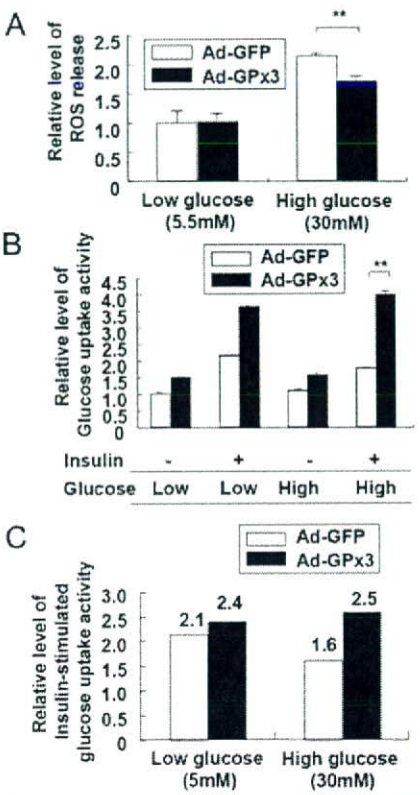

Fig. 7. GPx3 Overexpression Reduces ROS Accumulation and Improves High-Glucose-Induced Insulin Resistance in 3T3-L1 Adipocytes

3T3-L1 adipocytes were infected with a green fluorescent protein (GFP)- or GPx3-expressing adenovirus (multiplicity of infection = 50). Two days after infection (>80% of cells were GFP positive), the cells were incubated with low- (5.5 mM) or high-glucose (25 mM) DMEM. The medium was supplemented with sodium selenite (0.1 μM) throughout the experiments. A, The level of ROS released from the adipocytes was measured using the Amplex Red hydrogen peroxide assay kit, as described in *Materials and Methods*. **, $P < 0.01$. B, Glucose uptake assays. Twenty-four hours after incubation in low- or high-glucose media, the cells were incubated in the presence or absence of insulin for 1 h; [^{14}C]2-deoxy-glucose was added for 30 min and its uptake was measured. The values represent the means ± SEM (n = 3). *, $P < 0.05$. C, Insulin-stimulated glucose uptake activity. The relative activities were determined by normalizing the values in panel B in the presence of insulin with the values obtained in the absence of insulin. Ad-GFP, adenoviral GFP; Ad-GPx3, adenoviral GPx3.

suggesting that extracellular GPx activity is critical for maintaining plasma oxidative tone and normal vascular function. Therefore, it seems that down-regulation of adipose GPx3 expression and subsequent decrease in circulating GPx activity might be associated with the obesity-related rise in systemic oxidative stress and incidence of metabolic complications.

Because excessive levels of ROS play causative roles in the development of insulin resistance and diabetes (55, 56), it has been speculated that increased GPx activity could have beneficial effects on glucose metabolism. However, GPx1-overexpressing transgenic mice develop insulin resistance along with hampered insulin function, probably due to overquenching of the intracellular ROS burst required for insulin sensitization (57). An acute intracellular ROS burst after insulin stimulation is required for sensitizing insulin signaling to suppress protein tyrosine phosphatase activity (58). Thus, it appears that reducing the ROS accumulation in circulation while maintaining proper intracellular ROS tone may be critical for managing glucose homeostasis in diabetic subjects. In this regard, we highlight the use of GPx3 as a potential target for intervention in insulin resistance. GPx3 functions as a major extracellular antioxidant enzyme, and its overexpression in adipocytes was observed to reduce ROS accumulation, diminish proinflammatory gene expression, and ameliorate hyperglycemia-induced insulin resistance (Figs. 6 and 7). Additionally, we observed that glucose tolerance was improved in *db/db* mice by administering the antioxidant NAC (data not shown), suggesting that increased systemic antioxidative activity may reverse obesity-related glucose intolerance. Therefore, it is likely that GPx3 would participate in controlling ROS-induced stress in circulation as well as in adipose tissue, thus modulating the energy homeostasis of the whole body, and that it would play a protective role in obesity-related metabolic disorders

Interestingly, we observed that circulating GPx3 levels closely correlated with adipose GPx3 expression rather than that in the kidneys of obese animals (Figs. 1 and 3) (supplemental Figs. 1 and 2). GPx3 is expressed most abundantly in the kidneys (Fig. 2). Further, anephric individuals show reduced plasma GPx activity and GPx3 protein expression, which is reversed by kidney transplantation (59–61). Thus, it had been suspected that circulating GPx3 appears to be derived mainly from the kidneys. However, to our surprise, kidney GPx3 expression was not altered or even slightly increased when circulating GPx3 expression was substantially reduced in obese *ob/ob* and *db/db* mice (Figs. 1 and 3). With these findings, it would be feasible to propose that reduced circulating GPx activity in obese animals would be primarily correlated with decreased adipose GPx3 expression. However, it remains to be elucidated whether kidney GPx3 expression might also affect reduced plasma GPx3 in obesity with decreased secretion of GPx3 proteins.

