Glucocorticoid reamplification within cells intensifies NF-κB and MAPK signaling and reinforces inflammation in activated preadipocytes

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HSD1 is abundantly expressed in adipose tissue and preferentially cortisol and inactive cortisone (2). In particular, 11β-HSD1 and type 2 hydroxysteroid dehydrogenase type 1 (11β-HSD1) contribute to dysfunction of adipose tissue. Although the pathophysiologic role of 11β-HSD1 in mature adipocytes has long been investigated, its potential role in preadipocytes still remains obscure. The present study demonstrates that the expression of 11β-HSD1 in preadipocyte-rich stromal vascular fraction (SVF) cells in fat depots from ob/ob and diet-induced obese mice was markedly elevated compared with lean control. In 3T3-L1 preadipocytes, the level of mRNA and reductase activity of 11β-HSD1 was augmented by TNF-α, IL-1β, and LPS, with a concomitant increase in inducible nitric oxide synthase (iNOS), monocyte chemotactic protein-1 (MCP-1), or IL-6 secretion. Pharmacological inhibition of 11β-HSD1 and RNA interference against 11β-HSD1 reduced the mRNA and protein levels of iNOS, MCP-1, and IL-6. In contrast, overexpression of 11β-HSD1 further augmented TNF-α-induced iNOS, IL-6, and MCP-1 expression. Moreover, 11β-HSD1 inhibitors attenuated TNF-α-induced phosphorylation of NF-κB p65 and p38-, JNK-, and ERK1/2-MAPK. Collectively, the present study provides novel evidence that inflammatory stimuli-induced 11β-HSD1 in adipocyte differentiation pathways and results in further induction of proinflammatory molecules. Not limited to 3T3-L1 preadipocytes, we also demonstrated that the notion was reproducible in the primary SVF cells from obese mice. These findings highlight an unexpected, proinflammatory role of reamplified glucocorticoids within preadipocytes in obese adipose tissue.

11β-hydroxysteroid dehydrogenase type 1; preadipocyte; nuclear factor-κB; mitogen-activated protein kinase; adipose inflammation

OBESE ADIPOSE TISSUE IS CHARACTERIZED by low-grade, chronic inflammation (24, 58). In humans and rodents, it has been shown that intracellular glucocorticoid reactivation is exaggerated in obese adipose tissue (38). Two isoenzymes, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and type 2 (11β-HSD2), catalyze interconversion between hormonally active cortisol and inactive cortisone (2). In particular, 11β-HSD1 is abundantly expressed in adipose tissue and preferentially reactivates cortisol from cortisone (2). In contrast, 11β-HSD2 inactivates cortisol mainly in tissues involved in water and electrolyte metabolism (60). Transgenic mice overexpressing 11β-HSD1 in adipose tissue display a cluster of fuel dyshomeostasis (61). Conversely, systemic 11β-HSD1 knockouts and adipose-specific 11β-HSD2 overexpressors, which mimic adipose-specific 11β-HSD1 knockouts, are completely protected against diabetes and dyslipidemia on a high-fat diet (14, 30, 31, 42). Interestingly, 11β-HSD1 knockout mice on a high-fat diet showed preferential accumulation of subcutaneous adipose tissue, whereas wild-type mice accumulated considerable fat pads also in visceral (mesenteric) adipose tissue (39). These findings suggest that increased activity of 11β-HSD1 in adipose tissue contributes to dysfunction of adipose tissue and subsequent metabolic derangement.

Adipose tissue is composed of mature adipocytes (~50–70% of total cells), preadipocytes (~20–40%), macrophages (~1–30%), and other cell types (22). Biopsy studies of human adipose tissue demonstrated that the distribution of adipocyte diameter is bimodal, consisting of populations of very small adipocytes (“differentiating preadipocytes”) and mature adipocytes (28, 35). Interestingly, the proportion of very small adipocytes was higher in obese people compared with lean controls (28). Notably, insulin resistance was associated with an expanded population of small adipocytes and decreased expression of differentiation marker genes, suggesting that impairment of adipocyte differentiation may contribute to obesity-associated insulin resistance (35). In this context, a potential link between preadipocyte function and pathophysiology of obese adipose tissue has recently attracted research interest (53, 57).

Many of the genes overexpressed in mature adipocytes are associated with metabolic and secretory function, whereas the most representative function of the genes overexpressed in nonmature adipocytes, i.e., stromal vascular fraction (SVF) cells, is related to inflammation and immune response (9). Macrophage infiltration into obese adipose tissue contributes to local and systemic inflammation in subjects with obesity (63, 65). Furthermore, recent research (12, 48) highlights a pathophysiological role of preadipocytes in obese adipose tissue. In the proinflammatory milieu, preadipocytes act as macrophages (11, 13), share in phagocytic activities (11), and secrete an array of inflammatory substances (13).

