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

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Ishii-Yonemoto T, Masuzaki H, Yasue S, Okada S, Kozuka C, Tanaka T, Noguchi M, Tomita T, Fujikura J, Yamamoto Y, Ebihara K, Hosoda K, Nakao K. Glucocorticoid reamplification within cells intensifies NF-κB and MAPK signaling and reinforces inflammation in activated preadipocytes. *Am J Physiol Endocrinol Metab* 298: E930–E940, 2010. First published September 23, 2009; doi:10.1152/ajpendo.00320.2009.—Increased expression and activity of the intracellular glucocorticoid-reactivating enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) contribute to dysfunction of adipose tissue. Although the pathophysiological 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 chemoattractant 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 activated preadipocytes intensifies NF-κB and MAPK signaling 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 the 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-

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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]azocine 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-benzothiazol-2-yl) benzenesulfonamide (BVT-3498; Biovitrum, Stockholm, Sweden; Ref. 25) were synthesized according to the patent information.

Polyclonal antibodies against NF-κB p65, phospho-p65, p38 MAPK, phospho-p38, 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% CO_2.

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; Funahashi Farm) or a high-fat diet (Research Diets D12493, 5.2 kcal/g, 60% of kcal from fat, source soybean/lard) under a 14:10-h light-dark cycle at 23°C. The high-fat diet was administered to the diet-induced obese (DIO) mice from 3 to 17 wk of age. Animals were allowed free access to food and water. All animal 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 chopped 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% CO_2. 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-^{3}H]$_2$-cortisone (Muromachi Yakuhin, Kyoto, Japan) for reductase activity and 250 nM cortisol with [1,2,6,7-^{3}H]$_4$-cortisol (Muromachi Yakuhin) 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 [^{3}H]cortisone for 2 h. The reaction was stopped by 18β-glycyrrhetinic 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 in sixwell plates and cultured overnight in DMEM containing 10% (vol/vol) FBS at 37°C under 10% CO_2. 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 Na_3VO_4, 0.1 mg/ml aprotinin, 1 mM PMSF, and 50 nM 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′-GAAAUGGCAUAUCAUCUGUTT-3′ and antisense: 3′-TTCUUUACCGUAUAGUAGACA-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 ransfection 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 (5×10^6) were mixed with 2 μg plasmid in the solution provided with the cell line Nucleofector Kit V (Amaxa). pcDNA3.1/11β-HSD1 or a control vector was introduced into the cells using electroporation with a Nucleofector (Amaxa) 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 littermates (Fig. 1, *A* and *B*). Levels of 11β-HSD1 mRNA in the MAF from obese mice were substantially elevated compared with their lean littermates (*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 littermates (*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. 2*iv*). 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. 2*iv*). 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. 2*v*). 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 therapeutical 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

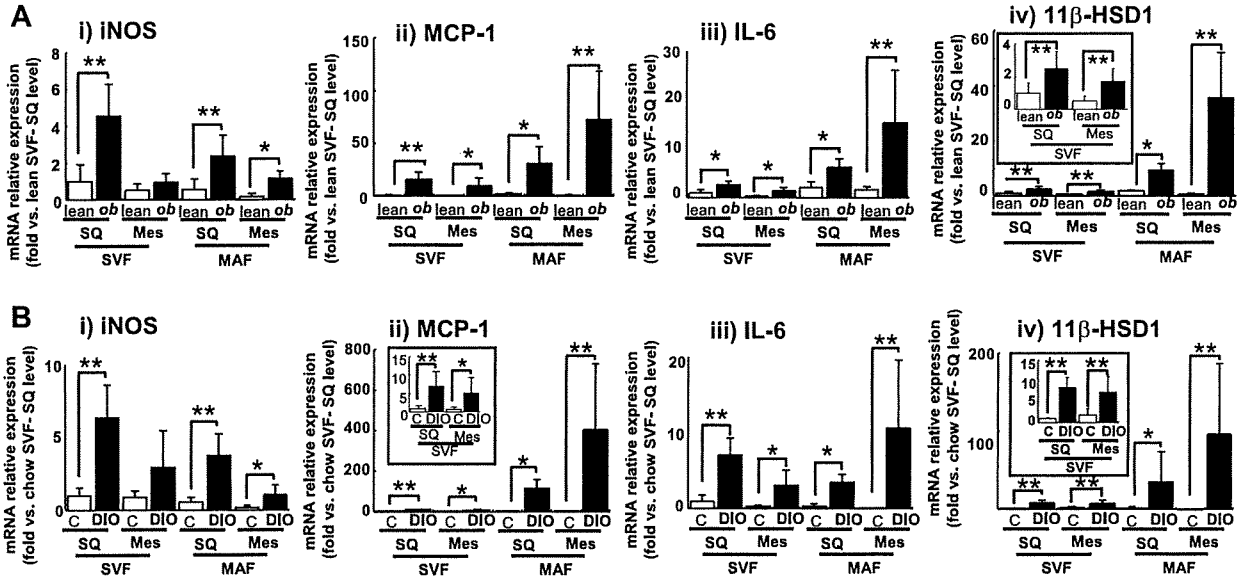

Fig. 1. 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) mRNA expression in stromal vascular fraction cells (SVF) and mature adipocytes fraction (MAF) isolated from obese adipose tissue of *ob/ob* mice and diet-induced obese (DIO) mice. *A*: *ob/ob* and lean littermates [control (C) 9 wk of age; $n = 6$]. *B*: DIO and littermates 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 littermates.

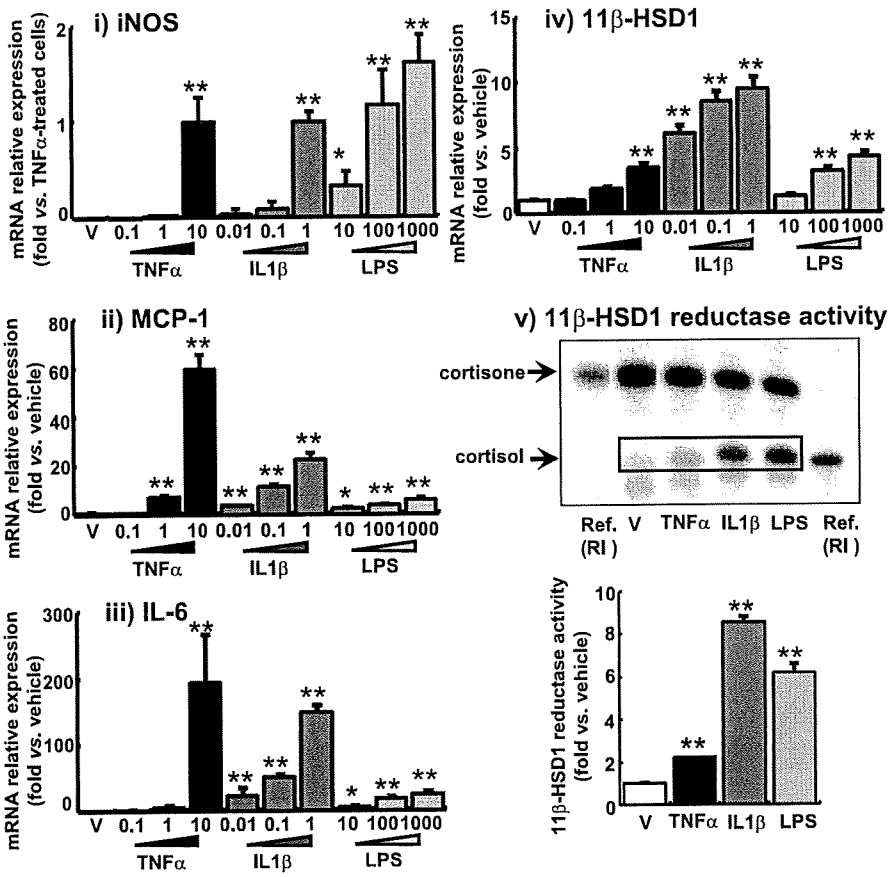

Fig. 2. TNF-α, IL-1β, and LPS augment the expression of proinflammatory mediators and 11β-HSD1 in 3T3-L1 preadipocytes. Cells were treated with TNF-α (0.1, 1, and 10 ng/ml), IL-1β, (0.01, 0.1, and 1 ng/ml) or LPS (10, 100, and 1,000 ng/ml) for 24 h. Levels of iNOS (*i*), MCP-1 (*ii*), IL-6 (*iii*), and 11β-HSD1 (*iv*) mRNA were quantified using real-time PCR. Values were normalized to that of 18S rRNA. *v*: 11β-HSD1 reductase activity (expressed as conversion ability of cortisone to cortisol) was assessed in the medium of 3T3-L1 cells treated with 10 ng/ml TNF-α, 1 ng/ml IL-1β, or 1,000 ng/ml LPS for 24 h. A reference of [^{3}H]cortisone or [^{3}H]cortisol was used as a size marker. A representative autoradiograph of thin-layer chromatography in 11β-HSD reductase activity assay (*top*) and quantification (*bottom*). Intensities of cortisol signals correspond to the enzyme activity of reductase. Ref. (RI), reference samples of [^{3}H]cortisone or [^{3}H]cortisol as size marker. Data are means ± SE of triplicate experiments. $*P < 0.05$, $**P < 0.01$, compared with vehicle (V)-treated group.

