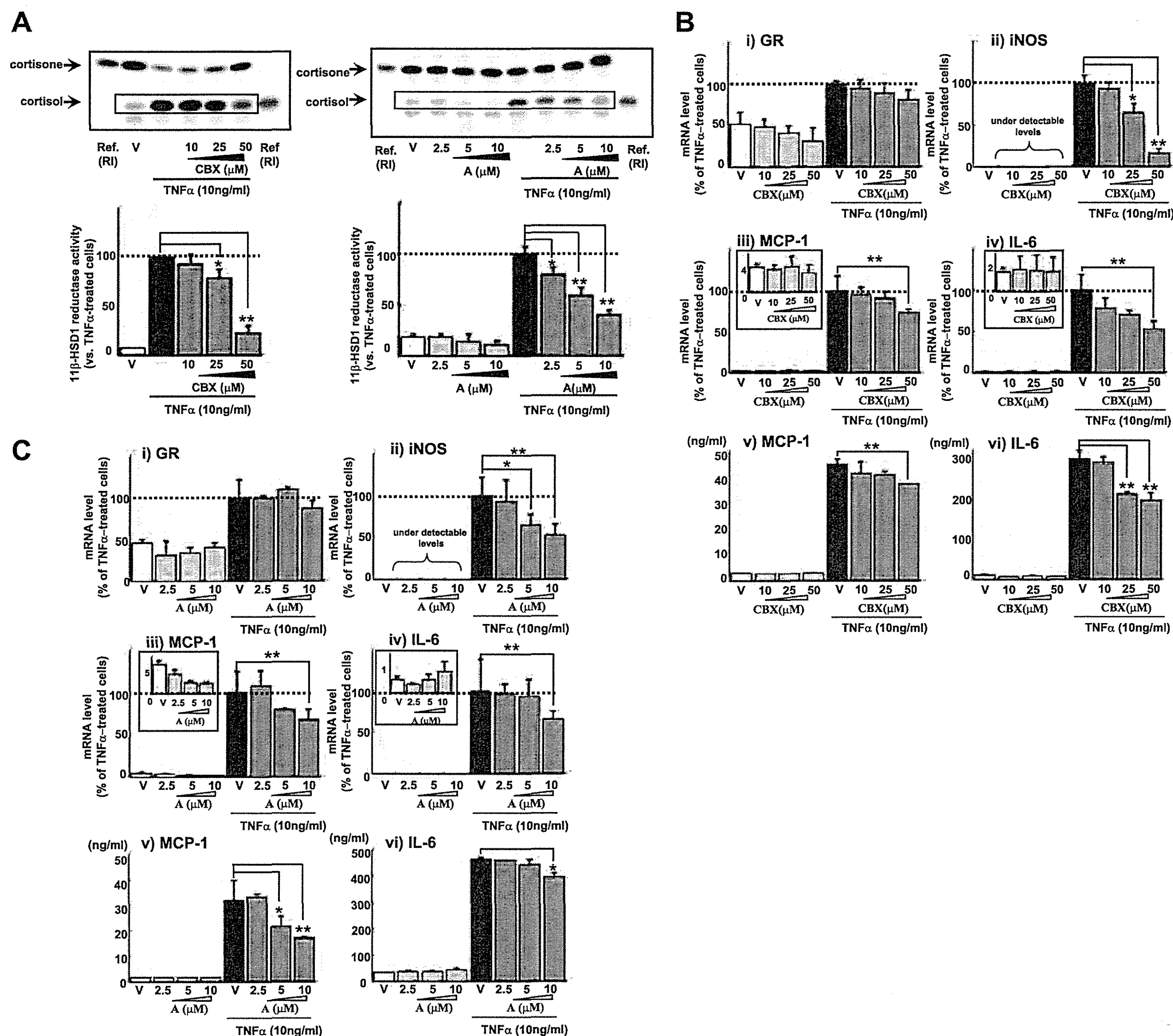

Fig. 3. Effects of pharmacological inhibition of 11β-HSD1 on glucocorticoid receptor (GR), MCP-1, IL-6, and iNOS expression in and secretion from TNF-α-treated 3T3-L1 preadipocytes. *A*: 11β-HSD1 activity assay for validation of 11β-HSD1 inhibitors. 3T3-L1 preadipocytes were incubated for 24 h in serum-free DMEM, adding 250 nM of cortisone with tritium-labeled cortisone. 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 mg/ml)-treated cells. carbenoxolone (CBX; 10–50 μM) and inhibitor A (A; 2.5–10 μM) substantially reduced 11β-HSD1 activity in 3T3-L1 preadipocytes. CBX (*B*; 10–50 μM) and inhibitor A (*C*; 2.5–10 μM) 3T3-L1 preadipocytes were treated with TNF-α (10 ng/ml) or cotreated with CBX and inhibitor A for 24 h. GR (*i*), iNOS (*ii*), MCP-1 (*iii*), and IL-6 (*iv*) mRNA levels were determined using real-time PCR. Values were normalized to that of 18S rRNA and expressed relative to TNF-α-treated cells. Concentrations of MCP-1 (*v*) and IL-6 (*vi*) in the medium were measured with ELISA. Data are means ± SE of triplicate experiments. $^*P < 0.05$, $^{**}P < 0.01$, compared with TNF-α-treated cells.

MCP-1: 13 ± 1% and IL-6: 17 ± 1% reduction; and si(2): MCP1: 19 ± 7% and IL-6: 30 ± 1% reduction; Fig. 4*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. 5*A*). 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. TNF-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 introduction of the vector (2-fold) or 10 ng/ml TNF-α (10-fold). Notably, TNF-α-induced enzyme activity was further augmented by the vector (1.3-fold vs. empty 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 ±

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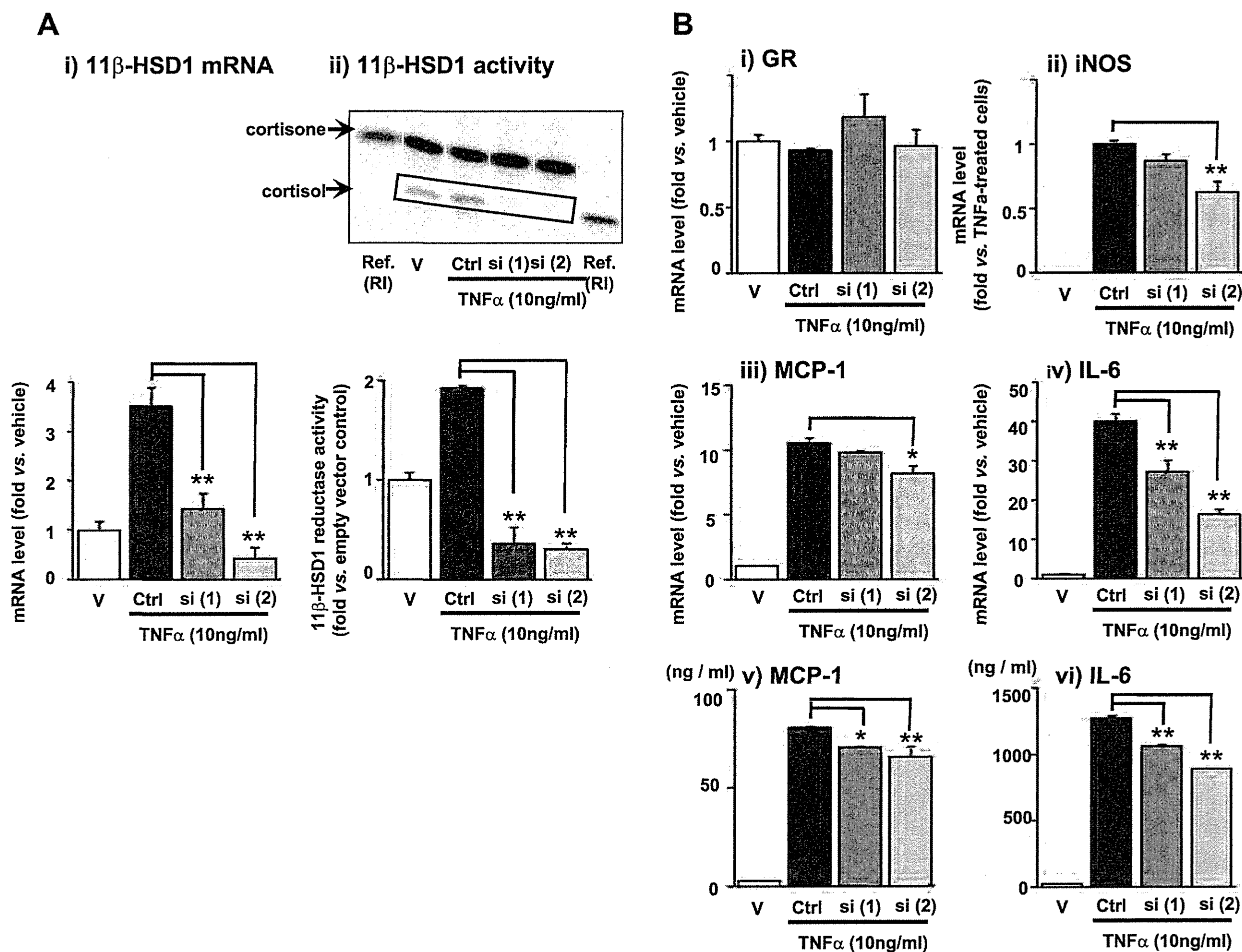

Fig. 4. Effects of 11β-HSD1 knockdown on TNF-α-induced expression of 11β-HSD1 in 3T3-L1 preadipocytes. Cells were transfected with either RNA interference for mouse 11β-HSD1 or a negative control (Ctrl). After 12 h incubation, cells were treated with 10 ng/ml TNF-α for 24 h. *A*: efficiency of 11β-HSD1 knockdown by small-interfering RNA. 11β-HSD1 mRNA (*i*) and reductase activity (*ii*). *B*: effects of knockdown of 11β-HSD1 on MCP-1, IL-6, and iNOS expression in and secretion from TNFα-treated 3T3-L1 preadipocytes. 11β-HSD1 (*i*), GR (*ii*), iNOS (*iii*), MCP-1 (*iv*), and IL-6 mRNA (*v*) levels were determined using real-time PCR. Values were normalized to that of 18S rRNA and expressed as a relative level vs. vehicle control (V). Concentrations of MCP-1 (*vi*) and IL-6 (*vii*) in the medium were measured with ELISA. Data are means ± SE of triplicate experiments. **P* < 0.05, ***P* < 0.01, compared with TNF-α-treated cells. siRNA for mouse 11β-HSD1: si(1): MSS205244 (Invitrogen) and si(2): sense: 5′-GAAAUGGCAUAUCAUCUGUTT-3′ and antisense: 3′-TTCUUUACCGUAUAGUAGACA-5′ (Takara).

88%, IL-6: 194 ± 64%, and iNOS: 187 ± 47% vs. the empty vector; Fig. 5*B*, *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. 5*B*, *v* and *vi*).

Pharmacological inhibition of 11β-HSD1 attenuated TNF-α-induced 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. 6*A*). 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. 6*B*).

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. 7*i*). 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 TNFα-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.