A pharmacological dose of glucocorticoids is widely used for anti-inflammatory therapies in human clinics (49). On the other hand, recent research is highlighting the stimulatory effects of glucocorticoids on inflammatory response. Such effects are observed at lower concentrations relevant to phys-
iological stress in vivo (35, 55, 66). Therefore, the potential role of 11β-HSD1 in a variety of inflammatory responses has stimulated academic interest (10, 26). Furthermore, it is known that mature adipocytes abundantly express 11β-HSD1, which is related to adipocyte dysfunction in obese adipose tissue (44, 61). On the other hand, the role of 11β-HSD1 in SVF cells remains largely unclear.

In this context, the present study was designed to explore the expression, regulation, and pathophysiological role of 11β-HSD1 in activated preadipocytes. The results demonstrate that inflammatory stimuli-induced 11β-HSD1 reinforces NF-κB and MAPK signals and results in induction of proinflammatory molecules.

MATERIALS AND METHODS

Reagents and chemicals. All reagents were of analytical grade unless otherwise indicated. TNF-α, IL-1β, LPS, and carbenoxolone (3, 52), a nonselective inhibitor for 11β-HSD1 and 11β-HSD2, were obtained from Sigma-Aldrich (St. Louis, MO). The recently developed 11β-HSD1 selective inhibitors 3-[(1-adamantyl)-5,6,7,8,9,10-hexahydro[1,2,4]triazolo[4,3-α]jazoline trifluoroacetate salt (WO03/065983, inhibitor A; Merck, Whitehouse Station, NJ; Ref. 23) and 2,4,6-trichloro-N-(5,5-dimethyl-7-oxo-4,5,6,7-tetrahydro-1,3-benzothiazole-2-yl) benzanesulfonylamide (BVT-3498; Biovitrum, Stockholm, Sweden; Ref. 25) were synthesized according to the patent information.

Polycyclonial antibodies against NF-κB p65, phospho-p65, ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, Akt, and phospho-Akt were purchased from Cell Signaling Technology (Beverly, MA). Polyclonal antibodies against SHIP1, PP2A, and MKP-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against β-actin was purchased from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase-conjugated anti-mouse, anti-rat, and anti-rabbit IgG antibodies and ECL Plus Western blotting detection kits were purchased from Amersham Biosciences (Piscataway, NJ).

Cell culture. 3T3-L1 cells (kindly provided by Dr. H. Green and Dr. M. Morikawa, Harvard Medical School, Boston, MA) were maintained in DMEM containing 10% (vol/vol) calf serum at 37°C under 10% CO2.

Animals. Seventeen-week-old male C57BL/6 and nine-week-old ob/ob mice were used for the experiments. Mice were maintained on a standard diet (F-2, 3.7 kcal/g, 12% of kcal from fat, source soybean; AIN-93M, 4.9 kcal/g, 60% of kcal from fat, source soybean/lard) under a 14:10-h light-dark cycle at 23°C. All experiments were undertaken in accordance with the guidelines for animal experiments of the Kyoto University Animal Research Committee.

Isolation of SVF and the mature adipocyte fraction. Subcutaneous (SQ), mesenteric (Mes), and epididymal (Epi) fat deposits were scraped using fine scissors and digested with 2 mg/ml collagenase (Type VIII; Sigma-Aldrich) in DMEM for 1 h at 37°C under continuous shaking (170 rpm). Dispersed tissue was filtered through a nylon mesh with a pore size of 250 μm and centrifuged. Digested material was separated by centrifugation at 1,800 rpm for 5 min. The sedimented SVF and cell supernatant [mature adipocyte fraction (MAF)] were both washed with DMEM. For primary culture experiments, SVF cells from epididymal fat pads were plated in sixwell plates and cultured overnight in DMEM containing 10% (vol/vol) FBS at 37°C under 10% CO2. After being rinsed with the medium three times, the cells were incubated with or without TNF-α, carbenoxolone, or inhibitor A for 24 h.

Quantitative real-time PCR. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer’s instruction. The sequences of probes and primers are summarized in Suppl. Table S1 (supplemental data for this article are available at the Am J Physiol Endocrinol Metab website). Taqman PCR was performed using an ABI Prism 7300 sequence detection system following the manufacturer’s instructions (Applied Biosystems, Foster City, CA). mRNA levels were normalized to those of 18S rRNA.