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-HSD 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. 3*A*). CBX (50 μM) and inhibitor A (10 μM) markedly attenuated 11β-HSD1 activity (78 and 60% reduction vs. TNF-α-treated cells, respectively; Fig. 3*A*).

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. 3*B*). 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. 3*C*).

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. 4*A*, *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. 4*A*, *ii*]. 11β-HSD2 mRNA levels and the corresponding dehydrogenase activity were under detectable with or without siRNA treatments in 3T3-L1 preadipocytes (data not sown). 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):

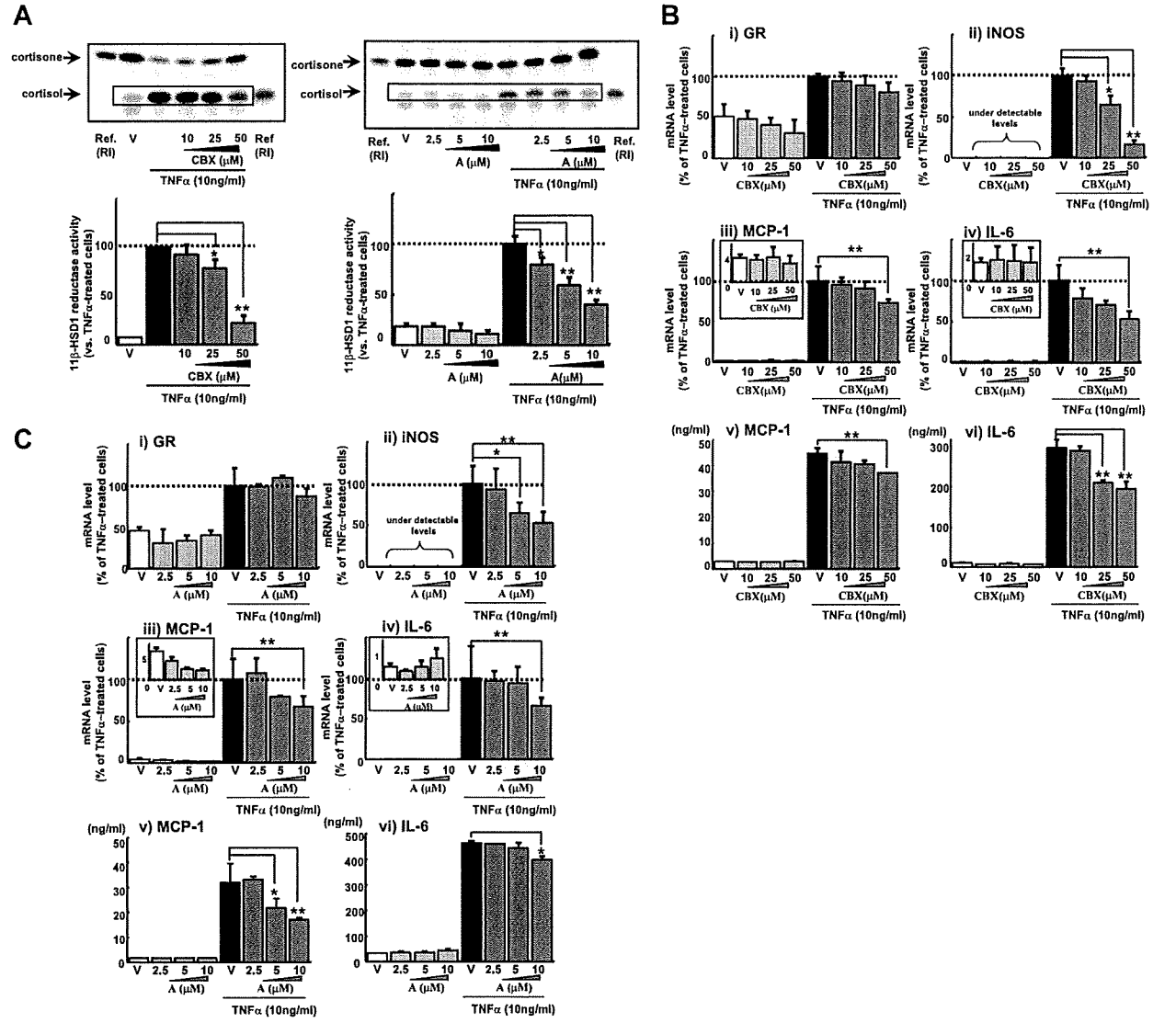

Fig. 3. Effects of pharmacological inhibition of 11β-HSD1 on glucocorticoid receptor (GR), MCP-1, IL-6, and iNOS expression in and secretion from TNF-α-treated 3T3-L1 preadipocytes. *A*: 11β-HSD1 activity assay for validation of 11β-HSD1 inhibitors. 3T3-L1 preadipocytes were incubated for 24 h in serum-free DMEM, adding 250 nM of 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 ±

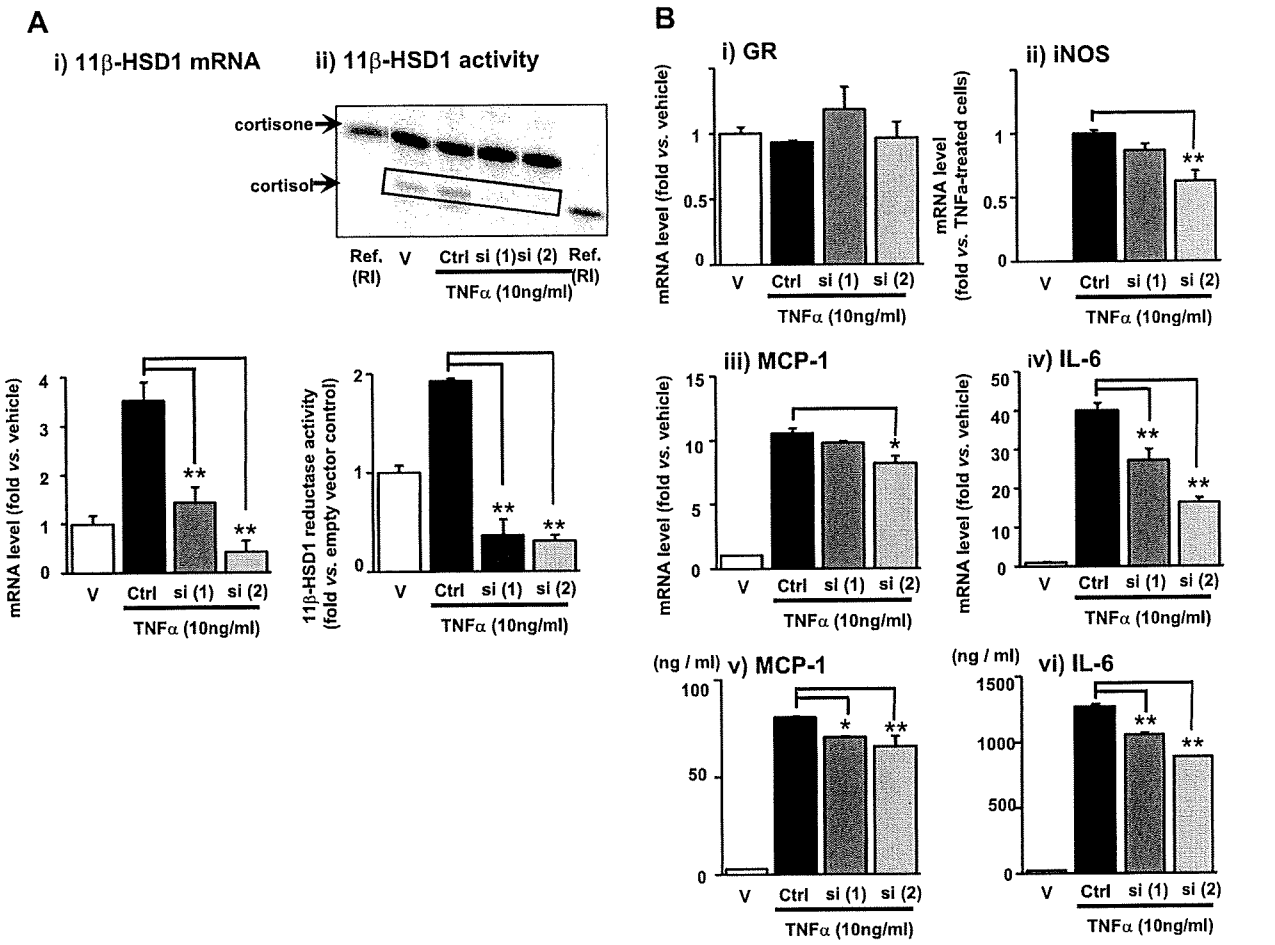

Fig. 4. Effects of 11β-HSD1 knockdown on TNF-α-induced expression of 11β-HSD1 in 3T3-L1 preadipocytes. Cells were transfected with either RNA interference for mouse 11β-HSD1 or a negative control (Ctrl). After 12 h incubation, cells were treated with 10 ng/ml TNF-α for 24 h. *A*: efficiency of 11β-HSD1 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.