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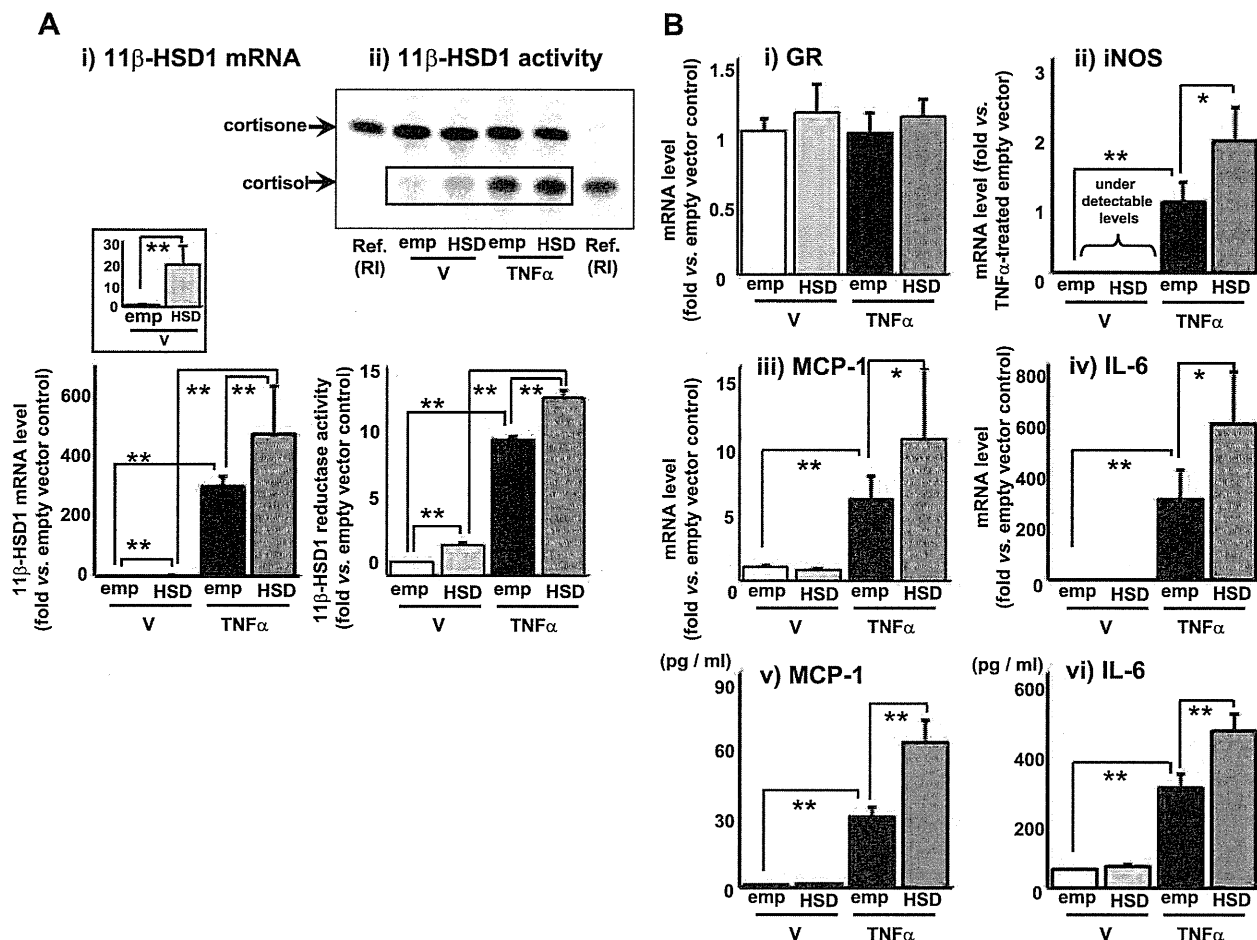

Fig. 5. Effects of overexpression of 11β-HSD1 on MCP-1, IL-6, and iNOS expression in and secretion from TNF-α-treated 3T3-L1 preadipocytes. *A*: efficiency of electroporation-mediated gene transfer. 3T3-L1 preadipocytes were transfected with the expression vector for 11β-HSD1 or a corresponding empty vector using electroporation. After 48 h, cells were treated with or without 10 ng/ml TNF-α for 24 h. Cells were assayed for 11β-HSD1 mRNA (*i*) and reductase activity (*ii*). *B*: effects of overexpression of 11β-HSD1 on MCP-1, IL-6, and iNOS expression in and secretion from TNF-α-treated 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were transfected as above, and 48 h after the infection, cells were treated with or without 10 ng/ml TNF-α for 24 h. Levels of mRNA for GR (*i*), iNOS (*ii*), MCP-1 (*iii*), and IL-6 (*iv*) were determined using real-time PCR. Values were normalized to those of 18S rRNA and expressed as a relative level vs. the vehicle control (V). Concentrations of MCP-1 (*v*) and IL-6 (*vi*) in the medium were measured with ELISA. Data are means ± SE of triplicate experiments. $*P < 0.05$, $**P < 0.01$.

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.

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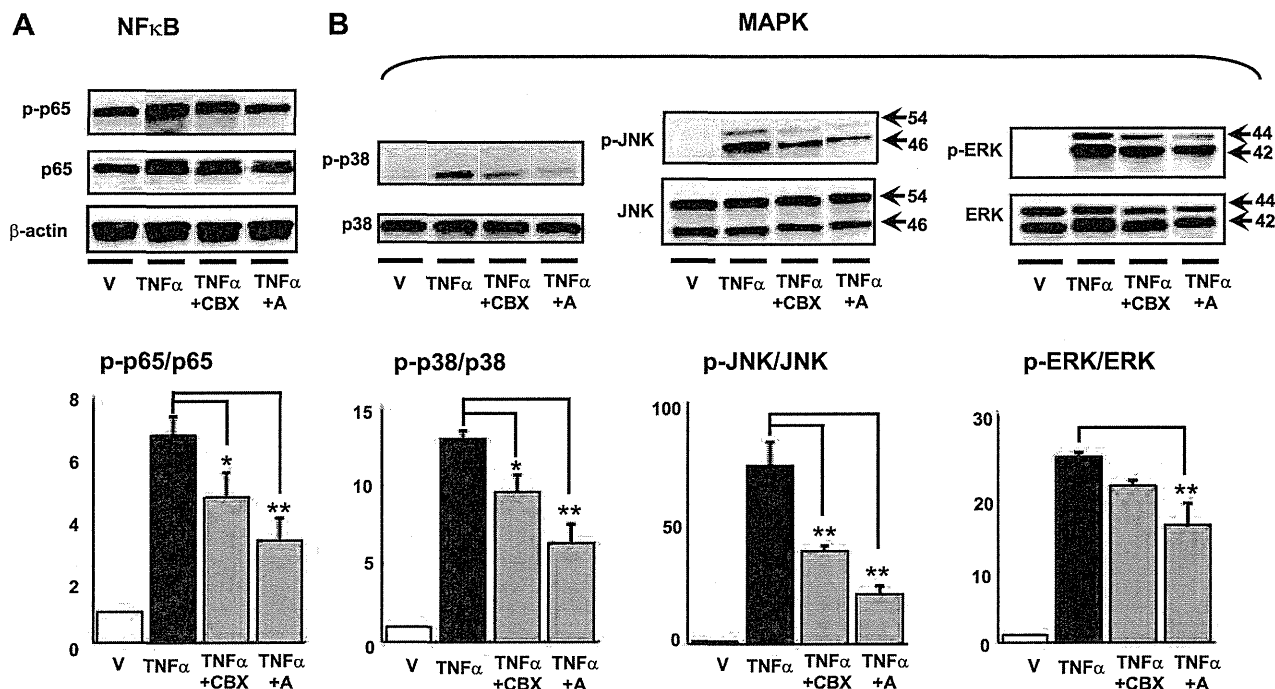

Fig. 6. Effects of inhibition of 11β-HSD1 on TNF-α-induced NFκB and MAPK signaling. NFκB (*A*) and MAPK (*B*) signaling pathways. 3T3-L1 preadipocytes were treated with 10 ng/ml TNF-α for 24 h in the presence or absence of 11β-HSD1 inhibitors (CBX or inhibitor A). After 2-h serum starvation, cells were 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 NFkB-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.

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. 5*B*). These findings led us to speculate that 11β-HSD1-mediated active glucoorticoids 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

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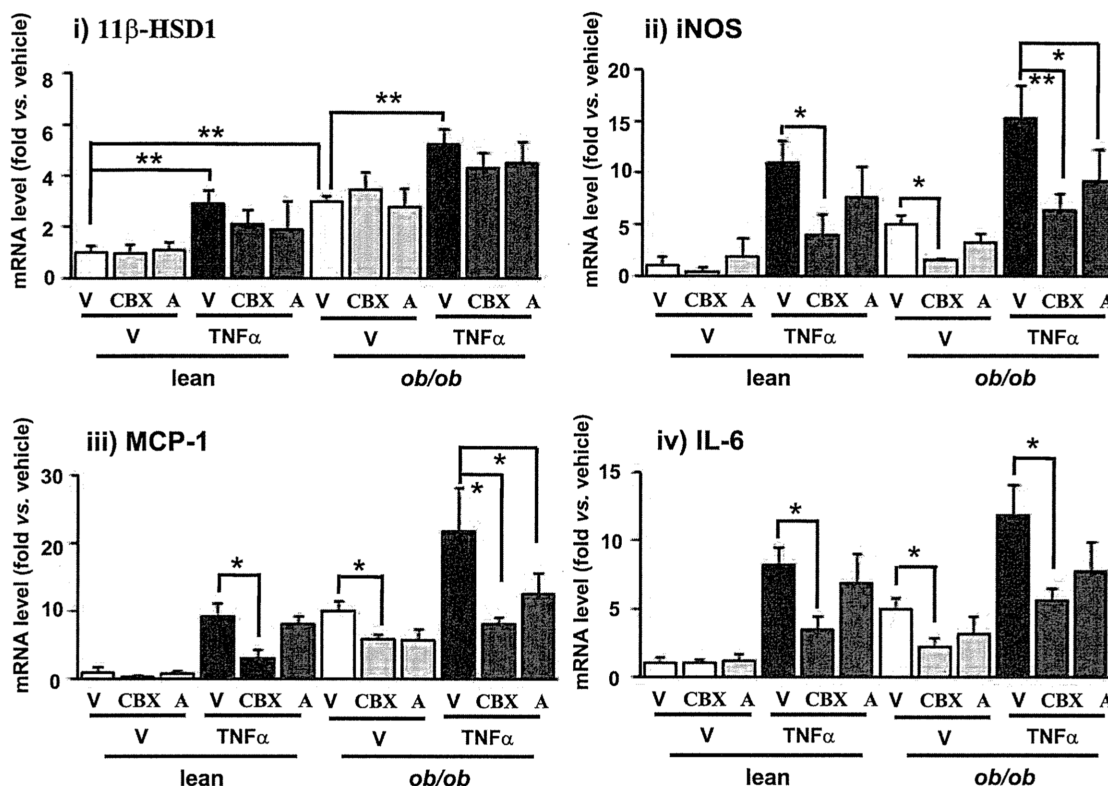

Fig. 7. Effects of pharmacological inhibition of 11β-HSD1 on iNOS, MCP-1, and IL-6 mRNA levels in SVF cells from *ob/ob* mice. SVF cells from *ob/ob* mice and lean control mice were treated with CBX (50 μM) or inhibitor A (10 μM), with or without TNF-α (10 ng/ml) for 24 h. 11β-HSD1 (*i*), iNOS (*ii*), 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. $^{*}P < 0.05$, $^{**}P < 0.01$.

hormonal milieu, it is therefore conceivable that 11β-HSD1 plays a role in both adipogenesis and inflammatory response in preadipoytes.

We assessed the expression of Pref-1 (a representative molecular marker for preadipocytes; Ref. 7) as well as aP2, PPARγ2, 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). In this context, it is possible that intracellular activation of glucocorticoids within physiological range would likely cause proinflammatory responses in certain 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

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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).