11β-HSD1 enzyme activity assay. 11β-HSD1 acts as a reductase and reactivates cortisol from cortisone in viable cells (54). In certain substrates, however, such as tissue homogenates or the microsome fraction, 11β-HSD1 acts as a dehydrogenase and inactivates cortisol to cortisone (8). 11β-HSD1 reductase activity in intact cells was measured as reported previously (8). Cells were incubated for 24 h in serum-free DMEM, with the addition of 250 nM cortisone and tritium-labeled tracer [1,2-3H]-cortisone (Muromachi Yukinhi, Kyoto, Japan) for reductase activity and 250 nM cortisol with [1,2,6,7-3H]-cortisol (Muromachi Yukinhi) for dehydrogenase activity. Cortisol and cortisone were extracted using ethyl acetate, evaporated, resuspended in ethanol, separated using thin-layer chromatography in 95:5 chloroform/methanol, and quantified using autoradiography.

To validate inhibitory potency of compounds against 11β-HSD1 with the use of FreeStyle 293 cells transiently transfected with human 11β-HSD1, the enzyme activity assay was carried out with 20 mM Tris·HCl at pH 7.0, 50 μM NADPH, 5 μg protein of microsomal fraction, and 300 nM [3H]cortisone for 2 h. The reaction was stopped by 18% glycerophosphate acid. The labeled cortisol product was captured by mouse monoclonal anti-cortisol antibody, bound to scintillation proximity assay beads coated with protein A, and quantified in a scintillation counter.

ELISA. Monocyte chemoattractant protein-1 (MCP-1) and IL-6 concentrations in the cultured media of 3T3-L1 preadipocytes were measured using ELISA according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).

Western blot analysis. Two days after confluence, 3T3-L1 preadipocytes were stimulated with 10 ng/ml TNF-α in the absence or presence of 11β-HSD1 inhibitors (50 μM carbenoxolone or 10 μM inhibitor A) for 24 h.

For primary culture experiments, SVF from epididymal fat pads were plated on sixwell plates and cultured overnight in DMEM containing 10% (vol/vol) FBS at 37°C under 10% CO2. After being rinsed with the medium three times, the cells were incubated with or without TNF-α, carbenoxolone, or inhibitor A for 24 h.

After 2-h serum starvation, cells were treated with TNF-α for 10 min to detect NF-κB and MAPK signals. Cells were washed with ice-cold PBS and harvested in lysis buffer (1% wt/vol SDS, 60 mM Tris·HCl, 1 mM Na3VO4, 0.1 mg/ml aprotinin, 1 mM PMSF, and 50 mM okadaic acid at pH 6.8) and boiled at 100°C for 10 min. After centrifugation, supernatants were normalized to the protein concentration via the Bradford method and then equal amounts of protein were subjected to SDS-PAGE and immunoblot analysis.

RNA interference. We tested four different small interfering RNA (siRNA) sequences. Stealth RNAi for mouse 11β-HSD1 (MSS205244, MSS205245, and MSS205246) (Invitrogen), and RNA interference (RNAi) for mouse 11β-HSD1 originally designed by an siRNA Design Support System (TaKaRa Bio, Shiga, Japan; sense: 5′-GAAUUUGCCAUAUCAUCGUTT-3′ and antisense: 3′-TTCTUUACGGUAUAUGAGACA-5′), MSS205245 and MSS205246 did not suppress the 11β-HSD1 mRNA level effectively in preliminary experiments. Therefore, we demonstrated the data of MSS205244 [si(1)] and of the originally designed siRNA [si(2)] in this study. According to the manufacturer’s protocol, 3T3-L1 preadipocytes were transfected with 10 nM siRNA in antibiotic-free medium using Lipofectamine RNAiMAX (Invitrogen). We assessed the transfection efficiency using green fluorescent protein (GFP) detection (pmaxGFP), according to the manufacturer’s instructions (Amaxa, Cologne, Germany). Fluorescent microscopic observa-
tion revealed that more than two-thirds of the cells expressed GFP (data not shown).

Expression vector. A mammalian expression vector encoding Hsd11b1 (Hsd11b1/pCDNA3.1) was constructed by inserting cDNA for mouse 11β-HSD1 into pCDNA3.1 (Invitrogen). 3T3-L1 preadipocytes were detached from culture dishes using 0.25% trypsin. Cells (5 × 10⁶) were mixed with 2 μg plasmid in the solution provided with the cell line Nucleofector Kit V (Amaza), pCDNA3.1/11β-HSD1 or a control vector was introduced into the cells using electroporation with a Nucleofector (Amaza) instrument according to the manufacturer’s instructions.

Statistical analysis. Data are expressed as the means ± SE of triplicate experiments. Data were analyzed using one-way ANOVA, followed by Student’s t-tests for each pair of multiple comparisons. Differences were considered significant if P < 0.05.

RESULTS

Expression of 11β-HSD1 was elevated in the MAF and in SVF isolated from fat depots in ob/ob mice and DIO mice. Genetic (ob/ob) and dietary (DIO) obese models were analyzed. Expression of iNOS, MCP-1, and IL-6, all of which are obesity-related proinflammatory mediators (19, 29, 45, 56), was elevated in the MAF and SVF from both ob/ob mice and DIO mice compared with lean littersmates (Fig. 1, A and B).