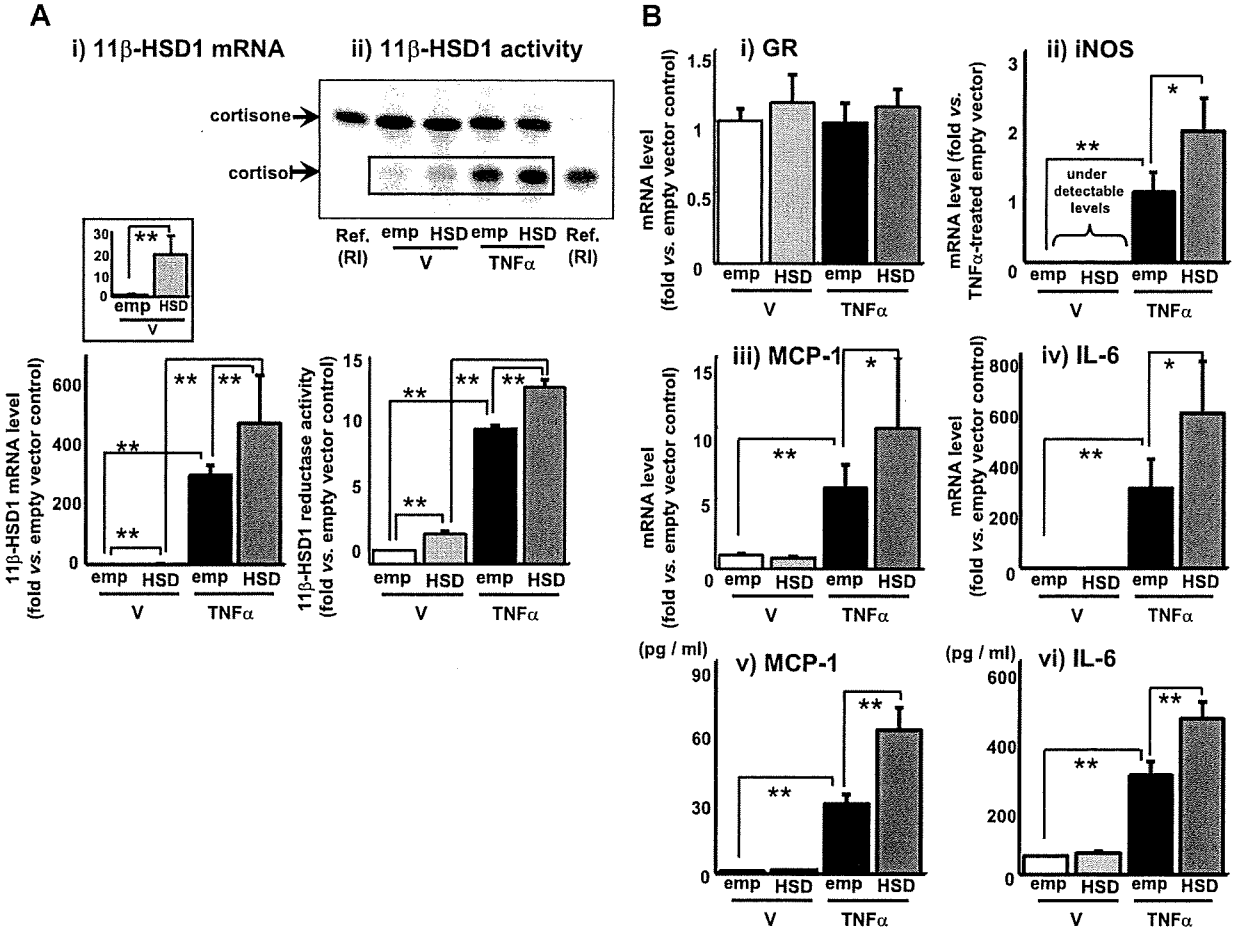

Fig. 5. Effects of overexpression of 11β-HSD1 on MCP-1, IL-6, and iNOS expression in and secretion from TNF-α-treated 3T3-L1 preadipocytes. *A*: efficiency of electroporation-mediated gene transfer. 3T3-L1 preadipocytes were transfected with the expression vector for 11β-HSD1 or a corresponding empty 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.

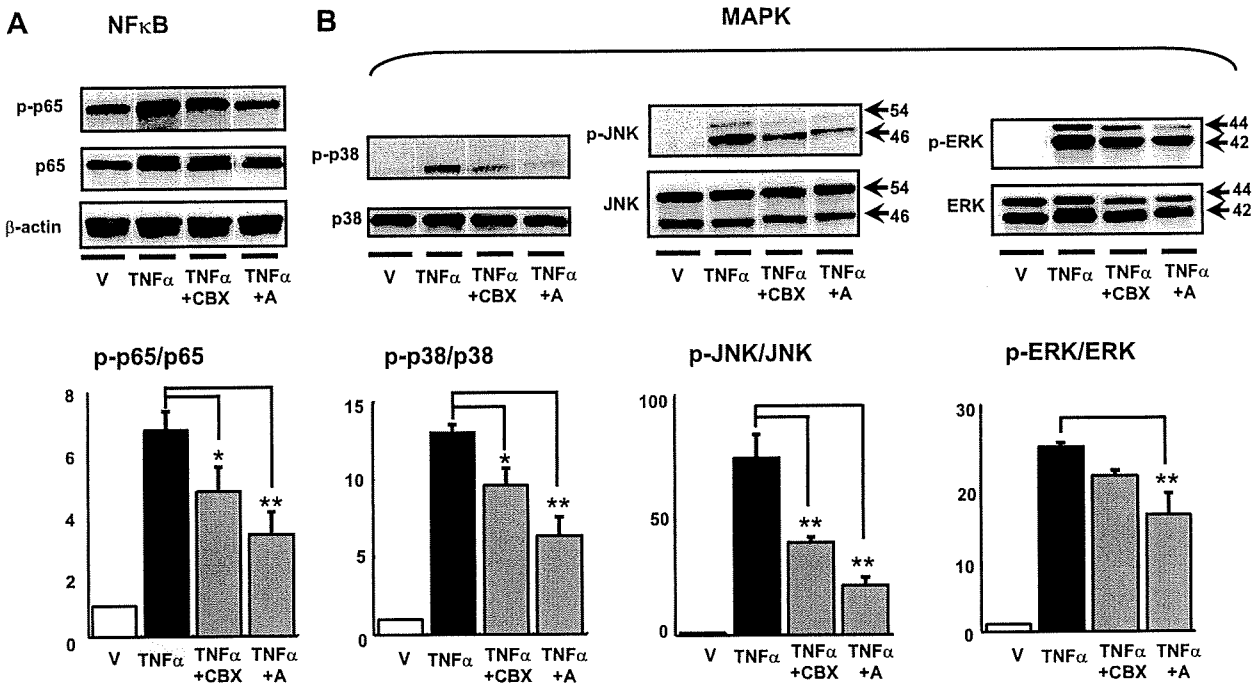

Fig. 6. Effects of inhibition of 11β-HSD1 on TNF-α-induced NFκB and MAPK signaling. NFκB (*A*) and MAPK (*B*) signaling pathways. 3T3-L1 preadipocytes were treated with 10 ng/ml TNF-α for 24 h in the presence or absence of 11β-HSD1 inhibitors (CBX or inhibitor A). After 2-h serum starvation, cells were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

