of 11βHSD1−/− mice exhibit attenuated hypoxia, induction of hypoxia-inducible factor (HIF-1α) activation of the TGF-β/Smad3/α-smooth muscle actin (α-SMA) signaling pathway, and fibrogenesis despite similar fat accretion with diet-induced obesity. Moreover, augmented 11βHSD1−/− adipose tissue angiogenesis is associated with enhanced peroxisome proliferator-activated receptor γ (PParγ)-inducible expression of the potent angiogenic factors VEGF-A, apelin, and angiopeptin-like protein 4. Improved adipose angiogenesis and reduced fibrosis provide a novel mechanism whereby suppression of intracellular glucocorticoid regeneration promotes safer fat expansion with weight gain.

In obesity, adipose tissue expansion may exceed compensatory vascular supply. Consequently, adipose tissue hypoxia is postulated to precipitate inflammatory macrophage infiltration and impair insulin signaling (1–4). Induction of hypoxia-inducible factors (HIFs)5 is a molecular hallmark of tissue hypoxia. Both HIF-1α and HIF-2α are involved in adipocyte differentiation and responses to hypoxia in vitro (5, 6). HIF-1α associates with hypoxic and inflammatory changes in adipose tissues in vivo, and transgenic overexpression of HIF-1α in adipose tissue causes fibrosis (7).

Altered glucocorticoid action has a major impact upon adipose tissue distribution and insulin sensitivity. Elevated adipose tissue levels of the intracellular glucocorticoid-regenerating enzyme 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) are found in human obesity (8, 9). Modeling this by adipose-specific overexpression in mice drives visceral obesity and a metabolic syndrome (10). In contrast, 11βHSD1 gene knock-out (11βHSD1−/−) mice are protected from high fat diet-induced metabolic disease (11, 12) and adipose inflammation (13). In addition, 11βHSD1 deficiency is associated with enhanced angiogenesis during wound healing and in the myocardium following ischemia (14, 15). We hypothesized that the metabolically “healthy” adipose tissue found with 11βHSD1 deficiency might have its origin in improved adipose tissue angiogenesis, with consequent improvement in hypoxia-induced fibrosis. We therefore examined the impact of 11βHSD1 deficiency on adipocyte paracrine, stromal proangiogenic responses, and the consequent effects on adipose tissue vascularization and fibrosis in dietary obesity.

The abbreviations used are: HIF, hypoxia-inducible factor; SVF, stromovascular fraction; ANGPTL4, angiopoietin protein-like 4; scAT, subcutaneous adipose tissue; mesAT, mesenteric adipose tissue; PHD, HIF prolyl hydroxylase; KO, 11βHSD1 knock-out; 11βHSD1−/−, 11β-hydroxysteroid dehydrogenase type 1; DIO, diet-induced obesity; PPAR, peroxisome proliferator-activated receptor; HF, high fat; α-SMA, α-smooth muscle actin; ANOVA, analysis of variance; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol.

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‡ This article contains supplemental Figs. S1–S4.

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EXPERIMENTAL PROCEDURES

Diet-induced Obesity (DIO)—Adult male 11\(\beta\)HSD1\(^{-/-}\) and congenic C57Bl/6J controls (12, 13) were single-housed (12-h light/12-h dark cycle) and given food and water ad libitum.

Mice (\(n = 6\)–8 per group) were randomized to standard chow or a high fat (HF) diet, 58% calories as fat (Research Diets D12331), for 12 weeks.

Pimonidazole Staining—To assess adipose tissue hypoxia \textit{in vivo}, mice were injected intraperitoneally with 60 mg/kg of pimonidazole (Hypoxyprobe\(^\text{TM}\)-1 Plus kit, Chemicon International), 30 min prior to sacrifice. Subcutaneous (scAT) and mesenteric (mesAT) adipose tissues were collected in formalin and paraffin-embedded. 4-\(\mu\)m sections were incubated with mAb1 conjugated with FITC at a 1:100 dilution for 30 min at room temperature. A secondary anti-FITC mAb was applied at a 1:100 dilution for 30 min (according to the manufacturer’s instructions). Pimonidazole adduct staining was visualized using the diaminobenzidine chromogen-A system (DAKO). Sections were rinsed and counterstained with hematoxylin and imaged using a Zeiss microscope.