In obesity, adipose tissue gradually develops hypoxia due to rapid growth in the overall fat cell size and fat mass. Recently, it has been shown that hypoxia potently enhances GPx3 expression in Caki-2 renal cells, and this expression is blocked by treatment with the antioxidant molecule NAC (62). Because hypoxia stimulates ROS production, it has been proposed that increased GPx3 expression may induce an adaptive response mechanism to oxidative stress under hypoxic conditions in the kidneys. However, in the current study, we observed that GPx3 expression evi-

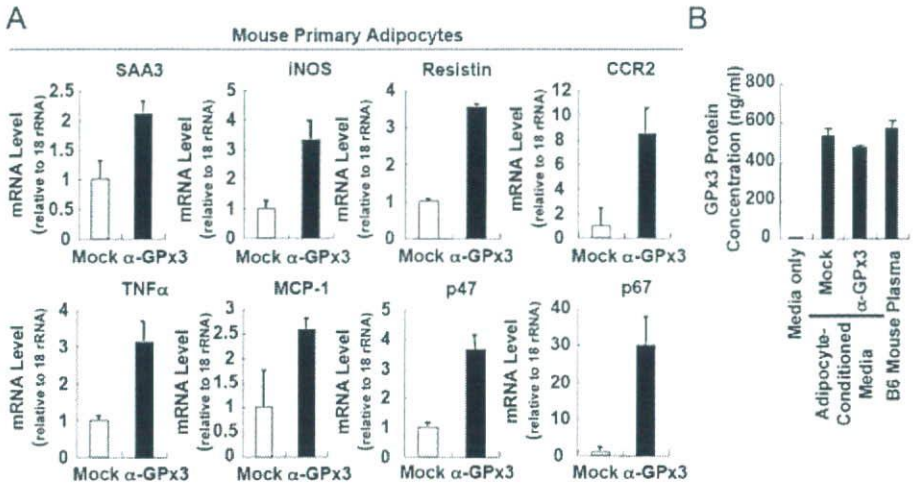

Fig. 8. Neutralization of GPx3 with Anti-GPx3 Antibodies Stimulates Proinflammatory Gene Expression in Mouse Primary Adipocytes

A, Mouse primary adipocytes were obtained by collagenase digestion of adipose tissue from lean C57/BL6 mice. Cells were incubated with a rabbit control IgG (mock) or purified anti-GPx3 antibodies (IgG fraction) for 24 h. Total RNA isolated from each group was analyzed by quantitative real-time RT-PCR. B, GPx3 protein concentration in each sample was assessed by using a GPx3 ELISA kit.

dently decreased in the adipose tissue but increased slightly in the kidneys of obese animals such as *ob/ob* and *db/db* mice (Fig. 3) (supplemental Fig. 1). Additionally, GPx3 expression was specifically diminished in the adipocytes but remained unaltered in the SVCs of the fat tissues in obese mice (Fig. 3C). Moreover, the GPx3 expression in adipocytes was reduced in $CoCl_2$-induced hypoxic conditions (Fig. 4F), indicating that cell type-specific regulation of GPx3 in adipocytes due to hypoxia could be at least partly responsible for the down-regulation of GPx3 mRNA in the adipose tissue of obese mice.

Another potential mechanism responsible for reduced GPx3 expression in the adipocytes of obese mice is related to chronically augmented local inflammation with increased macrophage infiltration into the adipose tissue. We observed that the adipose GPx3 mRNA level was diminished by inflammatory signals of TNFα and LPS *in vitro* and *in vivo* (Fig. 4). TNFα increases the ROS generation in adipose tissue by stimulating iNOS expression and the NADPH oxidase activity in adipocytes and macrophages (63, 64). Thus, it would be plausible that adipose GPx3 expression is reduced by prooxidative conditions such as inflammation and hypoxia, which may induce further ROS accumulation in the adipose tissue and serum of obese animals. Consistent with this hypothesis, administration of the antioxidant molecule NAC to *db/db* mice increased adipose GPx3 expression (Fig. 4G).