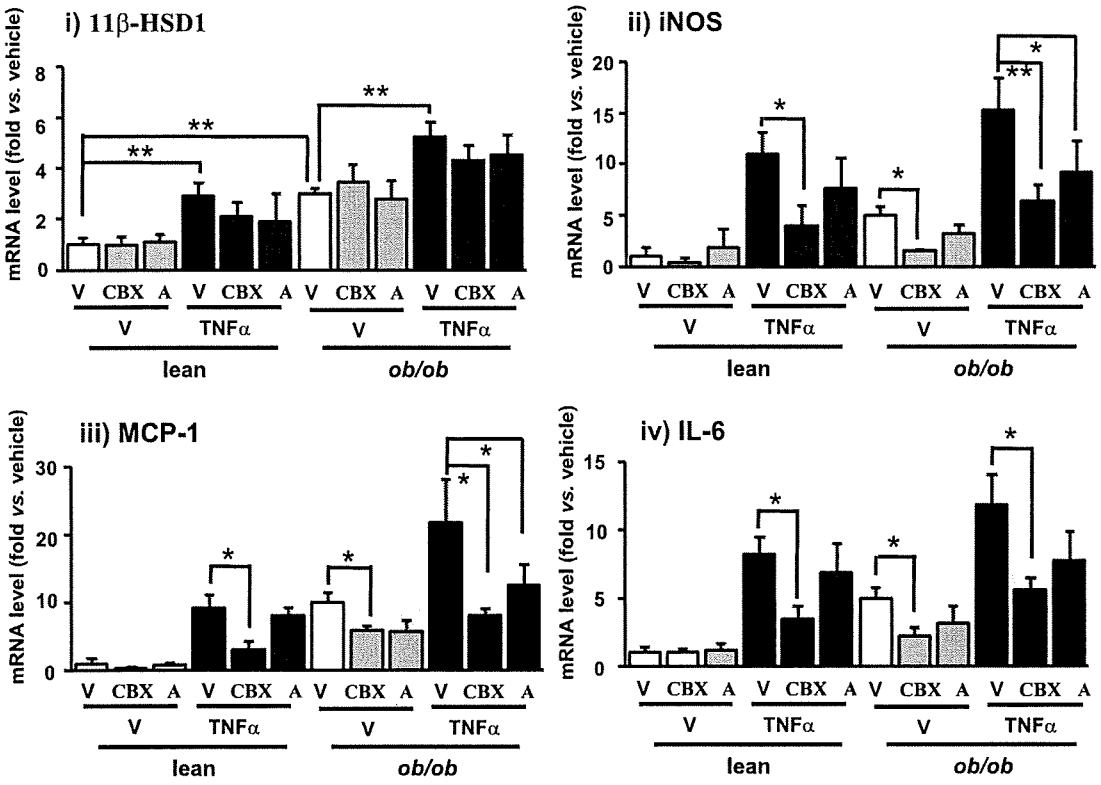

Fig. 7. Effects of pharmacological inhibition of 11β-HSD1 on iNOS, MCP-1, and IL-6 mRNA levels in SVF cells from *ob/ob* mice. SVF cells from *ob/ob* mice and lean control mice were treated with CBX (50 μM) or inhibitor A (10 μM), with or without TNF-α (10 ng/ml) for 24 h. 11β-HSD1 (*i*), iNOS (*ii*),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

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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ORIGINAL ARTICLE

Index of the systemic balance of end products of glucocorticoid metabolism in fresh urine from humans
Its potential usefulness in the evaluation of obesity-related diseases

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Abbreviations: 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; THF, tetrahydrocortisol; allo-THF, allo-tetrahydrocortisol; THE, tetrahydrocortisone; GC–MS, gas chromatography and mass spectrometry; GC–MS-SIM, gas chromatography and mass spectrometry selected ion monitoring; PPARγ, peroxisome proliferator-activated receptor gamma; AME, apparent mineralocorticoid excess; dl, deciliter; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; T-CHO, total cholesterol; HDL-CHO, high-density-lipoprotein-cholesterol; LDL-CHO, low-density-lipoprotein-cholesterol; TG, triglyceride; AST, aspartate 2-oxoglutarate aminotransferase; ALT, alanine 2-oxoglutarate aminotransferase; UA, uric acid; VFA, visceral fat area; SFA, subcutaneous fat area; FPG, fasting plasma glucose; IRI, fasting insulin; HOMA-IR, homeostasis model assessment of insulin resistance; TNF-α, tumor necrosis factor alpha; IL-6, interleukin-6; hsCRP, high sensitivity C-reactive protein; BMD-PFP, bismethylenedioxy-pentafluoropropionate; NASH, non-alcoholic steatohepatitis; CT scan, computed tomography imaging; log, logarithm; ELIZA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay; MRI, magnetic resonance imaging.

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KEYWORDS
Glucocorticoid;
Obesity;
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11β-Hydroxysteroid dehydrogenase;
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Summary:
Objective: Dysregulation of tissue-specific intracellular glucocorticoid reactivation is implicated in obesity and related metabolic diseases in humans. The ratio of end products of glucocorticoid metabolism in fresh urine sample, tetrahydrocortisol (THF) + allo-tetrahydrocortisol (allo-THF) *vs.* tetrahydrocortisone (THE), i.e., the urinary ratio is regarded as an index of the systemic balance underlying intracellular glucocorticoid metabolism, where the enzymes, 11β-hydroxysteroid dehydrogenase type 1 and type 2 as well as 5α- and 5β-reductase are involved in a tissue-specific manner.
Methods: To explore the clinical implications of the urinary ratio in obesity and related metabolic diseases, the urinary ratio was determined by gas chromatography and mass spectrometry.
Results: The urinary ratio was shown to be constant and reproducible in the same individuals. The ratio was found to inversely correlate with BMI ($P < 0.01$), waist circumference ($P < 0.01$), and liver transaminase ($P < 0.05$) in a large cohort of ~200 Japanese subjects. This finding suggests that the systemic balance underlying intracellular glucocorticoid reactivation was suppressed in obesity and liver dysfunction. Consistent with this notion, the ratio was decreased in patients with non-alcoholic steatohepatitis ($P < 0.01$). The urinary ratio was not altered in patients with type 2 diabetes on a 2-month mild calorie restriction. In contrast, the ratio was significantly reduced in patients who responded to the anti-diabetic pioglitazone ($P < 0.01$).
Conclusion: The present study provides novel evidence that the urinary ratio reflects the facet of adipose tissue and liver function in humans, thereby offering a unique opportunity to evaluate obesity-related diseases.

Introduction

Because a combination of glucose intolerance, hypertension, and dyslipidemia, as a result of obesity and insulin resistance, noticeably increases the risk of fatal cardiovascular events [1–4], novel diagnostic options for obesity and related metabolic diseases are strongly warranted [5,6]. In this context, recent research has highlighted a potential role for the dysregulation of tissue-specific intracellular glucocorticoid metabolism in obesity and associated diseases [7–14]. Tissue-specific transgenic and knockout studies suggest that increased reactivation of glucocorticoids in adipose tissue and the liver plays a role in the convergence of metabolic derangements in mouse experimental models [1,2,15]. Two isoenzymes, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and type 2 (11β-HSD2) catalyze interconversion between hormonally active cortisol and inactive cortisone [8]. 11β-HSD1 is expressed abundantly in adipose tissue and in the liver, and that reactivates cortisol from cortisone [8]. In contrast, 11β-HSD2 inactivates cortisol in tissues critically involved in water and electrolyte metabolism [16].

Recent studies demonstrated that enzyme activity of 11β-HSD1 was elevated in adipose tissue of obese humans and rodents [11,17–20]. On the other hand, a couple of studies reported that 11β-HSD1 activity decreased in the liver of obese individuals [11,21]. Furthermore, the activity and gene expression of 5α- and 5β-reductase, both of which catalyze the clearance of active glucocorticoid in liver [13], are known to increase in liver of obese rodents and humans [5,6], thereby contributing to a drop in the intracellular glucocorticoid metabolism throughout the body. Therefore, interpreting the systemic balance of intracellular glucocorticoid metabolism is complicated [5]. The ratio of end products of glucocorticoid metabolism in urine, i.e., the "urinary ratio" (tetrahydrocortisol (THF) + allo-tetrahydrocortisol (allo-THF)) *vs.* tetrahydrocortisone (THE), has long been regarded as a compelling index of the systemic balance underlying intracellular glucocorticoid metabolism, where 11β-hydroxysteroid dehydrogenase type 1 and type 2 (11β-HSDs) as well as 5α- and 5β-reductase are mainly involved in a tissue-specific manner [7,8,10–13].