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**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>C57Bl/6 CD</th>
<th>C57Bl/6 HF D</th>
<th>11(\beta)HSD1(^{-/-}) HF D</th>
<th>(p) values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Terminal BW (g)</strong></td>
<td>38.8 ± 3.2*</td>
<td>49.2 ± 1.7</td>
<td>53 ± 1.1</td>
<td>&lt;0.0001</td>
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<td><strong>BW gain (g)</strong></td>
<td>5.9 ± 0.3*</td>
<td>16.1 ± 1.2</td>
<td>17.1 ± 1.2</td>
<td>&lt;0.0001</td>
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<tr>
<td><strong>scAT (g)</strong></td>
<td>0.4 ± 0.03*</td>
<td>1.0 ± 0.06</td>
<td>1.2 ± 0.09</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>mesAT (g)</strong></td>
<td>0.7 ± 0.06*</td>
<td>1.5 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>scAT/BW ratio</strong></td>
<td>0.010 ± 0.0006*</td>
<td>0.020 ± 0.001</td>
<td>0.022 ± 0.001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>mesAT/BW ratio</strong></td>
<td>0.018 ± 0.0009*</td>
<td>0.030 ± 0.001</td>
<td>0.031 ± 0.001</td>
<td>0.0006</td>
</tr>
</tbody>
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**FIGURE 1. Less adipose hypoxia in 11\(\beta\)HSD1\(^{-/-}\) mice.** A and B, representative images of Hypoxyprobe staining of subcutaneous (A) and mesenteric (B) adipose tissue in chow-fed control (WT CD; quantification histogram: hatched bars), HF-fed control (WT HF; quantification histogram: white bars), and HF 11\(\beta\)HSD1\(^{-/-}\) (KO HF; quantification histogram: black bars) mice (\(n = 6\)). 30–40 high power fields (magnification \(\times 200\)) per section were quantified. Brown adducts indicate areas of low oxygen availability. Note the unstained blood vessel that acts as an internal negative control. \(n = 6/group\), *, \(p < 0.05\), **, \(p < 0.01\), ***, \(p < 0.001\) by ANOVA. HFD, high fat diet.

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(magnification ×200) per section were randomly selected for each slide by an assessor blind to genotype. Diaminobenzidine brown staining was quantified using Photoshop PS3 and normalized to the total number of pixels.

**RNA Extraction and Quantitative Real Time PCR—**scAT and mesAT were weighed and snap-frozen in liquid nitrogen, and RNA was extracted as described (12). 1 μg of RNA was reverse-transcribed using the SuperScript III cDNA synthesis kit (Invitrogen) according to the manufacturer’s instructions. Real time PCR was performed with the LightCycler (Roche Applied Science), using TaqMan assays for all genes measured and correcting for 18 S and β-actin expression.

**Immunoblotting—**Frozen adipose tissues were homogenized in a urea/SDS buffer supplemented with Complete protease inhibitor (Roche Applied Science), using TaqMan assays for all genes measured and correcting for 18 S and β-actin expression.

**Immunohistochemistry—**For the detection of collagen deposition, formalin-fixed, paraffin-embedded sections (4 μm) were stained with picrosirius red (Sigma). Myofibroblasts were detected with a mouse monoclonal antibody to α-SMA (Sigma). An anti-CD31 antibody (Abcam) was used for identifying endothelial cells/vessels. Secondary antibodies were all from Dako. Binding of primary antibody was visualized using dianinobenzidine chromogen-A (Dako) after counterstaining with hematoxylin.

**SVF Cell Preparation and in Vitro Hypoxia—**Adipose tissue was removed from HF-fed mice into warmed Krebs’ buffer and collagenase type 1 (Worthington Biochemicals)-digested as described (30). Briefly, after 1 h of digestion and centrifugation to separate from adipocytes, stromovascular fraction (SVF) cells were seeded at 2.5 × 10^5 cells/well in DMEM. On day 2, culture medium was replaced with 1% O2 preconditioned medium. Cells were left at 1% O2 in a hypoxic chamber (1% O2) for 6 h and then lysed in TRIzol, and RNA was extracted as above.