Levels of 11β-HSD1 mRNA in the MAF from obese mice were substantially elevated compared with their lean littersmates (ob/ob: SQ, 5-fold; Mes, 62-fold; DIO: SQ, 24-fold; Mes, 460-fold; Fig. 1, A and B). On the other hand, levels of 11β-HSD1 mRNA in SVF from ob/ob mice and DIO mice were also elevated compared with their lean littersmates (ob/ob: SQ, 3-fold; Mes, 3-fold; and DIO: SQ, 8-fold, Mes, 4-fold; Fig. 1, A and B).

TNF-α, IL-1β, and LPS augmented 11β-HSD1 mRNA expression and reductase activity in 3T3-L1 preadipocytes. When 3T3-L1 preadipocytes were treated with TNF-α (10 ng/ml) for 24 h, mRNA levels of 11β-HSD1 markedly increased (∼4-fold; Fig. 2iv). Levels of iNOS, MCP-1, and IL-6 mRNA were concomitantly increased (50-, 70-, and 200-fold, respectively; Fig. 2, i–iii). IL-1β (1 ng/ml) and LPS (1,000 ng/ml) substantially augmented 11β-HSD1 mRNA expression in 3T3-L1 preadipocytes (10- and 3-fold vs. control, respectively) (Fig. 2iv). Reductase activity of 11β-HSD1 was augmented by TNF-α, IL-1β, and LPS compared with the control (2-, 9-, and 6-fold vs. control, respectively; P < 0.05; Fig. 2v).

Based on the results of 11β-HSD1 activity, TNF-α was used at 10 ng/ml in subsequent experiments. On the other hand, 11β-HSD2 mRNA and the corresponding dehydrogenase activity were undetected not only at the baseline condition but with TNF-α, IL-1β, and LPS treatments (data not shown).

Dexamethasone decreased iNOS, MCP-1, and IL-6 mRNA and protein levels in TNF-α-treated 3T3-L1 preadipocytes. The effects of glucocorticoid on proinflammatory gene expression in TNF-α-treated 3T3-L1 preadipocytes were examined over a wide range of concentrations (10⁻¹⁰, 10⁻⁹, 10⁻⁸, and 10⁻⁷ M), representing physiological to therapeutic levels in vivo (5). Dexamethasone (10⁻⁷ M) decreased mRNA levels of iNOS, MCP-1, and IL-6 (iNOS: 85 ± 2%, MCP-1: 40 ± 16%, and IL-6: 97 ± 1% reduction vs. TNF-α-treated cells) and protein levels in the media (MCP-1: 48 ± 5% and IL-6: 83 ± 1% reduction) in TNF-α-treated 3T3-L1 preadipocytes (Suppl. Fig. S1).

Pharmacological inhibition of 11β-HSD1 attenuated iNOS, MCP-1, and IL-6 mRNA and protein levels in TNF-α-treated 3T3-L1 preadipocytes. The effects of pharmacological inhibition of 11β-HSD1 on proinflammatory gene expression were examined in TNFα-treated 3T3-L1 preadipocytes. In previous in vitro studies, carbenoxolone (CBX), a nonselective inhibitor of 11β-HSD1 and 11β-HSD2, was used at concentrations from 5 to 300 μM (16, 17, 26). To date, an 11β-HSD1-specific

![Fig. 1. 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) mRNA expression in stromal vascular fraction cells (SVF) and mature adipocytes fraction (MAF) isolated from obese adipose tissue of ob/ob mice and diet-induced obese (DIO) mice. A: ob/ob and lean littersmates [control (C) 9 wk of age; n = 6]. B: DIO and littersmates on a chow diet (17 wk of age; n = 6). Levels of inducible nitric oxide synthase (iNOS, i), monocyte chemoattractant protein-1 (MCP-1; ii), IL-6 (iii), and 11β-HSD1 (iv) mRNA in SVF and MAF in subcutaneous abdominal fat depots (SQ) and mesenteric fat depots (Mes). *P < 0.05, **P < 0.01 compared with lean littersmates.](http://ajpendo.physiology.org/)
inhibitor, inhibitor A, has not been used for in vitro studies; however, another 11β-HSD1-specific inhibitor (compound 544) sharing almost the same structure as inhibitor A was used at a concentration of 5 μM (62). Therefore, in the present study, 10–50 μM CBX and 2.5–10 μM inhibitor A were used.

Before using these inhibitors in intact cells, we validated inhibitory potency of compounds against 11β-HSD1 in the microsome fraction assay. We verified that inhibitor A (10 nM) and CBX (1 μM) inhibited 11β-HSD1 activity as little as 25% vs. control, respectively, and that both of the 11β-HSD1 inhibitors suppressed 11β-HSD activity in a dose-dependent manner (Suppl. Fig. S2).