REFERENCES

1. **Alberts P, Nilsson C, Selen G, Engblom LO, Edling NH, Norling S, Klingstrom G, Larsson C, Forsgren M, Ashkzari M, Nilsson CE, Fiedler M, Bergqvist E, Ohman B, Bjorkstrand E, Abrahmsen LB.** Selective inhibition of 11 beta-hydroxysteroid dehydrogenase type 1 improves hepatic insulin sensitivity in hyperglycemic mice strains. *Endocrinology* 144: 4755–4762, 2003.
2. **Andrew R, Phillips DI, Walker BR.** Obesity and gender influence cortisol secretion and metabolism in man. *J Clin Endocrinol Metab* 83: 1806–1809. 1998.
3. **Andrews RC, Rooyackers O, Walker BR.** Effects of the 11 beta-hydroxysteroid dehydrogenase inhibitor carbenoxolone on insulin sensitivity in men with type 2 diabetes. *J Clin Endocrinol Metab* 88: 285–291, 2003.
4. **Asensio C, Muzzin P, Rohner-Jeanrenaud F.** Role of glucocorticoids in the physiopathology of excessive fat deposition and insulin resistance. *Int J Obes Relat Metab Disord* 28 *Suppl* 4: S45–52, 2004.
5. **Balachandran A, Guan H, Sellan M, van Uum S, Yang K.** Insulin and dexamethasone dynamically regulate adipocyte 11beta-hydroxysteroid dehydrogenase type 1. *Endocrinology* 149: 4069–4079, 2008.
6. **Berger J, Tanen M, Elbrecht A, Hermanowski-Vosatka A, Moller DE, Wright SD, Thieringer R.** Peroxisome proliferator-activated receptor-gamma ligands inhibit adipocyte 11beta-hydroxysteroid dehydrogenase type 1 expression and activity. *J Biol Chem* 276: 12629–12635, 2001.
7. **Boney CM, Fiedorek FT Jr, Paul SR, Gruppuso PA.** Regulation of preadipocyte factor-1 gene expression during 3T3–L1 cell differentiation. *Endocrinology* 137: 2923–2928, 1996
8. **Bujalska IJ, Kumar S, Stewart PM.** Does central obesity reflect "Cushing's disease of the omentum"? *Lancet* 349: 1210–1213, 1997.
9. **Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C, Coupaye M, Pelloux V, Hugol D, Bouillot JL, Bouloumie A, Barbatelli G, Cinti S, Svensson PA, Barsh GS, Zucker JD, Basdevant A, Langin D, Clement K.** Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes* 54: 2277–2286, 2005.
10. **Chapman KE, Coutinho AE, Gray M, Gilmour JS, Savill JS, Seckl JR.** The role and regulation of 11beta-hydroxysteroid dehydrogenase type 1 in the inflammatory response. *Mol Cell Endocrinol* 301: 123–131, 2009.
11. **Charriere G, Cousin B, Arnaud E, Andre M, Bacou F, Penicaud L, Casteilla L.** Preadipocyte conversion to macrophage. Evidence of plasticity. *J Biol Chem* 278: 9850–9855, 2003.
12. **Chung S, Lapoint K, Martinez K, Kennedy A, Boysen Sandberg M, McIntosh MK.** Preadipocytes mediate lipopolysaccharide-induced inflammation and insulin resistance in primary cultures of newly differentiated human adipocytes. *Endocrinology* 147: 5340–5351, 2006.
13. **Cousin B, Munoz O, Andre M, Fontanilles AM, Dani C, Cousin JL, Laharrague P, Casteilla L, Penicaud L.** A role for preadipocytes as macrophage-like cells. *FASEB J* 13: 305–312, 1999.
14. **De Sousa Peixoto RA, Turban S, Battle JH, Chapman KE, Seckl JR, Morton NM.** Preadipocyte 11beta-hydroxysteroid dehydrogenase type 1 is a keto-reductase and contributes to diet-induced visceral obesity in vivo. *Endocrinology* 149: 1861–1868, 2008.
16. **Dembinska-Kiec A, Pallapies D, Simmet T, Peskar BM, Peskar BA.** Effect of carbenoxolone on the biological activity of nitric oxide: relation to gastroprotection. *Br J Pharmacol* 104: 811–816, 1991.
17. **Elsen FP, Shields EJ, Roe MT, Vandam RJ, Kelty JD.** Carbenoxolone induced depression of rhythmogenesis in the pre-Botzinger complex. *BMC Neurosci* 9: 46, 2008.
18. **Entingh-Pearsall A, Kahn, CR.** Differential roles of the insulin and insulin-like growth factor-i (igf-i) receptors in response to insulin and IGF-I. *J Biol Chem* 279: 38016–38024, 2004.
19. **Fernandez-Real JM, Vayreda M, Richart C, Gutierrez C, Broch M, Vendrell J, Ricart W.** Circulating interleukin 6 levels, blood pressure, and insulin sensitivity in apparently healthy men and women. *J Clin Endocrinol Metab* 86: 1154–1159, 2001.
20. **Fried SK, Bunkin DA, Greenberg AS.** Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *J Clin Endocrinol Metab* 83: 847–850, 1998.
21. **Fukao T, Koyasu S.** PI3K and negative regulation of TLR signaling. *Trends Immunol* 24: 358–363, 2003.
22. **Hauner H.** Secretory factors from human adipose tissue and their functional role. *Proc Nutr Soc* 64: 163–169, 2005.
23. **Hermanowski-Vosatka A, Balkovec JM, Cheng K, Chen HY, Hernandez M, Koo GC, Le Grand CB, Li Z, Metzger JM, Mundt SS, Noonan H, Nunes CN, Olson SH, Pikounis B, Ren N, Robertson N, Schaeffer JM, Shah K, Springer MS, Strack AM, Strowski M, Wu K, Wu T, Xiao J, Zhang BB, Wright SD, Thieringer R.** 11beta-HSD1 inhibition ameliorates metabolic syndrome and prevents progression of atherosclerosis in mice. *J Exp Med* 202: 517–527, 2005.
24. **Hotamisligil GS.** Inflammation and metabolic disorders. *Nature* 444: 860–867, 2006.
25. **Hult M, Shafqat N, Elleby B, Mitschke D, Svensson S, Forsgren M, Barf T, Vallgarda J, Abrahmsen L, Oppermann U.** Active site variability of type 1 11beta-hydroxysteroid dehydrogenase revealed by selective inhibitors and cross-species comparisons. *Mol Cell Endocrinol* 248: 26–33, 2006.
26. **Ishii T, Masuzaki H, Tanaka T, Arai N, Yasue S, Kobayashi N, Tomita T, Noguchi M, Fujikura J, Ebihara K, Hosoda K, Nakao K.** Augmentation of 11beta-hydroxysteroid dehydrogenase type 1 in LPS-activated J774.1 macrophages–role of 11beta-HSD1 in pro-inflammatory properties in macrophages. *FEBS Lett* 581: 349–354, 2007.
27. **Jamieson PM, Chapman KE, Edwards CR, Seckl JR.** 11 beta-hydroxysteroid dehydrogenase is an exclusive 11 beta- reductase in primary cultures of rat hepatocytes: effect of physicochemical and hormonal manipulations. *Endocrinology* 136: 4754–4761, 1995.
28. **Julien P, Despres JP, Angel A.** Scanning electron microscopy of very small fat cells and mature fat cells in human obesity. *J Lipid Res* 30: 293–299, 1989.
29. **Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M.** MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest* 116: 1494–1505, 2006.
30. **Kershaw EE, Morton NM, Dhillon H, Ramage L, Seckl JR, Flier JS.** Adipocyte-specific glucocorticoid inactivation protects against diet-induced obesity. *Diabetes* 54: 1023–1031, 2005.
31. **Kotelevtsev Y, Holmes MC, Burchell A, Houston PM, Schmoll D, Jamieson P, Best R, Brown R, Edwards CR, Seckl JR, Mullins JJ.** 11beta-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. *Proc Natl Acad Sci USA* 94: 14924–14929, 1997.
32. **Lane MD, Tang QQ, Jiang MS.** Role of the CCAAT enhancer binding proteins (C/EBPs) in adipocyte differentiation. *Biochem Biophys Res Commun* 266: 677–683, 1999.

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33. **Liu Y, Sun Y, Zhu T, Xie Y, Yu J, Sun WL, Ding GX, Hu G.** 11β HSD1 promotes differentiation of 3T3-L1 preadipocyte. *Acta Pharmacol Sin* 28: 1198–204, 2007.
34. **McEwen BS, Biron CA, Brunson KW, Bulloch K, Chambers WH, Dhabhar FS, Goldfarb RH, Kitson RP, Miller AH, Spencer RL, Weiss JM.** The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions. *Brain Res Brain Res Rev* 23: 79–133, 1997.
35. **McLaughlin T, Sherman A, Tsao P, Gonzalez O, Yee G, Lamendola C, Reaven GM, Cushman SW.** Enhanced proportion of small adipose cells in insulin-resistant vs insulin-sensitive obese individuals implicates impaired adipogenesis. *Diabetologia* 50: 1707–1715, 2007.
36. **Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S, Coppack SW.** Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J Clin Endocrinol Metab* 82: 4196–4200, 1997.
37. **Moller DE, Berger JP.** Role of PPARs in the regulation of obesity-related insulin sensitivity and inflammation. *Int J Obes Relat Metab Disord* 27 *Suppl* 3: S17–21, 2003.
38. **Montague CT, O'Rahilly S.** The perils of portliness: causes and consequences of visceral adiposity. *Diabetes* 49: 883–888, 2000.
39. **Morton NM, Paterson JM, Masuzaki H, Holmes MC, Staels B, Fievet C, Walker BR, Flier JS, Mullins JJ, Seckl JR.** Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11β-hydroxysteroid dehydrogenase type 1-deficient mice. *Diabetes* 53: 931–938, 2004.
40. **Muller S, Martin S, Koenig W, Hanifi-Moghaddam P, Rathmann W, Haastert B, Giani G, Illig T, Thorand B, Kolb H.** Impaired glucose tolerance is associated with increased serum concentrations of interleukin 6 and co-regulated acute-phase proteins but not TNF-alpha or its receptors. *Diabetologia* 45: 805–812, 2002.
41. **Napolitano A, Voice MW, Edwards CR, Seckl JR, Chapman KE.** 11beta-hydroxysteroid dehydrogenase 1 in adipocytes: expression is differentiation-dependent and hormonally regulated. *J Steroid Biochem Mol Biol* 64: 251–260, 1998.
42. **Ohara Y, Peterson TE, Harrison DG.** Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest* 91: 2546–2551, 1993.
43. **Pape ME, Kim KH.** Effect of tumor necrosis factor on acetyl-coenzyme A carboxylase gene expression and preadipocyte differentiation. *Mol Endocrinol* 2: 395–403, 1988.
44. **Paulmyer-Lacroix O, Boullu S, Oliver C, Alessi MC, Grino M.** Expression of the mRNA coding for 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue from obese patients: an in situ hybridization study. *J Clin Endocrinol Metab* 87: 2701–2705, 2002.
45. **Perreault M, Marette A.** Targeted disruption of inducible nitric oxide synthase protects against obesity-linked insulin resistance in muscle. *Nat Med* 7: 1138–1143, 2001.
46. **Petruschke T, Hauner H.** Tumor necrosis factor-alpha prevents the differentiation of human adipocyte precursor cells and causes delipidation of newly developed fat cells. *J Clin Endocrinol Metab* 76: 742–747, 1993.
47. **Pickup JC, Mattock MB, Chusney GD, Burt D.** NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. *Diabetologia* 40: 1286–1292, 1997.
48. **Poulain-Godefroy O, Froguel P.** Preadipocyte response and impairment of differentiation in an inflammatory environment. *Biochem Biophys Res Commun* 356: 662–667, 2007.
49. **Rhen T, Cidlowski JA.** Antiinflammatory action of glucocorticoids–new mechanisms for old drugs. *N Engl J Med* 353: 1711–1723, 2005.
50. **Roberge C, Carpentier AC, Langlois MF, Baillargeon JP, Ardilouze JL, Maheux P, Gallo-Payet N.** Adrenocortical dysregulation as a major player in insulin resistance and onset of obesity. *Am J Physiol Endocrinol Metab* 293: E1465–E1478, 2007.
51. **Sakaue H, Ogawa W, Matsumoto M, Kuroda S, Takata M, Sugimoto T, Spiegelman BM, Kasuga M.** Posttranscriptional control of adipocyte differentiation through activation of phosphoinositide 3-kinase. *J Biol Chem* 273: 28945–28952, 1998.
52. **Sandeep TC, Andrew R, Homer NZ, Andrews RC, Smith K, Walker BR.** Increased in vivo regeneration of cortisol in adipose tissue in human obesity and effects of the 11beta- hydroxysteroid dehydrogenase type 1 inhibitor carbenoxolone. *Diabetes* 54: 872–879, 2005.
53. **Schaffler A, Scholmerich J, Buchler C.** Mechanisms of disease: adipocytokines and visceral adipose tissue–emerging role in intestinal and mesenteric diseases. *Nat Clin Pract Gastroenterol Hepatol* 2: 103–111, 2005.
54. **Seckl JR, Walker BR.** Minireview: 11beta-hydroxysteroid dehydrogenase type 1- a tissue-specific amplifier of glucocorticoid action. *Endocrinology* 142: 1371–1376, 2001.
55. **Smoak KA, Cidlowski JA.** Mechanisms of glucocorticoid receptor signaling during inflammation. *Mech Ageing Dev* 125: 697–706, 2004.
56. **Straub RH, Hense HW, Andus T, Scholmerich J, Riegger GA, Schunkert H.** Hormone replacement therapy and interrelation between serum interleukin-6 and body mass index in postmenopausal women: a population-based study. *J Clin Endocrinol Metab* 85: 1340–1344, 2000.
57. **Tchkonia T, Giorgadze N, Pirtskhalava T, Thomou T, DePonte M, Koo A, Forse RA, Chinnappan D, Martin-Ruiz C, von Zglinicki T, Kirkland JL.** Fat depot-specific characteristics are retained in strains derived from single human preadipocytes. *Diabetes* 55: 2571–2578, 2006.
58. **Tilg H, Moschen AR.** Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nat Rev Immunol* 6: 772–783, 2006.
59. **Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM.** mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. *Genes Dev* 8: 1224–1234, 1994.
60. **Ulick S, Tedde R, Mantero F.** Pathogenesis of the type 2 variant of the syndrome of apparent mineralocorticoid excess. *J Clin Endocrinol Metab* 70: 200–206, 1990.
61. **Wake DJ, Rask E, Livingstone DE, Soderberg S, Olsson T, Walker BR.** Local and systemic impact of transcriptional up-regulation of 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue in human obesity. *J Clin Endocrinol Metab* 88: 3983–3988, 2003.
62. **Wamil M, Andrew R, Chapman KE, Street J, Morton NM, Seckl JR.** 7-oxysterols modulate glucocorticoid activity in adipocytes through competition for 11beta-hydroxysteroid dehydrogenase type. *Endocrinology* 149: 5909–5918, 2008.
63. **Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr.** Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112: 1796–1808, 2003.
64. **Xing H, Northrop JP, Grove JR, Kilpatrick KE, Su JL, Ringold GM.** TNF alpha-mediated inhibition and reversal of adipocyte differentiation is accompanied by suppressed expression of PPARg without effects on Pref-1 expression. *Endocrinology* 138: 2776–2783, 1997.
65. **Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H.** Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 112: 1821–1830, 2003.
66. **Yeager MP, Guyre PM, Munck AU.** Glucocorticoid regulation of the inflammatory response to injury. *Acta Anaesthesiol Scand* 48: 799–813, 2004.
67. **Zhang TY, Daynes RA.** Glucocorticoid conditioning of myeloid progenitors enhances TLR4 signaling via negative regulation of the phosphatidylinositol 3-kinase-Akt pathway. *J Immunol* 178: 2517–2526, 2007.