**Rosiglitazone Treatment of Primary Adipocytes—**The ceil-cultured adipocytes from collagenase digestion (see above) were filtered through a 200-μm size exclusion mesh and then centrifuged. Equal volumes of fractionated adipocytes were cultured in DMEM medium (Lonza, Berkshire, UK) for 16 h at 37 °C, 5% CO2 in the presence or absence of 1 μM rosiglitazone. Adipocytes were lysed in TRIZol, and total RNA was extracted as above.

**Angiogenesis Bioassays—**Strips of periaortic adipose tissue (immediately adjacent to the adventitia) were isolated from the thoracic aortas of C57Bl/6 or 11βHSD1−/− mice and incubated...
(20 mg of periaortic adipose/ml; 24 h at 37 °C) in DMEM containing 4.5 g/liter glucose, 584 mg/liter L-glutamine, 75 mg/liter L-ascorbic acid, 10 mg/liter heparin, 100 units/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B, and 0.2% BSA. Adipose tissue was removed from the conditioned medium, which was aliquoted and stored at −80 °C. Control medium (vehicle) was obtained following the same procedure without adipose tissue. Tube formation from mouse aortic rings was performed as described (14). Briefly, aortic rings (1 mm in length) from C57Bl/6 mice were embedded in Matrigel and incubated (DMEM, 37 °C; 5% CO2) in 50% DMEM; 50% periaortic fat conditioned medium or 50% DMEM; 50% control medium for 7 days, with medium refreshed every 48 h. Aortic rings cultured under these conditions in Matrigel spontaneously generated endothelial tube-like structures. The impact of periaortic fat conditioned medium on angiogenesis was quantified by counting the number of tube-like structures every other day (by an operator blinded to treatment).

**Statistical Analysis**—Values are means ± S.E. Simple comparisons were analyzed using Student’s t test. For multiple comparisons, differences between genotypes and the effect of treatment(s) were analyzed by two-way ANOVA with subsequent Tukey’s post hoc test. Significance was set at \( p < 0.05 \).

**RESULTS**

**11βHSD1−/− Adipose Tissue Is Resistant to Hypoxia with Obesity**—Weight gain and fat mass were similar after 12 weeks of HF feeding in both genotypes (Table 1). This intermediate time point suggests that a convergent phase in fat accumulation follows an earlier generalized reduction in fat mass (13) and precedes a later beneficial redistribution of fat away from visceral depots and into peripheral fat depots (12).

Despite similar scAT depot expansion in 12-week HF-fed 11βHSD1−/− mice, there was markedly less adipose tissue pimonidazole staining and hence hypoxia than in HF-fed control (WT) mice (Fig. 1). Importantly, mesAT from chow-fed animals exhibited 14-fold \( (p = 0.005) \) greater pimonidazole staining than scAT, suggesting that marked hypoxia occurs under basal circumstances in this depot. HF feeding significantly induced adipose HIF-1α in control mice, whereas HIF-1α remained very low in similarly obese HF-fed 11βHSD1−/− mice (Fig. 2A and supplemental Fig. S1). HIF-2α protein was unaffected by HF or 11βHSD1 genotype (Fig. 2A), suggesting that it does not play a role in adipose hypoxic responses in vivo.

HIF prolyl hydroxylases (PHD 1–3 (also known as EGLNs)) degrade HIFs under normoxic conditions by hydroxylation of the two proline residues in the oxygen-dependent degradation domain of HIF-1α (16–18). The role of PHDs in adipose tissue and obesity is currently unknown. Adipose tissue PHD mRNAs were unaffected by HF feeding or genotype (supplemental Fig. S2). However, PHD1 and PHD2 protein levels were suppressed by HF feeding (Fig. 2B). Thus, although lower PHD1 and PHD2 (and hence reduced HIF-1α degradation) may contribute to HIF-1α elevation with DIO in controls, variation in PHDs does not explain the lower levels of HIF-1α in adipose tissue with 11βHSD1 deletion.