In 3T3-L1 preadipocytes, although CBX and inhibitor A did not change the level of 11β-HSD1 reductase activity, both of them suppressed TNF-α-induced reductase activity of 11β-HSD1 in a dose-dependent manner (Fig. 3A). CBX (50 μM) and inhibitor A (10 μM) markedly attenuated 11β-HSD1 activity as little as 25% vs. control, respectively, and that both of the 11β-HSD1 inhibitors suppressed 11β-HSD activity in a dose-dependent manner (Suppl. Fig. S2).

Without TNF-α-treatment, CBX and inhibitor A did not affect mRNA or protein levels of iNOS, MCP-1, and IL-6. On the other hand, in TNF-α-treated cells, these inhibitors reduced the mRNA and protein levels of proinflammatory genes. CBX decreased iNOS, MCP-1, and IL-6 mRNA levels (50 μM; iNOS: 83 ± 5%, MCP-1: 27 ± 4%, and IL-6: 47 ± 10% reduction vs. TNF-α-treated cells without compounds) and protein levels in the media (MCP-1: 17 ± 1% and IL-6: 34 ± 6% reduction) in TNF-α-treated 3T3-L1 preadipocytes (Fig. 3B). Similarly, inhibitor A reduced iNOS, MCP-1, and IL-6 mRNA (10 μM; iNOS: 47 ± 13%, MCP-1: 32 ± 12%, and IL-6: 33 ± 9% reduction) and protein levels in the media (MCP-1: 47 ± 3% and IL-6: 14 ± 3% reduction) (Fig. 3C).

Effect of 11β-HSD1 knockdown on proinflammatory properties in 3T3-L1 preadipocytes. To explore the potential role of 11β-HSD1 in cytokine release from activated preadipocytes, 11β-HSD1 was knocked down using siRNA. We tested four different siRNA sequences as described in MATERIALS AND METHODS; however, two of them did not suppress 11β-HSD1 mRNA level significantly in the preliminary experiments. Thus we demonstrated the data on si(1) and si(2).

When 3T3-L1 preadipocytes were transfected with 11β-HSD1 siRNA, TNF-α-induced expression of 11β-HSD1 was markedly attenuated [si(1): 60 ± 9% and si(2): 88 ± 7% reduction vs. negative control siRNA; Fig. 4A, i]. 11β-HSD1 reductase activity was also decreased by 11β-HSD1 siRNA [si(1): 81 ± 9% and si(2): 84 ± 3% reduction vs. negative control siRNA; Fig. 4A, ii]. 11β-HSD2 mRNA levels and the corresponding dehydrogenase activity were under detectable with or without siRNA treatments in 3T3-L1 preadipocytes (data not shown). Negative control RNAi did not influence the expression of 11β-HSD1. Knockdown of 11β-HSD1 in TNF-α-treated 3T3-L1 preadipocytes effectively reduced iNOS, MCP-1, and IL-6 mRNA levels [si(1): IL-6: 32 ± 7% reduction; and si(2): iNOS: 37 ± 8%, MCP-1: 22 ± 5%, and IL-6: 59 ± 3% reduction] and protein levels in the media [si(1):
Fig. 3. Effects of pharmacological inhibition of 11β-HSD1 on glucocorticoid receptor (GR), MCP-1, IL-6, and iNOS expression in and secretion from TNF-α-treated 3T3-L1 preadipocytes. A: 11β-HSD1 activity assay for validation of 11β-HSD1 inhibitors. 3T3-L1 preadipocytes were incubated for 24 h in serum-free DMEM, adding 250 nM of cortisol with tritium-labeled cortisol. A representative autoradiograph of TLC for the 11β-HSD1 activity assay (top) and quantification of 11β-HSD1 activities (bottom). Intensities of cortisol signals correspond to the reductase activity. The y-axis shows percent 11β-HSD1 reductase activity compared with TNF-α (10 ng/ml)-treated cells.