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Originally, blots in Figures 2, 3, 4, 5 and 6 were adjusted to show representative blots without demarcation. Revised Figures 2, 3, 4, 5, and 6 are now presented showing representative blots that are clearly separated. 11β-HSD1 activity analyses were performed by running the samples in triplicate under the same conditions as done previously. Independent experiments were performed to confirm the reproducibility of the results. These new figures appear online, linked directly to the article (http://ajpendo.physiology.org/cgi/content/full/ajpendo.00320.2009/DC2). The authors apologize for the previous errors, none of which have altered the conclusions reached in this study.

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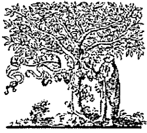
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Adipose tissue–specific dysregulation of angiotensinogen by oxidative stress in obesity

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Abstract

Adipose tissue expresses all components of the renin-angiotensin system including angiotensinogen (AGT). Recent studies have highlighted a potential role of AGT in adipose tissue function and homeostasis. However, some controversies surround the regulatory mechanisms of AGT in obese adipose tissue. In this context, we here demonstrated that the AGT messenger RNA (mRNA) level in human subcutaneous adipose tissue was significantly reduced in obese subjects as compared with nonobese subjects. Adipose tissue AGT mRNA level in obese mice was also lower as compared with their lean littermates; however, the hepatic AGT mRNA level remained unchanged. When 3T3-L1 adipocytes were cultured for a long period, the adipocytes became hypertrophic with a marked increase in the production of reactive oxygen species. Expression and secretion of AGT continued to decrease during the course of adipocyte hypertrophy. Treatment of the 3T3-L1 and primary adipocytes with reactive oxygen species (hydrogen peroxide) or tumor necrosis factor α caused a significant decrease in the expression and secretion of AGT. On the other hand, treatment with the antioxidant *N*-acetyl cysteine suppressed the decrease in the expression and secretion of AGT in the hypertrophied 3T3-L1 adipocytes. Finally, treatment of obese *db/db* mice with *N*-acetyl cysteine augmented the expression of AGT in the adipose tissue, but not in the liver. The present study demonstrates for the first time that oxidative stress dysregulates AGT in obese adipose tissue, providing a novel insight into the adipose tissue–specific interaction between the regulation of AGT and oxidative stress in the pathophysiology of obesity.
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1. Introduction

Overactivity of the systemic renin-angiotensin system (RAS) is one of the central mechanisms for obesity-related metabolic disorders [1,2]. Notably, the major components of the RAS are expressed in various tissues including the heart, blood vessels, adipose tissue, and brain [3]; these comprise tissue RAS. A series of products are produced locally from

The authors of this manuscript have nothing to declare.

Institutional approval: The human study was approved by the ethics committee for human research of the Kyoto University Graduate School of Medicine (2004, no. 553). Written informed consent was obtained from all subjects prior to the study. All animal experimental procedures were approved by the Kyoto University Graduate School of Medicine Animal Research Committee and the Seoul National University Animal Experiment Ethics Committee.

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angiotensinogen (AGT), the unique precursor of angiotensin peptides, and play a critical role in cardiovascular homeostasis [3,4].

Although AGT is produced mainly by the liver, adipose tissue is also considered as a source of AGT production [5]. In agreement with this notion, the adipose tissue expresses all components of the RAS, including AGT, renin, angiotensin I–converting enzyme, and angiotensin II type 1 receptor, in humans and rodents [6,7]. A previous study has demonstrated that AGT-deficient mice are low in blood pressure and body fat mass [8]. Moreover, adipocyte-specific transgenic overexpression of AGT on an AGT-deficient background was shown to augment plasma AGT level and rescue hypotension and leanness [9]. These results indicate that adipose tissue–derived AGT does contribute to the circulating AGT level and adipogenesis.

In rodent experiments, the AGT messenger RNA (mRNA) level in white adipose tissue has been shown to be regulated by the nutritional status; however, that in the liver was independent of the nutritional status [10,11]. In human cross-sectional studies, the AGT mRNA level in adipose tissue was shown to be higher in obese subjects [6,12]. On the other hand, another study reported that the AGT mRNA level in adipose tissue was significantly lower in obese individuals [13]. Elevation of AGT expression in adipose tissue in obese individuals thus remains controversial [14].

Several studies have shown that increased oxidative stress is a manifestation of obesity-related metabolic derangement [15-17]. In fact, in humans, oxidative stress is critically associated with atherosclerosis, hypertension, and diabetes mellitus [18,19]. Oxidative stress is also related with the RAS. Angiotensin II is a potent inducer of reactive oxygen species (ROS) in a variety of tissues [20-22]. In the liver and kidney, increased ROS has been reported to increase AGT gene expression [23-26]. Also in obese adipose tissue, generation of ROS is exaggerated and is involved in adipose tissue dysfunction [17,27]. However, whether increased ROS may affect adipose AGT production remains to be elucidated.

In the present study, we demonstrated that the AGT mRNA level was reduced in obese adipose tissue in humans and mice and in hypertrophied 3T3-L1 adipocytes. In this context, we tested the hypothesis that increased oxidative stress would modulate AGT in obese adipose tissue.

2. Materials and methods

2.1. Subcutaneous abdominal adipose tissue biopsies in human subjects

The present study was performed according to the Declaration of Helsinki and approved by the Ethical Committee on Human Research of Kyoto University Graduate School of Medicine (2004, no. 553). Written informed consent was obtained from all subjects before the study.

Subcutaneous abdominal adipose tissue biopsies were obtained from 46 Japanese subjects (24 men and 22 women; age [mean ± SD], 46 ± 2.1 years). The body mass index (BMI) of the subjects ranged from 19 to 52 (mean ± SD, 30 ± 1.6) kg/m^2. All subjects had been on stable therapy with lipid-lowering, antihypertensive, or hypoglycemic agents for at least 1 month before admission and continued with the same doses throughout the study period. Patients who received angiotensin I–converting enzyme inhibitors, angiotensin II receptor blockers, and steroid-related drugs were carefully excluded. For the study, subcutaneous abdominal adipose depots of the study subjects were excised from the periumbilical region under local anesthesia. The samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

2.2. Mouse experiments

Male *ob/ob* mice (age, 12 weeks) were purchased from Oriental BioService (Kyoto, Japan) and housed in the animal facility of Kyoto University. Male *db/db* mice (age, 10 weeks) were purchased from Japan SLC (Hamamatsu, Japan) and housed in Seoul National University. The mice were allowed free access to food and water. For in vivo antioxidant treatment, the *db/db* mice were injected with *N*-acetyl cysteine (NAC; 150 mg/kg body weight; Sigma-Aldrich Japan, Tokyo, Japan) or the vehicle (phosphate-buffered saline) into the peritoneal cavity once daily for 1 week. All experimental procedures were approved by the Kyoto University Graduate School of Medicine Animal Research Committee and the Seoul National University Animal Experiment Ethics Committee.

2.3. Cell culture and isolation of primary adipocytes

3T3-L1 fibroblasts were cultured and differentiated into adipocytes as described previously [28]. Briefly, the 2-day postconfluent cells (designated as day 0) were incubated for 2 days with 10% fetal bovine serum (FBS)/Dulbecco modified Eagle medium (DMEM), 0.5 mmol/L 3-isobutyl-1-methylxanthine, 0.25 μmol/L dexamethasone, and 1 μg/mL insulin. The cells were then incubated for 2 days in 10% FBS/DMEM with insulin and, thereafter, incubated in 10% FBS/DMEM that was changed on every alternate day. Oil red O staining was performed as described [29].

Primary adipocytes were isolated from epididymal fat pads of 9-week-old male C57BL/6J mice (purchased from Oriental BioService, Kyoto, Japan). Epididymal fat pads were harvested, minced into 2- to 3-mm pieces, and digested using 0.8 mg/mL collagenase (Sigma-Aldrich Japan) in DMEM for 30 minutes at 37°C in a shaking water bath. After the digestion with collagenase, cells were filtered through a 250-μm nylon filter and centrifuged at 1000 rpm for 30 seconds. The suspended mature adipocytes were separated from the pelleted stromovascular fraction and washed 3 times in DMEM for experiments.

2.4. Determination of adipocyte size

The cells were fixed with 2% osmium tetroxide and passed through a 250-μm nylon filter to remove the fibrous elements, and the cells were washed extensively with isotonic saline. A total of 10 000 cells was analyzed using the Coulter Multisizer III (Beckman Coulter, High Wycombe, England) [30].

2.5. Quantitative real-time polymerase chain reaction

Total RNA was extracted from human and mouse adipose tissue by using a QIAGEN RNeasy Mini Kit (QIAGEN Japan, Tokyo, Japan) and from cultured adipocytes by using Trizol Reagent (Invitrogen, Carlsbad, CA). Complementary DNA was then synthesized by using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Taqman polymerase chain reactions (PCRs) for human AGT, mouse AGT, mouse monocyte chemoattractant protein 1 (MCP-1), mouse interleukin 6 (IL-6), and mouse tumor necrosis factor α (TNFα) were performed using the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA). The sequences of probes and primers are summarized in Table 1.