From a diagnostic viewpoint, the urinary ratio was substantially elevated in patients with apparent mineralocorticoid excess (AME) in which defective 11β-HSD2 activity leads to impaired inactivation of cortisol and results in excessive mineralocorticoid-receptor action in the collecting ducts [16]. However, there are a couple of

controversies regarding the clinical implications of the urinary ratio in common metabolic diseases [7–11,22]. Moreover, in all the previous studies, end products of glucocorticoid metabolism in urine were assessed in 24-h pooled urine samples [7–11,22]. On the basis of these complicated backgrounds, the present study was designed to explore the clinical implications of the urinary ratio in a Japanese large cohort. Here, we provide novel evidence of the association between the urinary ratio and the metabolic status in the case of obesity, liver function, and glucose homeostasis, thus resolving a series of unanswered questions.

Methods

The clinical investigation was performed according to the Declaration of Helsinki. The present study was approved by the ethical committee on human research of Kyoto University Graduate School of Medicine (No. 494). Subjects were recruited from Kyoto University Hospital and affiliated hospitals (August 2003 to January 2005) consecutively. Written informed consent was obtained from all subjects. Subjects with renal dysfunction (serum creatinine level > 1.4 mg/deciliter (dl)) were excluded, because renal dysfunction is known to affect the level of end products of glucocorticoid metabolism in urine [23]. The present study comprises 4 studies as follows.

Study 1

Urinary ratio in fresh urine samples from healthy volunteers

The ratio of end products of glucocorticoid metabolism in fresh urine sample, i.e., (THF + allo-THF)/THE, is regarded as the "urinary ratio" [7–11,22]. To examine possible diurnal variation in the urinary ratio, we analyzed the ratio in 18 healthy volunteers (8 males (age, 28–42 years old; BMI, $21.9 \pm 0.4\,kg/m^2$) and 10 females (age, 26–43 years old; BMI, $20.9 \pm 0.5\,kg/m^2$)). To examine possible diurnal variation in the urinary ratio, samples were obtained at 09:00 h, 16:00 h, and 20:00 h in the case of 16 subjects (8 males and 8 females) out of the 18 healthy volunteers. To verify the reproducibility and stability of the urinary ratio in the same individual, samples were collected twice at an interval of 4 weeks at 09:00 h in the case of 7 subjects (4 males and 3 females). We also compared the ratio between 1 ml of fresh urine and 24-h pooled urine samples in the 18 healthy volunteers (8 males and 10 females). None of the 18 healthy volunteers were under any medication.

Study 2

Relation between the urinary ratio in fresh urine samples and metabolic parameters

Subjects receiving health check ups were recruited in the present study (group A; $n = 201$; age, 28–76 years old). They were not under medication. Anthropometric parameters (BMI, waist circumference, systolic blood pressure (SBP), and diastolic blood pressure (DBP)) and metabolic parameters (total cholesterol (T-CHO), high-density-lipoprotein-cholesterol (HDL-CHO), low-density-lipoprotein-cholesterol (LDL-CHO), triglyceride (TG), aspartate 2-oxoglutarate aminotransferase (AST), alanine 2-oxoglutarate aminotransferase (ALT), and uric acid (UA)) were analyzed. In group B ($n = 72$; age, 28–64 years old; a subgroup of group A), visceral fat area (VFA), subcutaneous fat area (SFA) [24], fasting plasma glucose (FPG), fasting insulin (IRI), and glycosylated hemoglobin (HbA1c) were measured. In group C ($n = 62$; age, 28–64 years old; a subgroup of group B), serum leptin, adiponectin, tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and high sensitivity C-reactive protein (hsCRP) were analyzed.

Study 3

Urinary ratio in fresh urine samples from subjects with non-alcoholic steatohepatitis (NASH)

The urinary ratio was assessed in male patients with non-alcoholic steatohepatitis (NASH) ($n = 7$; age, 29–55 years old). NASH was diagnosed by liver biopsies in all patients [25]. Both the grade of steatosis and stage of fibrosis ranged from class 1 to 3 in the present study. As a control, the urinary ratio of the 8 male healthy volunteers (age, 28–42 years old) from study 1 was employed.

Study 4

Urinary ratio in fresh urine samples from patients with type 2 diabetes who were treated with mild calorie restriction or pioglitazone

To explore the potential impact of thiazolidinedione treatment on the systemic balance underlying intracellular glucocorticoid metabolism, we analyzed the urinary ratio in patients with type 2 diabetes who were being treated with 15 mg/day pioglitazone for 2 months ($n = 17$, 6 males and 11 females; age, 26–73 years old; BMI, $29.1 \pm 1.2\,kg/m^2$; HbA1c, $8.2 \pm 0.3\%$; FPG, $176 \pm 15\,mg/dl$) [26]. We also assessed the urinary ratio in diabetic patients treated with

mild calorie restriction for the same period of time (20 kcal/kg per day; from 1200 kcal/day to 2000 kcal/day) without any glycemic agents ($n = 10$, 3 males and 7 females; age, 25–58 years old; BMI, 30.7 ± 1.3 kg/m^2; HbA1c, 8.0 ± 0.3%; FPG, 151 ± 17 mg/dl). Patients with type 2 diabetes were randomly divided into two groups without any statistical difference in fasting plasma glucose level.

According to the improvement in glycemic control, we divided the 17 diabetic patients treated with pioglitazone into 2 groups. Patients whose glycemic control considerably improved (0.7% or more improvement in HbA1c levels over 2 months) were defined as group Y ($n = 9$, 5 males and 4 females; age, 26–73 years old; BMI, 31.0 ± 1.7 kg/m^2; HbA1c, 8.3 ± 0.6%; FPG, 183 ± 28 mg/dl), while the others (HbA1c improved by less than 0.7%) were defined as group X ($n = 8$, 1 male and 7 females; age, 32–70 years old; BMI, 27.2 ± 1.4 kg/m^2; HbA1c, 8.1 ± 0.3%; FPG, 170 ± 15 mg/dl). There was no statistical difference in fasting plasma glucose level between responded group (group Y) and non-responded group (group X). HbA1c levels and the urinary ratios were measured at the baseline and at 2 months after the initiation of the treatment. The change in HbA1c levels (delta HbA1c) was defined as follows: [HbA1c at 2 months after the initiation of treatment] – [HbA1c at the baseline]. The change in the urinary ratio (delta urinary ratio) was defined as follows: [the ratio at 2 months after initiation of therapy] – [the ratio at the baseline].

Anthropometric measurements

The VFA and SFA at the umbilical level were evaluated by computed tomography imaging (CT scan) (Toshiba Medical Systems, Tokyo, Japan) as previously reported [24].

Blood examination

Blood samples were obtained at 09:00 h after an overnight fast. Serum leptin, adiponectin, and hsCRP were determined using the radioimmunoassay (RIA) (LINCO Research Inc., MO, USA), enzyme-linked immunosorbent assay (ELISA) (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) or nephelometric analysis (Dade Behring, Marburg, Germany). The serum levels of TNF-α and IL-6 were determined by ELISA (R&D Systems, MN, USA).

Measurement of urinary glucocorticoid end-metabolites within cells

After an overnight fast, urine samples were obtained at 09:00 h in the morning. Intracellular end products of glucocorticoid metabolism, namely, THF, allo-THF, and THE, were analyzed by gas chromatography and mass spectrometry [27,28]. Capillary gas chromatography and mass spectrometry selected ion monitoring (GC–MS-SIM) analyses were carried out on an HP 6890-5973MSD gas chromatograph-mass spectrometer equipped with a data processing system (Agilent, CA, USA). Gas chromatography was performed on an SPB-1 fused-silica capillary column (15 m × 0.25 mm internal diameter (I.D.)) with a 0.25 μm film thickness (Supelco, Bellefonte, PA, USA). Details are shown in Fig. 1.

Statistical analyses

The two-tailed Student's t test (studies 1, 3, and 4) and Pearson's correlation coefficient (study 2) were used where applicable. Values that were not distributed normally were transformed into logarithms (base e) (log) and subsequently analyzed by Pearson's correlation coefficient. Values that were not distributed normally even if they were transformed into logarithms (base e) (log), Spearman's correlation coefficient (study 2) were used (statistical package for social sciences, SPSS Ver. 12.0J Inc. IL, USA). Two-way repeated measures analysis of variance (ANOVA) (studies 1 and 2) were used where applicable (Stat-View-J 5.0). Values are presented as the mean ± SEM. $P < 0.05$ was considered statistically significant.

Results

Study 1

Fig. 1A and B show selected ion-monitoring of the bismethylenedioxy-pentafluoropropionate (BMD-PFP) derivatives of unlabeled and labeled tetrahydrocorticoids after processing the spiked urine. The recorded profiles for the urinary glucocorticoid metabolites and the internal standards showed coincided closely, validating the accuracy of the assays in the present study.