B: Overexpression of 11β-HSD1 augmented iNOS, MCP-1, and IL-6 in TNF-α-treated 3T3-L1 preadipocytes. We examined whether overexpression of 11β-HSD1 is relevant to the augmentation of proinflammatory molecules in activated preadipocytes. The extent of 11β-HSD1 overexpression in 3T3-L1 preadipocytes was assessed by 11β-HSD1 mRNA levels and reductase activity (Fig. 5A). As expected, 11β-HSD1 mRNA level was increased by treatment of the 11β-HSD1 vector (~20-fold) or 10 ng/ml TNF-α (~300-fold) compared with the vehicle. TFN-α-induced expression of 11β-HSD1 was further augmented by the introduction of the 11β-HSD1 vector (1.6-fold vs. empty vector). Reductase activity of 11β-HSD1 was also increased by the vector (1.6-fold). Notably, TNF-α-induced enzyme activity was further augmented by the vector. Expression of iNOS, MCP-1, and IL-6 did not differ between the 11β-HSD1 vector and the empty vector. On the other hand, TNF-α-induced expression of iNOS, MCP-1, and IL-6 was augmented in 11β-HSD1 transfectants (MCP-1: 172 ±...
88%, IL-6: 194 ± 64%, and iNOS: 187 ± 47% vs. the empty vector; Fig. 5B, ii-iv). Similarly, protein levels of MCP-1 and IL-6 in the media were increased in transfectants (MCP-1: 206 ± 32% and IL-6: 156 ± 17% vs. the empty vector; Fig. 5B, v and vi).

Pharmacological inhibition of 11β-HSD1 attenuated NF-κB and MAPK signaling in 3T3-L1 preadipocytes. We examined the possible involvement of 11β-HSD1 in proinflammatory signaling pathways. 3T3-L1 preadipocytes were incubated with TNF-α (10 ng/ml), with or without CBX (50 μM) and inhibitor A (10 μM) for 24 h. After a 2-h serum starvation, the cells were incubated with TNF-α (10 ng/ml), with or without CBX (50 μM) and inhibitor A (10 μM) for 10 min. TNF-α-induced p-65 phosphorylation was markedly attenuated by CBX (30 ± 12% decrease vs. TNF-α-treated cells) and inhibitor A (51 ± 11% decrease vs. TNF-α-treated cells; Fig. 6A). Regarding MAPK signaling, augmented phosphorylation of p-38, JNK, and ERK with the TNF-α treatment was substantially attenuated by CBX (p-38: 26 ± 8% decrease and JNK: 48 ± 3% decrease vs. TNF-α-treated cells) and inhibitor A (p-38: 51 ± 9% decrease, JNK: 72 ± 5% decrease, and ERK: 36 ± 11% decrease vs. TNF-α-treated cells; Fig. 6B).

Pharmacological inhibition of 11β-HSD1 attenuated iNOS, MCP-1, and IL-6 mRNA levels in SVF cells from ob/ob mice. We examined the effects of pharmacological inhibition of 11β-HSD1 on proinflammatory gene expression in primary cultured SVF cells isolated from epididymal fat depots in obese ob/ob mice or lean control mice. CBX (50 μM) and inhibitor A (10 μM) did not change the expression level of 11β-HSD1 (Fig. 7i). CBX decreased mRNA level of iNOS, MCP-1, and IL-6 in both the basal state (iNOS: 69 ± 4%, MCP1: 42 ± 7%, and IL-6: 56 ± 14% reduction vs. vehicle control) and TNF-α-stimulated state (iNOS: 58 ± 11%, MCP-1: 63 ± 5%, and IL-6: 53 ± 8% reduction vs. TNFα-treated cells without compounds) in SVF cells from ob/ob mice.
Without TNF-α-treatment, CBX did not change mRNA levels of iNOS, MCP-1 and IL-6 in SVF cells from lean control mice. However, CBX reduced the mRNA levels of iNOS, MCP-1, and IL-6 (iNOS: 64 ± 18%, MCP-1: 67 ± 14%, and IL-6: 58 ± 12% reduction vs. TNF-α-treated cells without compounds) in TNF-α-treated SVF cells from lean control mice (Fig. 7).

**Pharmacological inhibition of 11β-HSD1 attenuated NF-κB and MAPK signaling in SVF cells from ob/ob mice.** SVF cells from ob/ob or lean control mice were incubated with TNF-α (10 ng/ml), with or without CBX (50 μM) and inhibitor A (10 μM) for 24 h. After a 2-h serum starvation, the cells were incubated with TNF-α (10 ng/ml), with or without CBX (50 μM) and inhibitor A (10 μM) for 10 min. Activation of NF-κB (p65) and MAPK (p38, JNK, and ERK) signaling did occur in SVF cells from ob/ob mice compared with lean control (Suppl. Fig. S3). In ob/ob mice, phosphorylation of these signaling without TNF-α treatment was attenuated by CBX and inhibitor A. TNF-α-induced p-65, p38, JNK, and ERK phosphorylation was also attenuated by CBX and inhibitor A in SVF cells from both ob/ob and lean control mice (Suppl. Fig. S3).