2.6. Enzyme-linked immunosorbent assay

The AGT protein level in the culture media was measured by sandwich-type enzyme-linked immunosorbent assay (ELISA) as described [31]. Similarly, the MCP-1 and IL-6 protein levels were detected by using an ELISA kit (R&D Systems, Minneapolis, MN).

2.7. Determination of ROS

The ROS activity was determined by the nitroblue tetrazolium (NBT) assay [32]. Reduced NBT (formazan) was dissolved in 50% acetic acid, and the absorbance of the supernatant was determined at 560 nm.

2.8. Statistical analysis

The data are presented as the mean ± SE. Unpaired Student *t* test was used for comparisons with the control group. The differences were accepted as significant at a level of $P < .05$.

3. Results

3.1. AGT mRNA expression level in adipose tissue from obese humans and mice

To explore the impact of obesity on AGT gene expression in human adipose tissue, we performed subcutaneous abdominal adipose tissue biopsies from 46 subjects with a wide range of BMI. The AGT mRNA level was significantly reduced by 61% in the obese subjects as compared with the nonobese subjects (Fig. 1A).

To verify the obesity-related decrease in adipose AGT expression, we analyzed adipose tissue from genetically obese mice. In 12-week-old male *ob/ob* mice (mean body weight, 60 ± 0.7 g), the AGT mRNA level was significantly decreased in both epididymal (29%) and subcutaneous (57%) adipose depots as compared with their lean littermates (mean body weight, 29 ± 0.3 g) (Fig. 1B). On the other hand, the AGT mRNA levels in the liver remained unaltered in both groups (Fig. 1C).

Similar results were observed in case of the diet-induced obese (DIO) mice (12-week-old male C57BL/6J mice fed with a high-fat/high-sucrose diet for 4 weeks). The AGT mRNA level in the adipose tissue of the DIO mice (mean body weight, 40 ± 0.8 g) was significantly lower than that in the adipose tissue of their lean littermates (mean body weight, 30 ± 0.4 g) ($P < .05$); however, the hepatic AGT mRNA level remained unchanged in both groups (Yasue et al, unpublished observations). These results indicate that the AGT mRNA level was decreased exclusively in the obese adipose tissue in both humans and mice.

3.2. AGT expression during the course of hypertrophy in the 3T3-L1 adipocytes

To explore the mechanism by which AGT is decreased in obese adipose tissue, we analyzed hypertrophied adipocytes. 3T3-L1 fibroblasts were completely differentiated into

Table 1
Sequences of Taqman PCR primers and probes

Gene name Genbank accession no.	Forward primer Reverse primer	Probe (5′-FAM, 3′-TAMRA)
Human AGT	GGTGGAGGGTCTCACTTTCCA	CCCTCAACTGGATGAAGAAACTGTCTCC
NM_000029	ATGGTCAGGTGGATGGTCCG	
Mouse Agt	ACACCTACGTTCACTTCCAAG	ATGAGAGGTTTCTCTCAGCTGCCTGGA
NM_007428	CCGAGATGCTGTTGTCCAC	
Mouse Ccl2 (MCP-1)	TTGGCTCAGCCAGATGC	CCCCACTCACCTGCTGCTACTCATTCA
NM_011333	CCAGCCTACTCATTGGGATCA	
Mouse Il6 (IL-6)	ATGAAGTTCCTCTCTGCAAGAG	CACCAGCATCAGTCCCAAGAAGGCA
NM_031168	GTAGGGAAGGCCGTGGTTG	
Mouse Tnf (TNFα)	TCTCTTCAAGGGACAAGGCTG	CCCGACTACGTGCTCCTCACCCA
NM_013693	ATAGCAAATCGGCTGACGGT	

The sequences of primers and probes for each gene used in the present study are summarized.

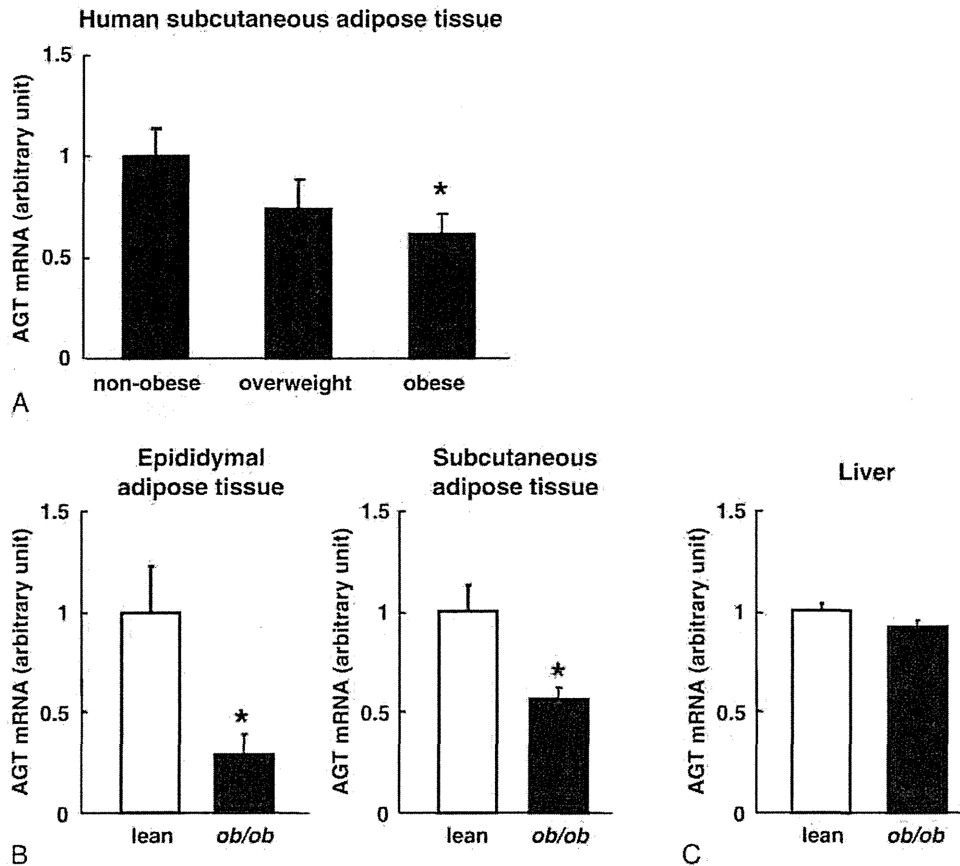

Fig. 1. The AGT mRNA levels in obese adipose tissue from humans and mice. A, The relation between the AGT mRNA level in subcutaneous abdominal adipose tissue and the degree of obesity in humans: nonobese (BMI <25), n = 20; overweight (25 ≤ BMI < 30), n = 13; obese (BMI ≥30), n = 13. B, Comparison of the adipose tissue AGT mRNA levels in 12-week-old male *ob/ob* mice (n = 4; mean body weight, 60 ± 0.7 g) and their lean littermates (n = 4; mean body weight, 29 ± 0.3 g). Left: epididymal adipose tissue depots. Right: subcutaneous abdominal adipose tissue depots. C, Comparison of the hepatic AGT mRNA level between the *ob/ob* mice (n = 4) and their lean littermates (n = 4). The mRNA level was examined by real-time PCR and normalized to that of 18S ribosomal RNA (rRNA). The data are expressed as the mean ± SE. *$P < .05$ as compared with the nonobese subjects or the lean littermates.

adipocytes for 8-day incubation with induction media [28]. Consistent with a previous report [33], the AGT mRNA level in differentiated 3T3-L1 adipocytes (day 8) was significantly elevated by 15-folds in comparison with 3T3-L1 fibroblasts (day 0). For generating hypertrophied adipocytes, 3T3-L1 adipocytes were cultured up to 30 days after the induction of differentiation. Oil red O staining exhibited a gradual increase in lipid accumulation from day 8 to day 28. The adipocytes displayed unilocular lipid droplets on days 18 and 28 (Fig. 2A).

The mean diameter of the adipocytes as assessed by the Coulter Multisizer III was 20.2 μm on day 8 and 37.5 μm on day 30 (Fig. 2B). During the course of adipocyte hypertrophy, ROS production increased 2.7-folds (day 18) and 4.3-folds (day 28) in comparison with the levels on day 8 (Fig. 2C). The mRNA and protein levels of MCP-1 and IL-6 were elevated substantially on days 18 and 28 (Fig. 2D and E, respectively). The AGT mRNA level was significantly lower on days 18 (48%) and 28 (42%) than that on day 8 (Fig. 2D). The AGT concentration in the culture media was decreased on days 18 (59% of the initial value) and 28 (42% of the initial value) (Fig. 2E). These results suggest that AGT expression and secretion were decreased in the hypertrophied adipocytes.

3.3. Impact of TNFα on the expression and secretion of AGT in adipocytes

Tumor necrosis factor α plays a critical role in the pathophysiology of inflammation and oxidative stress [27,34,35]. To explore the impact of TNFα on the expression and secretion of AGT in adipocytes, the differentiated 3T3-L1 adipocytes (day 8) were treated with TNFα (Sigma-Aldrich Japan) for 24 hours. Treatment with TNFα decreased the AGT mRNA level in a dose-dependent manner along with a concomitant increase in the MCP-1 and IL-6 mRNA levels (Fig. 3A). The AGT protein level in the culture media decreased in parallel to the AGT mRNA level (Fig. 3B).

We also investigated the effects of TNFα on primary adipocytes. Similarly to 3T3-L1 adipocytes, treatment with TNFα (10 ng/mL) for 24 hours slightly but significantly decreased AGT mRNA level and substantially increased MCP-1 mRNA level (Fig. 3C).