It is noteworthy that no apparent circadian variation was observed in the urinary ratio within the same individuals (1.01 ± 0.07 (09:00 h), 0.96 ± 0.07 (16:00 h), and 1.01 ± 0.09 (22:00 h)) (Fig. 1C). No significant difference was observed in the urinary ratio at the baseline (1.11 ± 0.10) and at 4

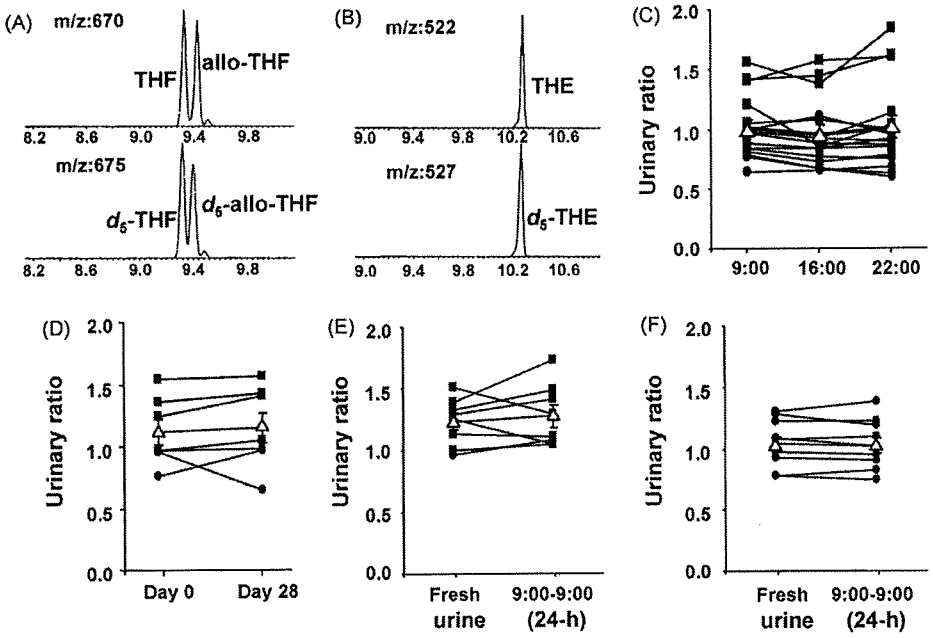

Figure 1 (A and B) Fresh urine samples (1 ml) were used to analyze the intracellular end products of glucocorticoid metabolism, namely, THF, allo-THF, and THE by gas chromatography and mass spectrometry [8–11]. Internal standards [1,2,3,4,5-$^2H_5$] tetrahydrocortisol (THF-d_5) [1,2,3,4,5-$^2H_5$] allo-tetrahydrocortisol (allo-THF-d_5), and [1,2,3,4,5-$^2H_5$] tetrahydrocortisone (THE-d_5) were obtained from Shionogi Pharmaceuticals (Osaka, Japan) and SPEC® C18 (Varian, Lake Forest, CA, USA). The initial column temperature was 150 °C. The mass spectrometer was operated in the electron impact mode with energies of 70 eV, and the ion source temperature was set at 280 °C. Urine samples (2 ml) were placed in glass tubes and 40 μl of combined I.S. solution (25 μg/ml) was added to each tube. Selected ion-monitoring of the bismethylenedioxy-pentafluoropropionate (BMD-PFP) derivatives of unlabeled and labeled tetrahydrocorticoids was conducted after processing the spiked urine. In the assay, solid-phase was utilized extraction for clean-up; double derivatization (BMD-PFP) and gas chromatography were used for separation of analytes, with on-line detection by electroimpact mass spectrometry in the selected-ion monitoring mode. The mixture was analyzed for the BMD-PFP derivatives of allo-THF, THF, and THE by monitoring [M–30]+ion intensities at *m/z* 670 (THF and allo-THF), *m/z* 675 (THF-d_5, allo-THF-d_5), *m/z* 522 (THE), and *m/z* 527 (THE-d_5). Recording patterns closely coincided between the urinary glucocorticoids and their internal standards. Vertical and horizontal axes indicate the ion intensity and recording time (arbitrary unit), respectively. (C) No diurnal pattern of change was observed in the urinary ratio ((THF + allo-THF)/THE) at 09:00 h, 16:00 h, and 22:00 h in the case of 16 healthy subjects (8 males and 8 females). (D) Comparison of the urinary ratio twice after a 4-week interval in the case of 7 subjects (4 males and 3 females). No difference was observed between the initial value and that at 4 weeks. No difference in the urinary ratio was observed between the fresh urine and 24-h pooled urine samples in 8 males (E) or 10 females (F). Black squares indicate the value in the case of males, black circles indicate the value in the case of females, and white triangles indicate the mean ± SEM.

weeks (1.14 ± 0.12) (Fig. 1D). Importantly, no difference was observed in the urinary ratio between fresh urine and 24-h pooled urine samples from 8 males (1.23 ± 0.07 *vs.* 1.28 ± 0.09) (Fig. 1E) and 10 females (1.04 ± 0.06 *vs.* 1.02 ± 0.06) (Fig. 1F). The urinary ratio was slightly but significantly higher in the males than in the females in the case of both fresh urine ($P = 0.045$) and 24-h pooled urine ($P = 0.036$) samples.

Study 2

The present study is the first to demonstrate that the urinary ratio showed a normal distribution curve (Fig. 2A). The ratio ranged from 0.57 to 1.93 (mean: 1.20 ± 0.02). Slight but significant inverse correlations were observed between the urinary ratio and BMI ($r = -0.27$, $P = 0.0001$) (Fig. 2B), waist circumference ($r = -0.21$, $P = 0.0030$) (Fig. 2C), log AST ($r = -0.16$, $P = 0.018$) (Fig. 2D) and log ALT ($r = -0.022$, $P = 0.0014$) (Fig. 2E). Both log AST and log ALT correlated with BMI ($r = 0.35$, $P < 0.0001$ and $r = 0.49$, $P < 0.0001$, respectively). An inverse correlation was observed between the urinary ratio and log hsCRP ($r = -0.30$, $P = 0.017$). No correlations were observed between the urinary ratio and other parameters (Table 1). It is important to note that there were no significant differences in groups A, B,

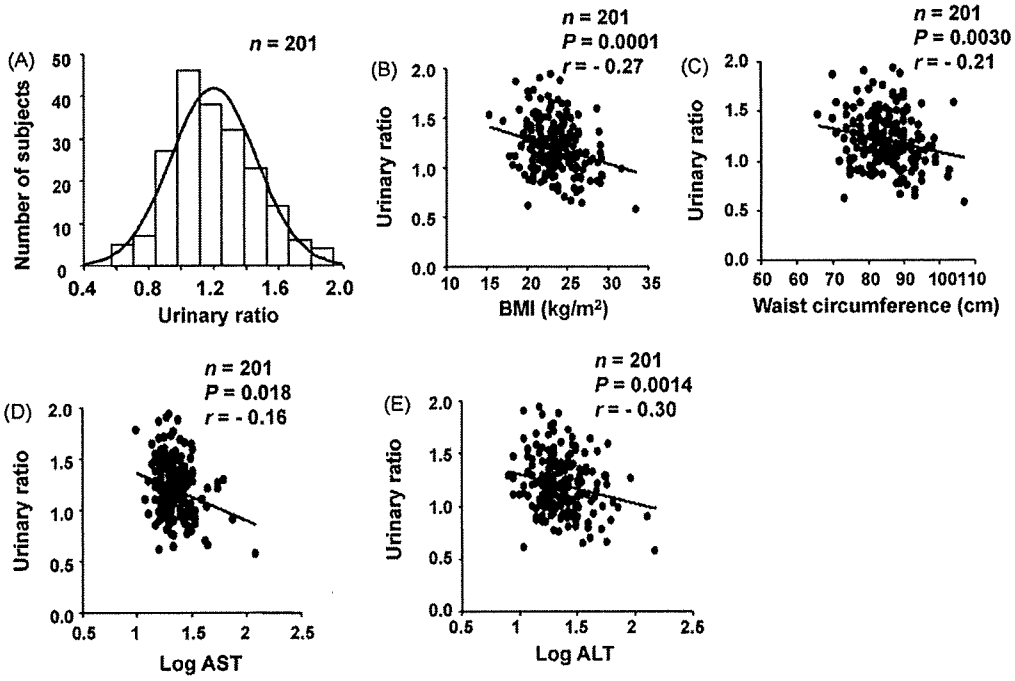

Figure 2 (A) Urinary ratio showed a normal distribution curve. Slight but significant inverse correlations were observed between the urinary ratio and BMI ($P < 0.001$) (B), waist circumference ($P < 0.01$) (C), log AST ($P < 0.05$) (D), and log ALT ($P < 0.01$) (E) in 201 Japanese males.

and C in the value of age, BMI, HbA1c, TG, T-CHO, SBP, or DBP.