**DISCUSSION**

Here we provide novel evidence that inflammatory stimuli-induced 11β-HSD1 in activated preadipocytes intensifies NF-κB and MAPK signaling pathways and the resultant augmentation of proinflammatory molecules. Not limited to 3T3-L1 preadipocytes, we also demonstrated the notion was reproducible in the primary SVF cells from obese mice. Previous works focused on the metabolically beneficial impact of 11β-HSD1 deficiency on adipose tissue distribution, fuel homeostasis, and insulin sensitivity. On the other hand, clearly distinct from previous works, our present study is the first to highlight an unexpected, proinflammatory role of reamplified glucocorticoids within activated preadipocytes in obese adipose tissue.
expression level of the glucocorticoid receptor did not vary by inhibitor A exerted similar effects to CBX (Fig. 3). Of note, the activated preadipocytes, we confirmed that an 11β-HSD1-specific inhibitor A exerted similar effects to CBX (Fig. 3). Of note, the expression level of the glucocorticoid receptor did not vary by inhibitor A exerted similar effects to CBX (Fig. 3). Of note, the activated preadipocytes, we confirmed that an 11β-HSD1-specific inhibitor A exerted similar effects to CBX (Fig. 3). Of note, the expression level of the glucocorticoid receptor did not vary by

Suppression and overexpression experiments with 11β-HSD1 in activated preadipocytes demonstrate that TNF-α-induced 11β-HSD1 further augments the expression of proinflammatory genes including iNOS, MCP-1, and IL-6. Elevation of iNOS, MCP-1, and IL-6 in adipose tissue is commonly observed in obese subjects, linking to dysfunction of adipose tissue (19, 29, 45, 56). For example, iNOS-deficient mice are protected against obesity-induced insulin resistance and glucose intolerance (45). Moreover, transgenic mice overexpressing MCP-1 in adipose tissue exemplify insulin resistance and exaggerated infiltration of macrophages into adipose tissue (29). Previous studies (20, 36) showed that adipose tissue is a primary production site for IL-6 in humans. In fact, circulating IL-6 levels are shown to elevate in patients with insulin-resistance (19, 56), impaired glucose tolerance (40), and type 2 diabetes (47). Taken together, the present study provides novel evidence for proinflammatory role of 11β-HSD1 in activated preadipocytes.

To optimize experimental condition, the present study was designed to eliminate possible toxic effects and nonspecific effects of 11β-HSD1 inhibitors. Because 11β-HSD2 mRNA and corresponding dehydrogenase enzyme activity (8, 27) were undetected in 3T3-L1 preadipocytes even after the treatment with TNF-α (unpublished observations), CBX virtually serves as a specific inhibitor against 11β-HSD1 in the present study. To further verify the effect of 11β-HSD1 inhibition on activated preadipocytes, we confirmed that an 11β-HSD1-specific inhibitor A exerted similar effects to CBX (Fig. 3). Of note, the expression level of the glucocorticoid receptor did not vary by the treatment with 11β-HSD1 inhibitors (unpublished observations). The notion that TNF-α-induced 11β-HSD1 would reinforce the expression of proinflammatory genes was endorsed by the results of RNAi experiments (Fig. 4) and overexpression experiments (Fig. 5). It should be emphasized that forced overexpression of 11β-HSD1 per se did not influence the expression level of proinflammatory genes in nonactivated preadipocytes (Fig. 5B). These findings led us to speculate that 11β-HSD1-mediated active glucocorticoids within cells reinforce inflammation under proinflammatory conditions commonly seen in obese adipose tissue.

The present study demonstrated that 11β-HSD1 was highly expressed in SVF cells from obese adipose tissue (Fig. 1). Although mature adipocytes abundantly express 11β-HSD1 (44, 61), a considerable amount of 11β-HSD1 expression was detected in SVF from adipose tissue (Fig. 1). Potential link between preadipocyte function and pathophysiology of obese adipose tissue has recently attracted research interest (53, 57). A recent study (14) using 11β-HSD1 knockout mice provided evidence that 11β-HSD1 in preadipocytes may affect fat distribution under overnutrition. In 3T3-L1 cells, the expression level of 11β-HSD1 is lower in preadipocytes but is dramatically increased during the course of differentiation into mature adipocytes (51). In fact, active glucocorticoids generated intracellularly by 11β-HSD1 are critical for normal adipose differentiation (33). On the other hand, TNF-α augments 11β-HSD1 expression in preadipocytes (Fig. 2). Of note, in proinflammatory milieu, TNF-α inhibits adipocyte differentiation by decreasing PPARγ expression (43, 46, 64). Depending on the

**Fig. 6. Effects of inhibition of 11β-HSD1 on TNF-α-induced NFκB and MAPK signaling. NFκB (A) and MAPK (B) signaling pathways. 3T3-L1 preadipocytes were treated with 10 ng/ml TNF-α for 24 h in the presence or absence of 11β-HSD1 inhibitors (CBX or inhibitor A). After 2-h serum starvation, cells were treated with TNF-α in the presence or absence of 11β-HSD1 inhibitors for 10 min to assess the activation of NFκB and MAPK signaling pathways. Western blot analyses were performed using antibodies against β-actin and NFκB-p65 (A), phospho-p65 (B), p38 MAPK (B, left), phospho-p38 (B, center) JNK, phospho-JNK (B, right) ERK 1/2, and phospho-ERK1/2. A representative Western blot (top) and quantification of p65, p38, JNK, and ERK phosphorylation (bottom). Data are means ± SE of triplicate experiments. *P < 0.05, **P < 0.01 compared with TNF-α-treated cells.**
hormonal milieu, it is therefore conceivable that 11β-HSD1 plays a role in both adipogenesis and inflammatory response in preadipocytes.