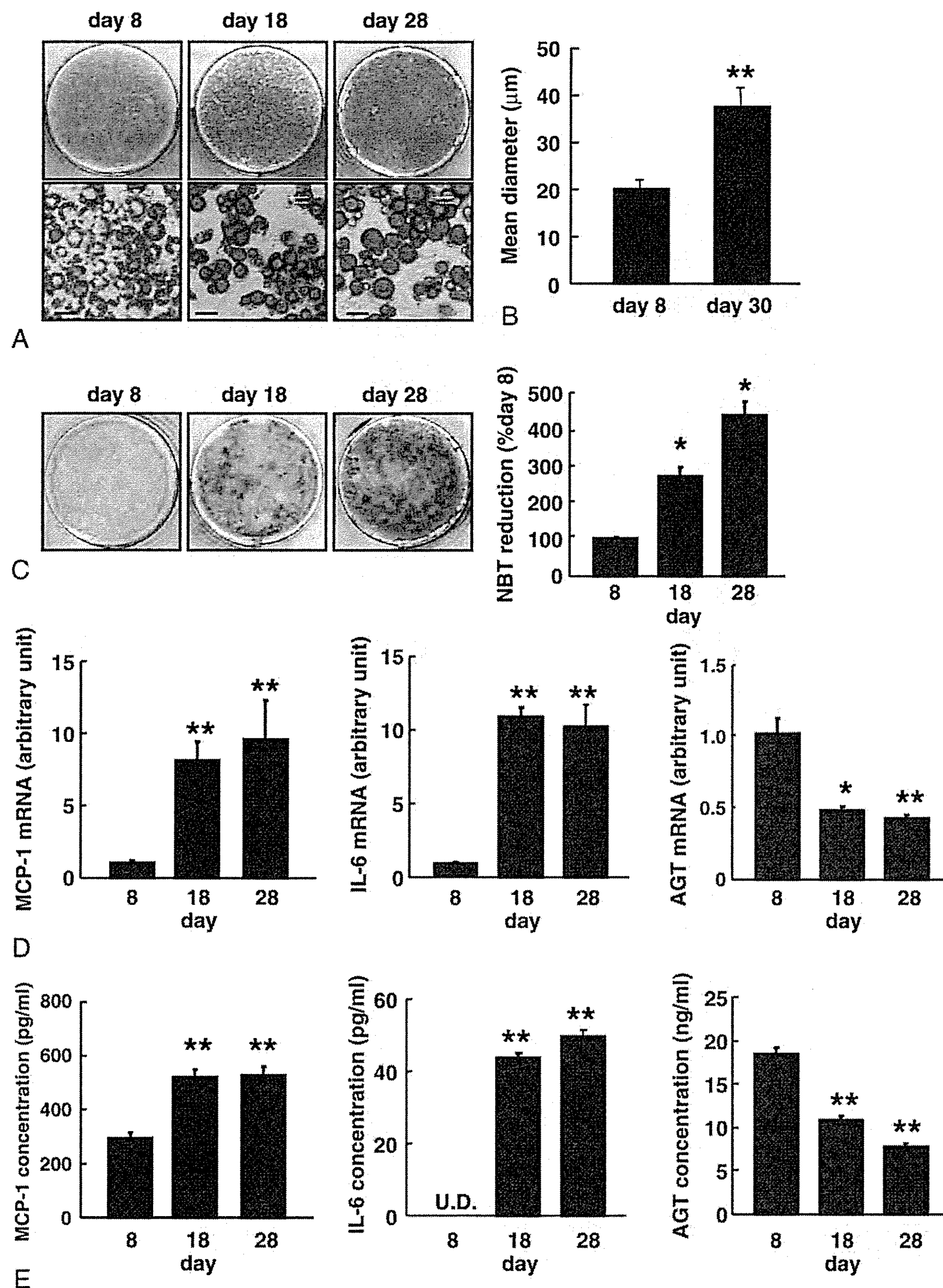

Fig. 2. The AGT expression during the course of hypertrophy in the 3T3-L1 adipocytes. A, Oil red O staining of the 3T3-L1 adipocytes on days 8, 18, and 28 after induction of differentiation. Bar = 30 μm. B, Size of the 3T3-L1 adipocytes on days 8 and 30. Adipocyte size was measured using a Coulter Multisizer III. C, The ROS production during adipocyte hypertrophy. The ROS production was assessed by the NBT assay. Dark blue formazan was dissolved, and the absorbance was determined at 560 nm (n = 3). D, The MCP-1, IL-6, and AGT mRNA levels in the 3T3-L1 adipocytes on days 8, 18, and 28 (n = 4). The mRNA level was examined by real-time PCR and normalized to that of 18S rRNA. E, The AGT protein concentration in the culture media. The MCP-1, IL-6, and AGT concentrations in the 3T3-L1 adipocytes on days 8, 18, and 28 were analyzed by ELISA (n = 4). Results are representatives of at least 3 independent experiments. The data are expressed as the mean ± SE. *$P < .05$ and **$P < .01$ as compared with the value of day 8. U.D. indicates undetectable.

3.4. *Impact of oxidative stress on the expression and secretion of AGT in adipocytes*

To explore the impact of oxidative stress on the expression and secretion of AGT in adipocytes, differentiated 3T3-L1 adipocytes (day 8) were exposed to a specific ROS molecule, hydrogen peroxide (H_2O_2), for 24 hours [17,36]. Incubation of adipocytes with H_2O_2 significantly increased the MCP-1 mRNA level, consistent with a previous report [17]. In contrast, H_2O_2 diminished the AGT mRNA level up to 35% of the initial value in a dose-dependent manner (Fig. 4A). The AGT protein level in the

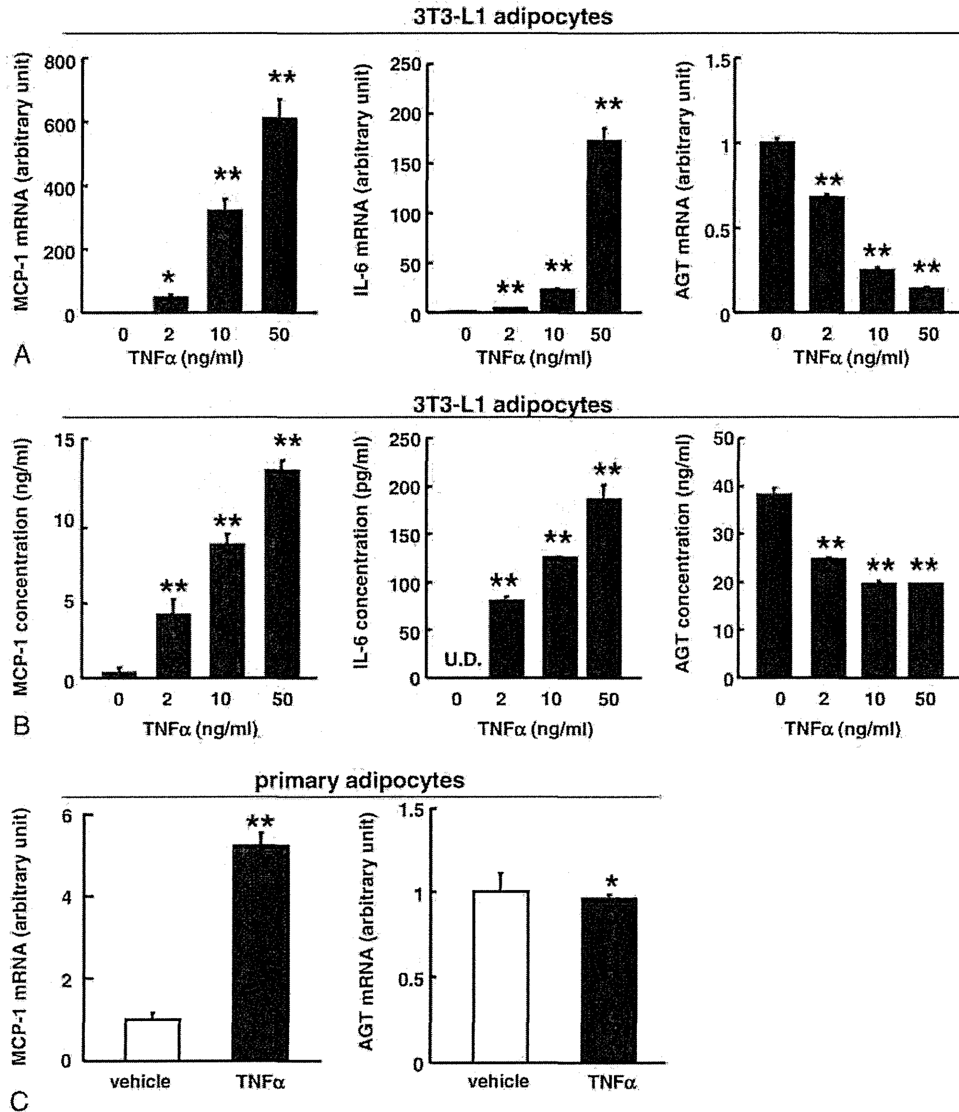

Fig. 3. Impact of TNFα on the expression and secretion of AGT in the 3T3-L1 adipocytes. A, The AGT, MCP-1, and IL-6 mRNA level in the 3T3-L1 adipocytes (day 8) treated with TNFα for 24 hours (n = 4). The mRNA level was examined by real-time PCR and normalized to that of 18S rRNA. B, The AGT protein concentration in the culture media in the 3T3-L1 adipocytes (day 8) treated with TNFα for 24 hours (n = 4). The protein level was assessed by ELISA. C, The AGT and MCP-1 mRNA level in the primary adipocytes treated with TNFα (10 ng/mL) for 24 hours (n = 4). The mRNA level was examined by real-time PCR and normalized to that of 18S rRNA. Results are representatives of at least 3 independent experiments. The data are expressed as the mean ± SE. *$P < .05$ and **$P < .01$ as compared with the control value.

culture media also decreased up to 23% of the initial value (Fig. 4B).

Similar to 3T3-L1 adipocytes, H_2O_2 treatment (1 mmol/L, 24 hours) significantly decreased AGT mRNA level in primary adipocytes (Fig. 4C). The H_2O_2 treatment tended to increase the MCP-1 mRNA level.

3.5. Effect of antioxidant treatment on the expression and secretion of AGT in adipocytes

We examined whether inhibition of ROS generation could nullify the decrease in AGT gene expression and AGT secretion in obese adipose tissue. First, we treated 3T3-L1 adipocytes with the antioxidant NAC (10 mmol/L) for 10 days (days 8-18). Without the NAC treatment, the adipocytes had become hypertrophic and increased ROS production in this period (Fig. 2B and C). The NBT assay revealed that NAC treatment significantly suppressed ROS production (Fig. 5A). Although ROS production was reported to potentiate adipocyte differentiation in early phase [37], the ROS suppression with the NAC treatment in our experiments did not cause morphologic changes in hypertrophied adipocytes compared with the vehicle treatment. The NAC treatment inhibited the increase in MCP-1 expression (Fig. 5B). The AGT mRNA level was significantly elevated with NAC treatment (Fig. 5B).

To test whether such phenomenon is reproducible in obese adipose tissue where ROS production is exaggerated

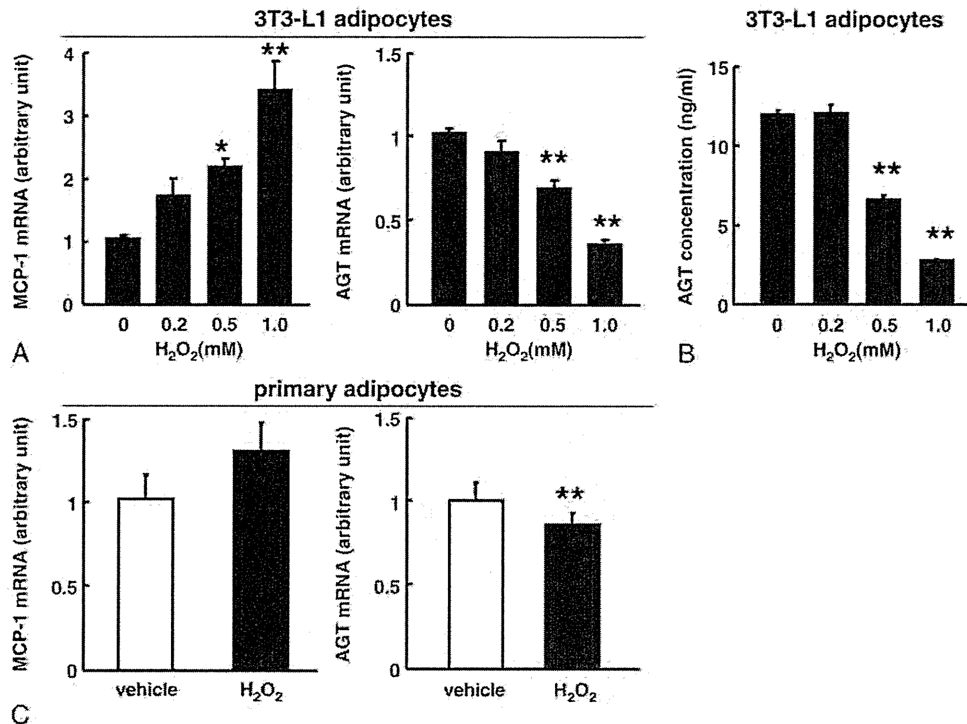

Fig. 4. Impact of oxidative stress on the expression and secretion of AGT in the 3T3-L1 adipocytes. A, The AGT and MCP-1 mRNA level in the 3T3-L1 adipocytes (day 8) treated with H_2O_2 for 24 hours (n = 4). The mRNA level was examined by real-time PCR and normalized to that of 18S rRNA. B, The AGT protein level in the culture media of the 3T3-L1 adipocytes (day 8) treated with H_2O_2 for 24 hours (n = 4). The protein concentration was assessed by ELISA. C, The AGT and MCP-1 mRNA level in the primary adipocytes treated with H_2O_2 (1 mmol/L) for 24 hours (n = 4). The mRNA level was examined by real-time PCR and normalized to that of 18S rRNA. Results are representatives of at least 3 independent experiments. The data are expressed as the mean ± SE. $*P < .05$ and $**P < .01$ as compared with the control value.

[17], we administered NAC to obese *db/db* mice once daily for 1 week. Similar to the obese *ob/ob* mice and DIO mice, in obese *db/db* mice (mean body weight, 48 ± 1.5 g), the AGT mRNA level in epididymal adipose tissue was markedly decreased to 22% as compared with their lean littermates (mean body weight, 28 ± 1.0 g) (Fig. 5C). In contrast, the TNFα mRNA level in epididymal adipose tissue was significantly higher in *db/db* mice than in their lean littermates (Fig. 5C). Both systemic and local (adipose tissue) oxidative stress was elevated substantially in obese *db/db* mice [38]. Notably, in *db/db* mice, NAC treatment significantly reduced the oxidative stress also in adipose tissue [38].