Study 3

In patients with NASH, the serum levels of AST (74 ± 13 U/l) and ALT (149 ± 39 U/l) were elevated. The BMI value (30.7 ± 2.8 kg/m^2) was higher in patients with NASH than in healthy volunteers (21.9 ± 0.4 kg/m^2) ($P = 0.0054$) (Fig. 3A). The urinary ratio in NASH patients (0.84 ± 0.07) was significantly lower than that in healthy volunteers (1.23 ± 0.07) ($P = 0.0014$) (Fig. 3B).

Study 4

To explore the potential relationship between the systemic balance of intracellular glucocorticoid reactivation and the metabolic consequences, the impact of mild calorie restriction or anti-diabetic pioglitazone treatment was investigated. At the baseline, no differences were noted in BMI, HbA1c and the urinary ratio between the calorie restriction group and the pioglitazone treatment group. Notably, the value of HbA1c at 2 months had equipotently decreased between the patients treated with calorie restriction (from $8.0 \pm 0.3\%$ to $7.4 \pm 0.3\%$; delta HbA1c $= 0.6 \pm 0.3$; $P = 0.048$ *vs.* initial value) and pioglitazone (from $8.2 \pm 0.3\%$ to $7.5 \pm 0.3\%$; delta HbA1c $= 0.7 \pm 0.2$; $P = 0.011$ *vs.* initial value) (Fig. 4A). Overall, the urinary ratio was slightly but significantly reduced in patients treated with pioglitazone (from 0.98 ± 0.08 to 0.88 ± 0.06; delta urinary ratio $= 0.10 \pm 0.05$; $P = 0.029$). In contrast, no significant change was observed in the urinary ratio in the mild calorie restriction group (from 0.92 ± 0.12 to 0.88 ± 0.09; delta urinary ratio $= 0.04 \pm 0.06$) (Fig. 4B).

On the basis of this result, we next investigated the impact of pioglitazone treatment on the urinary ratio in relation to glycemic control. Seventeen patients were divided into 2 groups according to the improvement in glycemia (groups X and Y). HbA1c was significantly decreased in group Y (from $8.3 \pm 0.6\%$ to $7.0 \pm 0.4\%$; delta HbA1c $= 1.3 \pm 0.2$; $P < 0.0001$ *vs.* initial value), while the value did not decrease in group X (Fig. 4C). Consequently, the decrease in HbA1c levels at 2 months in group Y (delta HbA1c $= 1.3 \pm 0.2$) was much greater compared to that in group X (delta HbA1c $= 0.05 \pm 0.3$) ($P = 0.0029$).

Although no differences were observed in the initial value of HbA1c and the urinary ratio between groups X and Y, a marked reduction in the urinary ratio was observed only in group Y (from 1.13 ± 0.12 to 0.92 ± 0.08; delta urinary ratio $= 0.21 \pm 0.05$; $P = 0.0014$) (Fig. 4D). Accordingly, the decrease in the urinary ratio in group Y (delta urinary

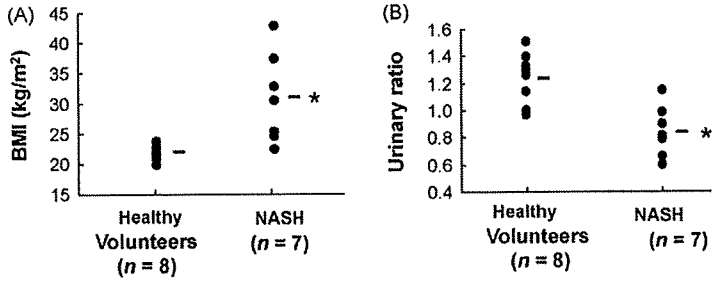

Figure 3 Values of BMI and the urinary ratio in each subject were shown as black circles. (A) The average value of BMI was significantly higher in patients with NASH than in healthy volunteers ($^{*}P < 0.01$). (B) The average value of the urinary ratio in NASH patients was significantly lower than that in healthy volunteers ($^{*}P < 0.01$). Black bars indicate the mean value.

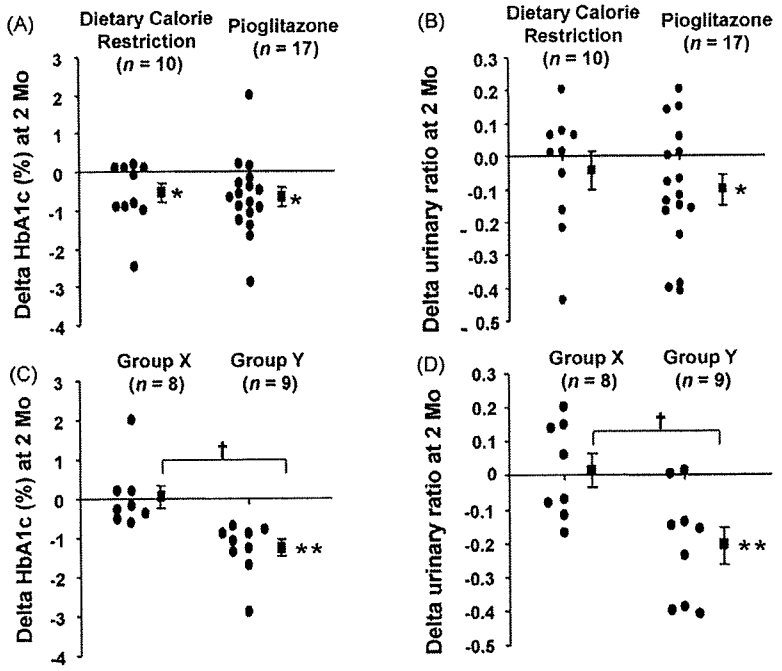

Figure 4 Urinary ratio from patients with type 2 diabetes who were treated with mild calorie restriction ($n = 10$, 3 males and 7 females; BMI, $30.7 \pm 1.3\,kg/m^2$; HbA1c, $8.0 \pm 0.3\%$) or pioglitazone ($n = 17$, 6 males and 11 females; BMI, $29.1 \pm 1.2\,kg/m^2$; HbA1c, $8.2 \pm 0.3\%$). (A) HbA1c levels significantly decreased at 2 months with calorie restriction treatment (from 1200 kcal/day to 2000 kcal/day) ($^{*}P < 0.05$) and pioglitazone treatment ($^{*}P < 0.05$). (B) The urinary ratio was significantly reduced in patients treated with pioglitazone as a whole ($^{*}P < 0.05$). We further investigated the relation between the urinary ratio and improvement in glycemic control as a result of pioglitazone treatment. Patients whose glycemic control improved (increased in HbA1c levels of 0.7% or more over 2 months) were defined as group Y ($n = 9$, 5 males and 4 females; BMI, $31.0 \pm 1.7\,kg/m^2$; HbA1c, $8.3 \pm 0.6\%$), and others (HbA1c levels improved by less than 0.7%) were defined as group X ($n = 8$, 1 male and 7 females; BMI, $27.2 \pm 1.4\,kg/m^2$; HbA1c, $8.1 \pm 0.3\%$). HbA1c levels and the urinary ratio were measured at the baseline and at 2 months after initiation of the treatment. The change in HbA1c levels (delta HbA1c) was defined as [HbA1c at 2 months after the initiation of treatment] − [HbA1c at the baseline]. The change in the urinary ratio (delta urinary ratio) was defined as [the ratio at 2 months after initiation of therapy] − [the ratio at the baseline]. (C) HbA1c levels decreased significantly only in the group Y patients ($^{**}P < 0.01$ *vs.* at the baseline). HbA1c levels were significantly decreased in group Y at 2 months compared to group X ($^{\dagger}P < 0.01$ *vs.* group Y). (D) In group Y, the delta urinary ratio was exaggerated at 2 months ($^{**}P < 0.01$ *vs.* at the baseline). On the other hand, no significant change in delta urinary ratio was observed in group X. The urinary ratio was significantly decreased compared to group X ($^{\dagger}P < 0.01$ *vs.* group Y). Black circles indicate the value in the case of each individual, and black squares indicate the mean ± SEM.

Table 1 Relation between the urinary ratio and metabolic parameters.