We assessed the expression of Pref-1 (a representative molecular marker for preadipocytes; Ref. 7) as well as aP2, PPARγ, and GLUT4 (a set of representative markers for differentiated adipocytes; Refs. 32 and 59) in preadipocytes overexpressing 11β-HSD1. Consequently, forced augmentation of 11β-HSD1 did not affect the expression level of these genes (Suppl. Fig. S4), supporting that a line of our observation was not a facet of mature adipocytes but of preadipocytes.

Previous studies demonstrated that chronic inflammation is closely associated with insulin resistance in insulin-sensitive organs (24, 64). Glucocorticoids are widely used as anti-inflammatory agents in a clinical setting (49). On the other hand, this hormone simultaneously causes insulin resistance (4, 50). Regarding this apparent paradox, recent studies (34, 55) suggest that reactivated glucocorticoids within cells have the potential to enhance inflammatory or immune responses in a variety of cells. In the present study, replenished dexamethasone in the culture media at pharmacological doses did decrease the synthesis and secretion of proinflammatory molecules in preadipocytes in a dose-dependent manner (Fig. 3). On the other hand, in activated preadipocytes, 11β-HSD1 intensifies TNF-α-induced activation of NF-κB and the MAPK signaling cascade (Fig. 6).

Fig. 7. Effects of pharmacological inhibition of 11β-HSD1 on iNOS, MCP-1, and IL-6 mRNA levels in SVF cells from ob/ob mice. SVF cells from ob/ob mice and lean control mice were treated with CBX (50 μM) or inhibitor A (10 μM), with or without TNF-α (10 ng/ml) for 24 h. 11β-HSD1 (i), iNOS (ii), MCP-1 (iii), and IL-6 mRNA (iv) levels were determined using real-time PCR. Values were normalized to that of 18S rRNA and expressed relative to lean control. Data are means ± SE of triplicate experiments. *P < 0.05, **P < 0.01.

cell types. It should be noted that preadipocytes possess very few insulin receptors (51). Instead, preadipocytes express a large number of IGF-1 receptors (18). Insulin can bind to the IGF-1 receptor only at supraphysiological concentrations. However, it is likely that increased release of inflammatory cytokines from activated preadipocytes may aggravate insulin receptor signaling in adjacent mature adipocytes in obese adipose tissue. This notion is supported by a line of mouse experiments showing that pharmacological inhibition of 11β-HSD1 ameliorated diabetes, dyslipidemia, and even arteriosclerosis (1, 23).

PPARγ agonists potently suppress the activity of 11β-HSD1 exclusively in adipose tissue (6). The present finding that amplified glucocorticoids within activated preadipocytes may enhance inflammatory responses does not contradict the notion that PPARγ agonists exert potent anti-inflammatory effects in a variety of cell types (37). Recent studies showed that phosphoinositide 3-kinase (PI3K)-Akt pathways, IL-1 receptor-associated kinase-M (IRAK-M), and suppressors of cytokine signaling-1 (SOCS-1) are negative regulators of NF-κB and MAPK signaling (21). Under inflammatory stimuli, a physiological dose of glucocorticoids positively regulates the expression of SHIP1, a phosphatase that negatively regulates PI3K signaling, resulting in the activation of NF-κB and MAPK in activated macrophages (67). Considering the close biological similarities between activated preadipocytes and activated macrophages (11, 13), we explored whether PI3K-Akt pathways, SHIP1, or other phosphatases could be...
involved in the 11β-HSD1-induced NF-κB and MAPK activation. Western blot analyses indicated that phosphorylation of Akt or protein levels of SHIP1, PP2A, or MKP-1 did not change significantly with inhibition or overexpression of 11β-HSD1 (Suppl. Fig. S5). Further studies are warranted to unravel the entire mechanism.

In summary, the present study provides novel evidence that inflammatory stimuli-induced 11β-HSD1 reinforces NF-κB and MAPK signaling pathways and results in further induction of proinflammatory molecules in activated preadipocytes. Our findings highlight an unexpected, inflammatory role of reactivated glucocorticoids within preadipocytes in obese adipose tissue.

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DISCLOSURES
No conflicts of interest are declared by the author(s).

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