In the NAC treatment group, the AGT mRNA level in the epididymal adipose depots increased significantly by 2.1-folds compared with that in the vehicle group, whereas the IL-6 ($P = .052$) and TNFα ($P = .10$) mRNA levels tended to decrease in the NAC treatment group (Fig. 5D). On the other hand, the hepatic AGT mRNA level remained unchanged in both groups (Fig. 5E).

4. Discussion

The major finding of the present study is that oxidative stress dysregulates AGT in adipose tissue in obese humans and rodents. The AGT mRNA level was decreased in both obese adipose tissue and hypertrophied adipocytes, in which oxidative stress was exaggerated. Exposure of oxidative stress decreased AGT expression not only in the adipocyte cell line but also in primary adipocytes. The decrease in AGT expression was rescued by treatment with the antioxidant both in vivo and in vitro. Such obesity-associated changes in AGT in the adipose tissue were not observed in the liver.

The AGT regulation in obese adipose tissue has long been analyzed, but results were inconsistent [6,12,13]. We here demonstrated that the AGT mRNA level in adipose tissue was reduced in both obese humans and mice (Fig. 1). In obese mice, there seem to be no apparent depot-specific (subcutaneous and epididymal adipose depots) or strain-specific (*ob/ob*, *db/db*, and DIO mice) differences in the fall of AGT in adipose tissue. The AGT mRNA level was decreased also in hypertrophied 3T3-L1 adipocytes (Fig. 2). These results are consistent with a previous report using differentiation system of human adipocytes in primary culture, where the AGT mRNA level increased in differentiation process, but decreased in further culture process [39].

In previous experiments, several hormonal and metabolic changes associated with obesity influence AGT expression in adipocytes; however, due to species differences and experimental conditions, there are controversies around the results [39-42]. On the other hand, our results indicate that

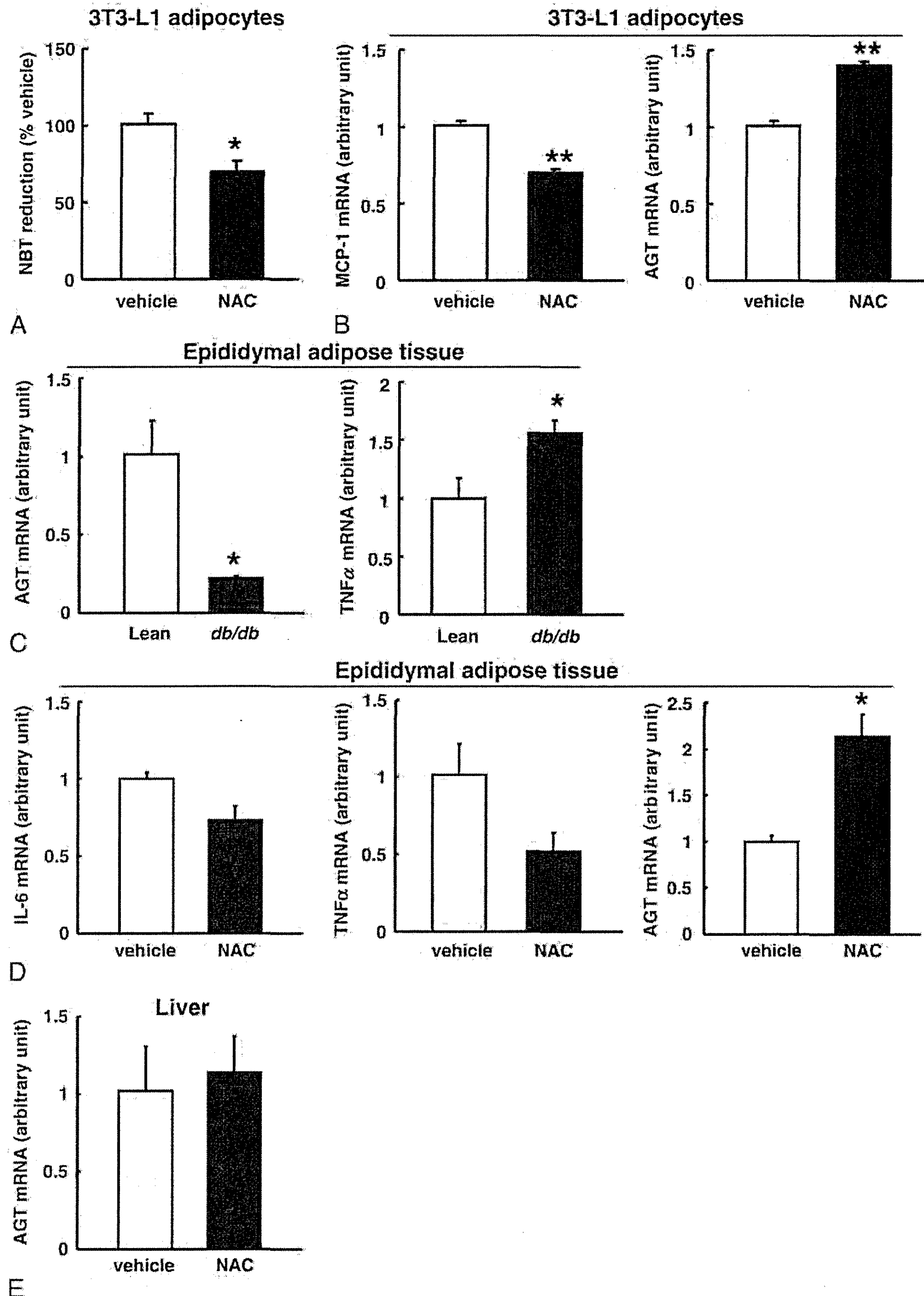

Fig. 5. Effect of antioxidant treatment on the expression and secretion of AGT in the adipocytes. A, Suppression of ROS generation in the 3T3-L1 adipocytes treated with NAC (10 mmol/L) for 10 days (n = 3). The ROS was estimated by the NBT assay. B, The MCP-1 and AGT mRNA levels in the 3T3-L1 adipocytes incubated with NAC (10 mmol/L) (n = 8). The mRNA level was examined by real-time PCR and normalized to that of 18S rRNA. Results are representatives of at least 3 independent experiments. C, Comparison of the AGT and TNFα mRNA levels between 10-week-old male *db/db* mice (n = 4; mean body weight, 48 ± 1.5 g) and their lean littermates (n = 4; mean body weight, 28 ± 1.0 g) in epididymal adipose tissue. D, The level of IL-6, TNFα, and AGT mRNA in the epididymal adipose tissue depots of obese *db/db* mice treated with NAC (150 mg/kg body weight) or vehicle (phosphate-buffered saline) once daily for 1 week (n = 3). E, The AGT mRNA level in the liver of obese *db/db* mice treated with NAC or vehicle for 1 week (n = 3). The mRNA level was examined by real-time PCR and normalized to that of cyclophilin mRNA. The data are expressed as the mean ± SE. $*P < .05$ and $**P < .01$ as compared with the control value.

AGT expression is decreased in obese adipose tissue. Reactive oxygen species (H_2O_2) decreased AGT expression in both 3T3-L1 adipocytes and primary adipocytes (Fig. 4). On the other hand, elimination of ROS with antioxidant increased AGT expression not only in hypertrophied 3T3-L1 adipocytes but also in adipose tissue from obese mice (Fig. 5). The oxidative stress–mediated decrease in adipose AGT is reproduced in our various experiments.

Several studies have suggested the augmentation of AGT by oxidative stress in the liver and kidney. In the liver, angiotensin II is known to enhance AGT expression via ROS generation [23], resulting in a positive feedback loop of AGT production [43]. In addition, oxidative stress mediated by hyperglycemia and hypertension has been shown to augment the expression of AGT in the rodent kidney [25,26]. In turn, elevated expression of AGT has been shown to activate renal RAS and considerably contribute to renal injury [26]. On the other hand, our data support a notion that oxidative stress "decreases" expression and secretion of AGT in obese adipose tissue, implying that regulation of AGT in adipose tissue may be distinct from other tissues in response to oxidative stress.

The clinical or pathophysiologic implications of decreased AGT in obese adipose tissue still remain unclear. Although further studies are warranted, the notion that adipose tissue RAS is involved in the control of adipogenesis and adipose tissue mass [44] tempts us to speculate that tissue-specific decrease of AGT in obese adipose tissue may serve as a defense against further exacerbation of adiposity. In obese adipose tissue, exaggerated oxidative stress affects the expression of a variety of genes [17]. Representatively, ROS induces the proinflammatory TNFα but suppresses the anti-inflammatory adiponectin in murine adipose tissue [17]. Glutathione peroxidase 3 (GPx3), an antioxidant enzyme secreted from the adipose tissue and kidney, is known to be decreased by oxidative stress exclusively in adipose tissue in obese *db/db* mice [38]. Notably, in vivo administration of an antioxidant was shown to rescue the decrease in GPx3 expression only in adipose tissue, but not in the kidney [38]. In this context, AGT shares close similarity with adiponectin and GPx3 in terms of the response to oxidative stress in adipose tissue.

Tissue-specific dysregulation of AGT has also been observed in inflammatory response [45,46]. Hepatic AGT is shown to increase by inflammatory stimuli via the acute-phase responsive element (APRE) on the promoter region of the AGT gene [43,47]. In rats treated with lipopolysaccharide, AGT mRNA level was shown to increase in the liver, aorta, and adrenal gland, but remained unchanged in the kidney [45]. Furthermore, in transgenic mice with cardioselective overexpression of TNFα, expression of AGT was decreased exclusively in the heart [46]. In the present study, we demonstrated that TNFα decreased the expression and secretion of AGT in 3T3-L1 adipocytes (Fig. 3). Considering that chronic, low-grade inflammation is a manifestation of obese adipose tissue [48,49], our results suggest that AGT is inversely regulated by inflammation in obese adipose tissue.

Previous works have raised a possibility that the inflammatory responses to AGT in adipose tissue and liver are controlled by distinct mechanisms [50]. In cultured adipocytes, inflammatory signals transcriptionally decrease AGT by the inhibition of APRE [50]; however, in cultured hepatocytes, nuclear factor–κB signaling augments AGT by the activation of APRE [47]. Importantly, the intracellular signaling involved in oxidative stress and inflammation interact and share, at least in part, common pathways in a tissue-specific manner [18,19]. In this context, tissue-specific dysregulation of AGT by oxidative stress is reminiscent of the case in inflammatory signals; and a possible link between the dysregulation of AGT and oxidative stress in obese adipose tissue may provide a fresh clue to dissect the pathophysiology of obesity. For example, we would suggest the one possibility that oxidative stress–induced suppression of adipose tissue RAS via the decrease in AGT may control adipose tissue function including adipocyte differentiation, lipolysis, and local blood flow [44].

In summary, the present study demonstrates for the first time that oxidative stress dysregulates AGT in obese adipose tissue in humans and rodents as well as in cultured adipocytes with hypertrophy. Our results support a concept that oxidative stress–dependent decrease in AGT may be a unique facet of dysfunction in obese adipose tissue.

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References

[1] Engeli S. Role of the renin-angiotensin-aldosterone system in the metabolic syndrome. Contrib Nephrol 2006;151:122-34.

[2] Rahmouni K, Correia ML, Haynes WG, Mark AL. Obesity-associated hypertension: new insights into mechanisms. Hypertension 2005;45:9-14.

[3] Dzau VJ. Circulating versus local renin-angiotensin system in cardiovascular homeostasis. Circulation 1988;77(6 Pt 2):I4-I13.

[4] Raizada V, Skipper B, Luo W, Griffith J. Intracardiac and intrarenal renin-angiotensin systems: mechanisms of cardiovascular and renal effects. J Investig Med 2007;55:341-59.