To determine whether 11βHSD1−/− mice have altered responses to hypoxia in the fibrogenic adipose SVF, HIFs and...
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PHDs were measured in SVF cells from HF-fed mice under normoxia (21% O₂) or acute hypoxia (1% O₂) in vitro. In both genotypes, HIF-1α, but not HIF-2α, mRNA levels were markedly and similarly up-regulated by hypoxia (Fig. 3, A and B). All three PHDs were expressed in SVF cells and were similarly up-regulated by hypoxia (Fig. 3, C–E). Thus, the attenuation of HIF-1α responses to HF in 11βHSD1−/− adipose tissue in vivo is likely due to the reduced tissue hypoxia rather than an intrinsic difference in hypoxia responses.

HF-fed 11βHSD1−/− Mice Have Reduced Adipose Collagen Deposition and Expression of Fibrogenic Mediators—To determine whether obesity-induced hypoxia impacted adipose fibrosis, as in liver (19), we analyzed collagen deposition. Staining for picrosirius red-reactive collagens I and III was low and comparable between genotypes in chow-fed scAT and mesAT. HF diet markedly induced collagen staining in adipose tissue, observed as broad strands transversing the tissue and around individual adipocytes (Fig. 4A). 11βHSD1−/− mice exhibited significantly less collagen deposition in response to HF in both fat depots with much thinner fibrillar streaks and little staining around adipocytes (Fig. 4A).

Tissue fibrosis is characterized by increased transdifferentiation of fibroblasts to activated myofibroblasts and collagen deposition (20). 11βHSD1−/− adipose tissue exhibited reduced α-SMA staining in both scAT and mesAT adipose in response to HF (Fig. 4B). Consistent with reduced fibrosis and fewer α-SMA-positive cells, glucocorticoid-regulated Col1α1 mRNA levels were lower in 11βHSD1−/− adipose tissue (Fig. 5C). Other genes involved in fibrosis were unaltered in HF-fed 11βHSD1−/− adipose tissues, including Col3α1 and Col6α1 (Fig. 5C). Activation of the TGF-β/Smad signaling pathway is critical in driving fibrogenesis in liver (21) and in adipose tissue (7). To investigate the impact of intra-adipose glucocorticoid deficiency on adipose fibrosis, we measured TGF-β1, TGF-β2, and TGF-β3 mRNA levels in adipose tissue (5C) and total Smad2/3 (Fig. 5A) levels were comparable across genotypes and diet. HF feeding significantly induced Smad3 phosphorylation in control mice (3-fold, p < 0.01, Fig. 5A and supplemental Fig. S3). However, Smad3 phosphorylation levels were lower in HF-fed 11βHSD1−/− adipose tissue (Fig. 5A). In vitro hypoxia rapidly up-regulated TGF-β in SVF cells from control mice, whereas TGF-β was unchanged in 11βHSD1−/− SVF cells (supplemental Fig. S4). Moreover, the fibrosis-associated 63-kDa MMP14 pro-enzyme was elevated by HF diet in adipose tissue of control but not 11βHSD1−/− mice (Fig. 5B). Other matrix remodeling enzymes were unaltered including TIMP-1, MMP2, and MMP13 mRNA levels (Fig. 5C).

Increased Angiogenesis in 11βHSD1−/− Adipose Tissue—We next explored whether the attenuation of adipose tissue hypoxia and fibrosis responses in HF-fed 11βHSD1−/− adipose...
tissues was associated with enhanced angiogenesis. The vessel-to-adipocyte ratio (correcting for adipose hypertrophy) was higher in HF-fed $11\beta$HSD1$^{-/-}$ mice than in control mice (Fig. 6A) despite comparable reductions in total vessel (Fig. 6B) and fat cell numbers (Fig. 6C). This appeared to be a direct effect of $11\beta$HSD1$^{-/-}$ adipose tissue on functional induction of angiogenesis because exposure of control aortic rings to $11\beta$HSD1$^{-/-}$ adipose tissue-conditioned medium induced greater angiogenesis (3-fold, $p < 0.01$) than control (1.5-fold, $p < 0.05$) adipose tissue medium (Fig. 7).