Parameter	*r*	*P* value	*n*
BMI (kg/m^2)	−0.27	0.0001**	201 (group A)
Waist circumference (cm)	−0.21	0.0030**	
SBP (mmHg)	−0.081	0.25	
log DBP	−0.060	0.40	
T-CHO (mg/dl)	−0.12	0.084	
log HDL-CHO	0.063	0.37	
LDL-CHO (mg/dl)	−0.086	0.22	
log TG	−0.073	0.30	
log AST	−0.16	0.018*	
log ALT	−0.22	0.0014**	
UA (mg/dl)	−0.073	0.30	
FPG (mg/dl)	−0.068	0.57	72 (group B)
log IRI	−0.074	0.53	
log HOMA-IR	−0.085	0.48	
log HbA1c	−0.14	0.22	
VFA (cm^2)	−0.041	0.73	
SFA (cm^2)	−0.22	0.062	
VFA + SFA (cm^2)	−0.18	0.13	
Ratio of VFA/SFA	0.14	0.24	
log leptin	−0.24	0.057	62 (group C)
Adiponectin (μg/dl)	0.24	0.061	
log hsCRP	−0.30	0.017*	
log TNF-α	−0.087	0.50	
log IL-6	−0.11	0.39	

Pearson's correlation coefficient was used to examine for association between the urinary ratio and each parameters (*$P < 0.05$, **$P < 0.01$ when compare with the urinary ratio).

Abbreviations: logarithm (base e) (log), systolic blood pressure (SBP), and diastolic blood pressure (DBP), total cholesterol (T-CHO), high-density-lipoprotein-cholesterol (HDL-CHO), low-density-lipoprotein-cholesterol (LDL-CHO), triglyceride (TG), aspartate 2-oxoglutarate aminotransferase (AST), alanine 2-oxoglutarate aminotransferase (ALT), uric acid (UA), visceral fat area (VFA), subcutaneous fat area (SFA), fasting plasma glucose (FPG), fasting insulin (IRI), homeostasis model assessment of insulin resistance (HOMA-IR), tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), high sensitivity C-reactive protein (hsCRP)

ratio = 0.21 ± 0.05) was much greater than that in group X (delta urinary ratio = 0.01 ± 0.05) ($P = 0.0086$) (Fig. 4D). It should be noted that no significant changes in BMI were observed among the mild calorie restriction group ($n = 10$, from 30.7 ± 1.3 kg/m^2 to 29.8 ± 1.3 kg/m^2), pioglitazone group ($n = 17$ patients, from 29.1 ± 1.2 kg/m^2 to 27.4 ± 1.3 kg/m^2), group X ($n = 8$, from 27.2 ± 1.4 kg/m^2 to 26.6 ± 1.4 kg/m^2), and group Y ($n = 9$, from 31.0 ± 1.7 kg/m^2 to 29.0 ± 1.7 kg/m^2). Values of transaminases did not change significantly in the treatment of pioglitazone in both group X (=non-responded group) and group Y (=responded group). The initial value of AST in group X was 20 ± 1 U/l and 20 ± 2 U/l in 2 months after the treatment, whereas ALT values were 24 ± 4 U/l and 21 ± 4 U/l, respectively. The initial values of AST in group Y was 34 ± 4 U/l and 32 ± 4 U/l in 2 months after the treatment, whereas ALT values were 51 ± 10 U/l and 43 ± 10 U/l, respectively.

Discussion

The present study provides evidence of the existence of significant associations between the urinary ratio and the metabolic status in the case of obesity, liver function, or glucose homeostasis in Japanese subjects. The ratio of end products of glucocorticoid metabolism in urine samples has been regarded as a compelling index of the systemic balance of intracellular glucocorticoid metabolism [7–9,11]. However, except for a few rare diseases such as AME [16], there are controversies regarding the clinical implications of the urinary ratio in common metabolic diseases [7–11,22]. For example, Valsamakis et al. showed that an "inverse" correlation exists between the urinary ratio and BMI [7], whereas Andrew et al. reported that a "positive" correlation exists between the urinary ratio and waist circumference in male subjects [8]. Rask et al. also demonstrated that a "positive" association exists between the urinary ratio and adiposity

in females [11]. Recently, they reported that the urinary ratio was elevated in subjects with a BMI ranging from 22 to 26, while it was decreased in subjects with a BMI ranging from 26 to 31, indicating a bell-shape correlation [10]. Thus, the relationship between the urinary ratio and the metabolic status has largely remained obscure.

In the present study, the urinary ratio in 24-h pooled urine samples (1.13 ± 0.7) from 18 healthy subjects (8 males and 10 females; age, 33.7 ± 1.3; BMI, 21.3 ± 0.7) was found to be equivalent to that obtained from patients in another laboratory [9] (urinary ratio, 1.06 ± 0.08; in 12 control subjects (6 males and 6 females) age, 27.9 ± 1.5; BMI, 22.3 ± 0.41). Similarly, the ratio in the case of male subjects in the present study (1.28 ± 0.09) was comparable to that in the case of a previous report [10] (urinary ratio, 1.18 ± 0.28 in 11 control males; age, 46.8 ± 8.7; BMI, 22.9 ± 1.4), validating the accuracy of measurement in the present study. On the basis of these results, using fresh urine samples obtained at 09:00 h, we investigated the clinical implications of the urinary ratio in metabolic diseases. The present study is the first to demonstrate that the urinary ratio in healthy volunteers showed no apparent circadian variation and show that there was no difference between fresh urine and 24-h pooled urine samples, suggesting that the ratio in fresh urine samples is constant and reproducible for the same individual. This is also the first report stating that the urinary ratio showed a normal distribution curve. Our findings raise the possibility that fresh urine sample, as little as 1 ml, can reveal the systemic balance of intracellular glucocorticoid metabolism.

Recent human studies showed a significantly positive association between the 11β-HSD1 mRNA level in subcutaneous adipose tissue and BMI [18–20]. Contrary to our initial prediction, however, the present study demonstrates a significantly inverse correlation between the urinary ratio and BMI or waist circumference in a large Japanese cohort. These results suggest that intracellular glucocorticoid metabolism in the whole body is decreased in the case of human obesity. Furthermore, when adjusted for BMI, there was no gender difference in the urinary ratio (data not shown). This suggests that sexual dimorphism in the urinary ratio is attributable, at least partly, to the gender difference in body fat mass.

As recent studies reported that NASH is critically associated with intra-abdominal obesity and insulin resistance [29], we investigated the urinary ratio in patients with histologically proven NASH. Diagnosis of NASH requires histopathologic evaluation because the lesions of parenchymal injury and fibrosis cannot be detected by imaging studies or laboratory tests [30]. The present study is the first to demonstrate that the urinary ratio was significantly lower in patients with NASH compared to healthy volunteers; this result was in agreement with those of a previous report where the urinary ratio in 24-h pooled urine was significantly lower in patients with non-alcoholic fatty liver diseases diagnosed by magnetic resonance imaging [13]. Therefore, it is reasonable to speculate that decreased activity of 11β-HSD1 as well as increased activity of 5α- and 5β-reductase in a steatotic liver may contribute to the decrease in the urinary ratio in NASH patients [11,13,21]. A higher BMI in patients with NASH may also be associated with the decrease in the urinary ratio. In this context, conducting liver biopsies to measure the activities and expression of 11β-HSDs and 5α- and 5β-reductase would be of considerable interest in future studies.

In previous cross-sectional studies, no significant difference was observed in the urinary ratio between patients with type 2 diabetes and the control subjects [7,22]. However, none of the studies have investigated the urinary ratio during the treatment of type 2 diabetes [7,22]. PPARγ is expressed abundantly in adipose tissue and plays a pivotal role in regulating a variety of adipocyte genes [26,31,32]. Importantly, PPARγ agonists are known to suppress 11β-HSD1 exclusively in adipose tissue [31]. In this context, we assessed the urinary ratio in patients with type 2 diabetes during the 2-month treatment with pioglitazone [26,31]. The present study demonstrates that mild calorie restriction without significant body weight changes did not affect the urinary ratio at all. In contrast, with an equipotent amelioration of HbA1c to calorie restriction therapy, the urinary ratio was slightly but significantly reduced in patients treated with pioglitazone. On the basis of this result, we further explored the impact of pioglitazone treatment on the urinary ratio in relation to drug responsiveness. In a recent report by Satoh et al., pioglitazone responders were defined as those who showed a >1% reduction in HbA1c with 3 months of treatment [33]. Since we evaluated the drug response for 2 months, patients who showed an improvement in glycemic control of 0.7% or more in terms of HbA1c levels over 2 months were defined as drug responders. In terms of glycemic control, our data demonstrates that the decrease in the urinary ratio was increased to a large extent in patients who responded to pioglitazone. Although the underlying mechanism is still obscure, a recent study demon-