[5] Cassis LA, Saye J, Peach MJ. Location and regulation of rat angiotensinogen messenger RNA. Hypertension 1988;11(6 Pt 2): 591-6.
[6] Giacchetti G, Faloia E, Mariniello B, Sardu C, Gatti C, Camilloni MA, et al. Overexpression of the renin-angiotensin system in human visceral adipose tissue in normal and overweight subjects. Am J Hypertens 2002;15:381-8.
[7] Engeli S, Negrel R, Sharma AM. Physiology and pathophysiology of the adipose tissue renin-angiotensin system. Hypertension 2000;35: 1270-7.
[8] Massiera F, Seydoux J, Geloen A, Quignard-Boulange A, Turban S, Saint-Marc P, et al. Angiotensinogen-deficient mice exhibit impairment of diet-induced weight gain with alteration in adipose tissue development and increased locomotor activity. Endocrinology 2001; 142:5220-5.
[9] Massiera F, Bloch-Faure M, Ceiler D, Murakami K, Fukamizu A, Gasc JM, et al. Adipose angiotensinogen is involved in adipose tissue growth and blood pressure regulation. FASEB J 2001;15:2727-9.
[10] Frederich Jr RC, Kahn BB, Peach MJ, Flier JS. Tissue-specific nutritional regulation of angiotensinogen in adipose tissue. Hypertension 1992;19:339-44.
[11] Boustany CM, Bharadwaj K, Daugherty A, Brown DR, Randall DC, Cassis LA. Activation of the systemic and adipose renin-angiotensin system in rats with diet-induced obesity and hypertension. Am J Physiol Regul Integr Comp Physiol 2004;287:R943-9.
[12] Van Harmelen V, Ariapart P, Hoffstedt J, Lundkvist I, Bringman S, Arner P. Increased adipose angiotensinogen gene expression in human obesity. Obes Res 2000;8:337-41.
[13] Engeli S, Bohnke J, Gorzelniak K, Janke J, Schling P, Bader M, et al. Weight loss and the renin-angiotensin-aldosterone system. Hypertension 2005;45:356-62.
[14] Engeli S, Schling P, Gorzelniak K, Boschmann M, Janke J, Ailhaud G, et al. The adipose-tissue renin-angiotensin-aldosterone system: role in the metabolic syndrome? Int J Biochem Cell Biol 2003;35:807-25.
[15] Keaney Jr JF, Larson MG, Vasan RS, Wilson PW, Lipinska I, Corey D, et al. Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham Study. Arterioscler Thromb Vasc Biol 2003;23:434-9.
[16] Urakawa H, Katsuki A, Sumida Y, Gabazza EC, Murashima S, Morioka K, et al. Oxidative stress is associated with adiposity and insulin resistance in men. J Clin Endocrinol Metab 2003;88:4673-6.
[17] Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest 2004;114:1752-61.
[18] Stocker R, Keaney Jr JF. Role of oxidative modifications in atherosclerosis. Physiol Rev 2004;84:1381-478.
[19] Grattagliano I, Palmieri VO, Portincasa P, Moschetta A, Palasciano G. Oxidative stress–induced risk factors associated with the metabolic syndrome: a unifying hypothesis. J Nutr Biochem 2008;19:491-504.
[20] Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ Res 1994;74:1141-8.
[21] Das DK, Maulik N, Engelman RM. Redox regulation of angiotensin II signaling in the heart. J Cell Mol Med 2004;8:144-52.
[22] Sachse A, Wolf G. Angiotensin II–induced reactive oxygen species and the kidney. J Am Soc Nephrol 2007;18:2439-46.
[23] Brasier AR, Jamaluddin M, Han Y, Patterson C, Runge MS. Angiotensin II induces gene transcription through cell-type–dependent effects on the nuclear factor–kappaB (NF-kappaB) transcription factor. Mol Cell Biochem 2000;212:155-69.
[24] Hsieh TJ, Zhang SL, Filep JG, Tang SS, Ingelfinger JR, Chan JS. High glucose stimulates angiotensinogen gene expression via reactive oxygen species generation in rat kidney proximal tubular cells. Endocrinology 2002;143:2975-85.
[25] Brezniceanu ML, Liu F, Wei CC, Tran S, Sachetelli S, Zhang SL, et al. Catalase overexpression attenuates angiotensinogen expression and apoptosis in diabetic mice. Kidney Int 2007;71:912-23.
[26] Miyata K, Ohashi N, Suzaki Y, Katsurada A, Kobori H. Sequential activation of the reactive oxygen species/angiotensinogen/renin-angiotensin system axis in renal injury of type 2 diabetic rats. Clin Exp Pharmacol Physiol 2008;35:922-7.
[27] Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 2006;440:944-8.
[28] Frost SC, Lane MD. Evidence for the involvement of vicinal sulfhydryl groups in insulin-activated hexose transport by 3T3-L1 adipocytes. J Biol Chem 1985;260:2646-52.
[29] Fujimoto M, Masuzaki H, Tanaka T, Yasue S, Tomita T, Okazawa K, et al. An angiotensin II AT1 receptor antagonist, telmisartan augments glucose uptake and GLUT4 protein expression in 3T3-L1 adipocytes. FEBS Lett 2004;576:492-7.
[30] Sakai T, Sakaue H, Nakamura T, Okada M, Matsuki Y, Watanabe E, et al. Skp2 controls adipocyte proliferation during the development of obesity. J Biol Chem 2007;282:2038-46.
[31] Kobori H, Katsurada A, Miyata K, Ohashi N, Satou R, Saito T, et al. Determination of plasma and urinary angiotensinogen levels in rodents by newly developed ELISA. Am J Physiol Renal Physiol 2008;294: F1257-63.
[32] Oliveira HR, Verlengia R, Carvalho CR, Britto LR, Curi R, Carpinelli AR. Pancreatic beta-cells express phagocyte-like NAD(P)H oxidase. Diabetes 2003;52:1457-63.
[33] Saye JA, Cassis LA, Sturgill TW, Lynch KR, Peach MJ. Angiotensinogen gene expression in 3T3-L1 cells. Am J Physiol 1989;256(2 Pt 1): C448-51.
[34] Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. Cell Death Differ 2003;10:45-65.
[35] Cawthorn WP, Sethi JK. TNF-alpha and adipocyte biology. FEBS Lett 2008;582:117-31.
[36] Kamigaki M, Sakaue S, Tsujino I, Ohira H, Ikeda D, Itoh N, et al. Oxidative stress provokes atherogenic changes in adipokine gene expression in 3T3-L1 adipocytes. Biochem Biophys Res Commun 2006;339:624-32.
[37] Lee H, Lee YJ, Choi H, Ko EH, Kim JW. Reactive oxygen species facilitate adipocyte differentiation by accelerating mitotic clonal expansion. J Biol Chem 2009;284:10601-9.
[38] Lee YS, Kim AY, Choi JW, Kim M, Yasue S, Son HJ, et al. Dysregulation of adipose glutathione peroxidase 3 in obesity contributes to local and systemic oxidative stress. Mol Endocrinol 2008;22:2176-89.
[39] Wang B, Jenkins JR, Trayhurn P. Expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture: integrated response to TNF-alpha. Am J Physiol Endocrinol Metab 2005;288:E731-40.
[40] Jones BH, Standridge MK, Taylor JW, Moustaid N. Angiotensinogen gene expression in adipose tissue: analysis of obese models and hormonal and nutritional control. Am J Physiol 1997;273(1 Pt 2): R236-42.
[41] Aubert J, Safonova I, Negrel R, Ailhaud G. Insulin down-regulates angiotensinogen gene expression and angiotensinogen secretion in cultured adipose cells. Biochem Biophys Res Commun 1998;250: 77-82.
[42] Harte A, McTernan P, Chetty R, Coppack S, Katz J, Smith S, et al. Insulin-mediated upregulation of the renin angiotensin system in human subcutaneous adipocytes is reduced by rosiglitazone. Circulation 2005;111:1954-61.
[43] Morgan L, Broughton Pipkin F, Kalsheker N. Angiotensinogen: molecular biology, biochemistry and physiology. Int J Biochem Cell Biol 1996;28:1211-22.
[44] Thatcher S, Yiannikouris F, Gupte M, Cassis L. The adipose renin-angiotensin system: role in cardiovascular disease. Mol Cell Endocrinol 2009;302:111-7.
[45] Nyui N, Tamura K, Yamaguchi S, Nakamaru M, Ishigami T, Yabana M, et al. Tissue angiotensinogen gene expression induced by lipopolysaccharide in hypertensive rats. Hypertension 1997;30: 859-67.

[46] Flesch M, Hoper A, Dell'Italia L, Evans K, Bond R, Peshock R, et al. Activation and functional significance of the renin-angiotensin system in mice with cardiac restricted overexpression of tumor necrosis factor. Circulation 2003;108:598-604.
[47] Ron D, Brasier AR, Habener JF. Transcriptional regulation of hepatic angiotensinogen gene expression by the acute-phase response. Mol Cell Endocrinol 1990;74:C97-C104.
[48] Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor–alpha in human obesity and insulin resistance. J Clin Invest 1995;95: 2409-15.
[49] Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 2003;112:1821-30.
[50] Ron D, Brasier AR, McGehee Jr RE, Habener JF. Tumor necrosis factor–induced reversal of adipocytic phenotype of 3T3-L1 cells is preceded by a loss of nuclear CCAAT/enhancer binding protein (C/EBP). J Clin Invest 1992;89:223-33.

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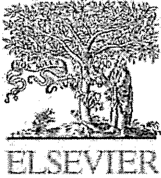

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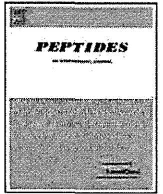

Review

Hypothalamic melanocortin signaling and leptin resistance—Perspective of therapeutic application for obesity-diabetes syndrome☆

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ABSTRACT

The adipocyte-derived hormone, leptin controls feeding behavior, augments fatty acid β-oxidation in the skeletal muscle, attenuates insulin secretion but enhances whole body insulin sensitivity and glucose disposal, thereby serving as a promising therapeutic candidate for the treatment of insulin resistance and dyslipidemia. Along with other researchers, we demonstrated the clinical efficacy and safety of leptin in the treatment of diabetes and dyslipidemia for patients with generalized lipodystrophy. However, the clinical application of leptin has been hampered by the notion that leptin does not fully exert its metabolic effects in human obesity and diet-induced obese rodents. We found that the activity of skeletal muscle AMP-activated protein kinase (AMPK) parallels hypothalamic leptin sensitivity and metabolic phenotype in transgenic mice overexpressing leptin. Our data indicate that the activation of skeletal muscle AMPK is mediated by the hypothalamic melanocortin pathway. In fact, intracerebroventricular administration of melanocortin agonist, MT-II in mice robustly overcomes high-fat diet-induced leptin resistance and ameliorates fuel dyshomeostasis and hyperphagia, with a concomitant recovery of AMPK activity in skeletal muscle. Conversely, AMPK/ACC phosphorylation by leptin was abrogated by the co-administration of melanocortin antagonist, SHU9119 and in the KKA^y mice, which centrally express endogenous melanocortin antagonist. Importantly, high-fat diet-induced attenuation of AMPK/ACC phosphorylation in leptin-overexpressing transgenic mice was not reversed by central leptin *per se*, but was markedly recovered by MT-II. Our data provide evidence for the critical role of the central melanocortin system in leptin-skeletal muscle AMPK axis, and highlight the system as a therapeutic target for leptin insuffciency in obese humans.

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1. Introduction

A variety of metabolic and molecular changes in adipose tissue contribute to the pathophysiology of a series of obesity-related diseases. Even in obese subjects with insulin resistance in glucose uptake into skeletal muscle or suppression of glucose output from liver, insulin action on adipose tissue remains intact or rather exaggerated [36], causing considerable difficulties in weight reduction [37]. In this context, in obesity-related type 2 diabetes, excessive insulin causes body fat gain as well as ectopic lipid overload in liver, skeletal muscle, pancreas, heart and perivascular regions [30]. In the liver, for example, the ability of insulin to suppress hepatic gluconeogenesis is impaired, whereas insulin-stimulated *de novo* lipogenesis and resultant VLDL secretion is increased [2,29]. The global concept of lipid deposition-associated

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