To explore the potential adipose tissue factors involved, we measured key angiogenic gene expression levels. VEGF-A, ANGPTL4, and apelin mRNA were more highly expressed in adipose tissue of $11\beta$HSD1$^{-/-}$ than in control mice (Fig. 8A). SVF endothelial cells are key to adipose tissue expansion (22). Consistent with the in vivo findings, normoxic SVF cells from $11\beta$HSD1$^{-/-}$ adipose tissue had higher VEGF-A and ANGPTL4 expression (Fig. 8, B and C). Hypoxia induced SVF cell VEGF-A and ANGPTL4 mRNA expression to a greater extent in $11\beta$HSD1$^{-/-}$ (Fig. 8B).

To determine whether the increased PPARγ sensitivity of $11\beta$HSD1$^{-/-}$ adipocytes (13) might contribute in a paracrine manner to enhanced SVF angiogenic responses, primary mesAT adipocytes from HF-fed mice were treated with the PPARγ agonist rosiglitazone (1 $\mu$m) for 24 h. As in vivo, ANGPTL4 (although not VEGF) was more highly expressed in $11\beta$HSD1$^{-/-}$ adipocytes in vitro (Fig. 9B). Rosiglitazone up-regulated adipose ANGPTL4 with a 2-fold greater effect in

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**FIGURE 5. Reduced profibrotic signaling in $11\beta$HSD1$^{-/-}$**. A and B, representative adipose Western blots of phosphorylated Smad3 (Smad3$^+$) (A) and unphosphorylated Smad2/3 protein pro-enzyme and active MMP14 (B). Lower panels, protein quantification histograms for control mice on chow diet (WT CD, hatched bars, $n = 4$), control HF (WT HFD, white bars, $n = 4$), and $11\beta$HSD1$^{-/-}$ HF mice (KO HFD, black bars, $n = 4$). AU, arbitrary units. C, mRNA of a panel of profibrotic genes ($n = 8$/genotype). White and black bars, differences between control on HF (white bars) and $11\beta$HSD1$^{-/-}$ on HF (black bars) by Student’s $t$ test. *, $p < 0.05$; **, $p < 0.01$. Reduced Adipose Fibrosis in $11\beta$HSD1 Deficiency
DISCUSSION

Here we show that relative glucocorticoid deficiency within adipose tissue is permissive for angiogenesis in response to DIO, thus preventing severe hypoxia and consequent inflammation, metabolic dysfunction, and fibrosis. We find that DIO induces more severe hypoxia in mesenteric AT than in subcutaneous AT. This is associated with HIF-1α induction through reduced PHD1- and PHD2-mediated HIF degradation. This cascade of responses associates with induction of TGF-β/Smad/s-SMA signaling, myofibroblast activation, increased collagen deposition, and MMP14 induction in HF-fed C57BL/6J but not 11βHSD1−/− mice. Increased PPARγ activation and induction of adipocyte angiogenic factors associates with the improved 11βHSD1−/− adipose tissue phenotype. This work suggests that a cascade of failed angiogenesis leads to tissue hypoxia and fibrosis in rapidly expanding adipose tissue in obesity. This is ameliorated with intra-adipose glucocorticoid deficiency.

Adipose tissue hypoxia mediates insulin resistance and inflammation in obesity (1–4). Moreover, hypoxia is profibrotic in adipose tissue, with transgenic overexpression of adipose-HIF-1α leading to exacerbated collagen deposition and fibrosis (7). Although persistent inflammation precedes fibrosis (23), it is unclear whether chronic low grade inflammation of obesity alters angiogenesis-dependent hypoxia and consequent adipose tissue fibrosis. Loss of the potent angiogenic factor VEGF in myeloid cells leads to excessive collagen deposition...
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and fibrosis, indicating that inflammatory cell-derived VEGF is required for vascular remodeling (24). Failure of extracellular matrix remodeling and/or neovascularization during adipose expansion could feasibly lead to adipose tissue fibrosis. Indeed, genetically obese mice and obese humans show significantly reduced adipose VEGF-A accompanied by lower capillary density (4, 25), suggesting reduced adaptive angiogenesis. Although hypoxia up-regulates VEGF-A, the protective angiogenic response observed in 11β/HSD1-/- mice did not follow the canonical hypoxia-HIF-dependent pathway. HIF-independent regulation of VEGF-A and angiogenesis through PPARγ coactivator PGC-1α-mediated responses occurs in skeletal muscle (26) and brain (27). Moreover, induction of HIF-1α in DIO or adipose HIF-1α overexpression does not increase VEGF-A (7). Glucocorticoids potently suppress angiogenesis through inhibition of VEGF-A (28, 29). Consistent with this, reduced hypoxia and fibrosis in adipose tissue from 11βHSD1-/- mice showed augmented PPARγ activation that is associated with induction of adipocyte VEGF-A and ANGPTL4 and sustained vessel density. Moreover, the SVF cells (fibroblast/endothelial population) are more responsive to DIO-mediated hypoxia in 11βHSD1-/- mice, driving beneficial adipose tissue remodeling. 11βHSD1 is highly expressed in the fibroblastic preadipocytes (30) and not the SVF macrophages (13), suggesting that

FIGURE 8. Higher expression of proangiogenic genes in 11βHSD1-/- in obesity. A, adipose mRNA levels of a panel of potent angiogenic factors, corrected for β-actin (n = 8). The bars indicate the following: SVF from HF control at 21% oxygen (white bars), 1% oxygen (diagonal hatched bars), HF 11βHSD1-/- 21% oxygen (black bars) and 1% oxygen (vertical hatched bars). Cells were kept in 1% oxygen for 6 h. Data are presented as means ± S.E. SVF was prepared from four individual mice. AU, arbitrary units. B and C, mRNA levels of VEGF-A (B) and ANGPTL4 (C). *, p < 0.05; **, p < 0.01, and ***, p < 0.001.

FIGURE 9. 11βHSD1-/- adipocytes are more sensitive to PPARγ-mediated angiogenic gene induction. A and B, VEGF-A (A) and ANGPTL4 (B) mRNA levels in adipocytes from HF-fed control mice (white bars, n = 4), rosiglitazone-treated mice (diagonal hatched bars, n = 6), 11βHSD1-/--untreated mice (black bars, n = 4) and rosiglitazone-treated mice (vertical hatched bars, n = 6). Two-way ANOVA, *, p < 0.05, **, p < 0.01. AU, arbitrary units.
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11βHSD1−/− preadipocytes and/or endothelial cells likely mediate the beneficial response to hypoxia/inflammation and fibrosis associated with obesity.

Distinct adipose depots exhibited different degrees of hypoxia, under basal or obesogenic conditions. Thus, mesAT is more hypoxic as compared with scAT depot. This suggests that hypoxia does not progress uniformly between depots, most probably due to differences in the initial degree of depot vascularization or in the rate/capacity of neovascularization during expansion. This is consistent with greater capillary density and angiogenic capacity in human subcutaneous versus visceral adipose tissue (25, 31). This suggests that individuals prone to visceral fat accumulation are at higher risk of hypoxia-induced metabolic dysfunction. Our finding that glucocorticoid deficiency is associated with fewer hypoxic areas, and consequently metabolic dysfunction. Our finding that glucocorticoid deficiency is associated with fewer hypoxic areas, and consequently metabolic dysfunction.

We propose that increased local glucocorticoid amplification exacerbates the metabolic insult caused by hypoxia and consequent fibrosis in adipose tissue. The detrimental impact on adipose tissue architecture may then lead to further metabolic compromise of adipocytes. Thus, selective 11βHSD1 inhibition might prove to be beneficial both by increasing adipocyte insulin sensitivity and function and by favoring enhanced angiogenesis signals, allowing escape from adipose hypoxia and fibrosis in obesity.

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