TZDs are prominent antidiabetic drugs that are widely used for decreasing the fasting glucose levels and improving insulin resistance (43, 44). Additionally, recent evidence indicates that TZDs also exert protective effects against cardiovascular diseases, including atherosclerosis, thrombosis, and stroke, due to their antiinflammatory and antioxidative properties (42). Of the TZDs, troglitazone reduces ROS accumulation by directly scavenging superoxide anions (65, 66). Moreover, pioglitazone and rosiglitazone reduce systemic oxidative stress in diet-induced obese mice by unknown mechanisms in the absence of direct ROS scavenging (65). Here, we demonstrated that rosiglitazone can reduce systemic ROS accumulation by inducing GPx3 expression via the stimulation of PPARγ in adipose tissue (Fig. 5). It is interesting to note that adipose GPx3 expression was suppressed under stressful conditions such as inflammation and hypoxia (Fig. 4), wherein PPARγ expression is substantially reduced in adipocytes (37, 67). Moreover, GPx3 down-regulation in obese subjects was shown to be restricted to the adipocytes where PPARγ is dominantly expressed, but not in SVCs. These results imply that PPARγ is one of the major transcriptional regulators of GPx3 expression, at least in adipocytes, and explain why GPx3 was decreased most severely and selectively in the adipose tissue of obese subjects.

In summary, we have provided the first evidence that defective GPx3 expression in adipose tissue is associated with reduced systemic GPx activity and increased oxidative stress in obesity. Furthermore, we demonstrated that hypoxia and TNFα regulate GPx3 in a tissue-specific manner that is possibly regulated by PPARγ; this may induce obesity-related down-regulation of adipose GPx3 expression, leading to augmented systemic oxidative stress and the onset of metabolic complications such as diabetes and cardiovascular diseases. In this respect, it is possible to propose that local ROS accumulation in the adipose tissue of obesity could be expanded into systemic oxidative stress by the vicious cycle wherein increasing local ROS

accumulation suppresses adipose GPx3 expression. Thus, these results support further exploration of GPx3 expression in adipose tissue as a therapeutic target for obesity-related metabolic complications.

MATERIALS AND METHODS

Cell Culture

3T3-L1 preadipocytes were grown to confluence in DMEM supplemented with 10% bovine calf serum. At 2 d postconfluence, the 3T3-L1 cells were incubated for 48 h with DMEM containing 10% fetal bovine serum, methylisobutylxanthine (500 μM), dexamethasone (1 μM), and insulin (5 μg/ml). Every alternate day, the culture medium was replaced with DMEM containing 10% fetal bovine serum and insulin (1 μg/ml).

Adipose Tissue Culture

Mouse epididymal adipose tissue was evenly minced and incubated in DMEM with 0.1% BSA and antibiotics (penicillin and streptomycin), in the presence or absence of 10 ng/ml recombinant murine TNFα for 8 h.

Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR was performed as previously described (68). rRNA (18S) was used as the invariant control. The primers used were as follows: GPx3-forward (f), 5′-TAATTTCCAGCTCTTTGAGAAA-3′; GPx3-reverse (r), 5′-GGAACTTCTCAAAGTTCCAGCG-3′; iNOS-f, 5′-AATCTTGGGCGAGTTGTGG-3′; iNOS-r, 5′-CAGGAAGTAGGTGAGGGCTTG-3′; SAA3-f, 5′-AGTGATGCCAGAGAGGCTGT-3′; SAA3-r, 5′-ACCCAGTAGTTGCCCCTCTT-3′; resistin-f, 5′-CAGAAGGCACAGCAGTCTTG-3′; resistin-r, 5′-GACCGGAGGACATCAGACAT-3′; $p47^{phox}$-f, 5′-AGTGTTCCCCATTGAGGCCCG-3′; $p47^{phox}$-r, 5′-GTTTCAGGTCATCAGGCCGC-3′; $p67^{phox}$-f, 5′-CTGGCTGAGGCCATCAGACT-3′; $p67^{phox}$-r, 5′-AGGCCACTGCAGAGTGCTTG-3′; adiponectin-f, 5′-ATGCTACTGTTGCAAGCTCTC-3′; and adiponectin-r, 5′-GTTGGTATCATGGAAGAGAAG-3′.

GPx Activity Assay

GPx activity was measured according to the manufacturer's protocol (Northwest Life Science Specialties, Vancouver, WA).

Lipid Peroxidation (LPO) and H_2O_2 Concentration

The tissue samples were homogenized and centrifuged, and the supernatant was used for the subsequent assays. The levels of LPO in the plasma and tissue homogenate were measured in terms of TBARS by performing the LPO test (Oxford Biomedical Research, Rochester Hills, MI). The H_2O_2 concentration was measured using an Amplex Red hydrogen peroxide assay kit (Invitrogen, Carlsbad, CA).

Gel Shift Assay

Gel shift assays were performed as described previously (69). One microgram of plasmid DNA expressing PPARγ or RXRα was used as template for *in vitro* translation. The DNA sequences of the double-stranded oligonucleotides used as probe or competitors were as follows (only one strand is shown): ARE7, 5′-GATCTGGAACTCTGATCCAGTAAG-3′; PPRE1, 5′-GCTGGAGGTTAGAAGTCAACTCT-3′; PPRE2, 5′-TAATGGGGTCACAGGTTATGCCA-3′; PPRE3, 5′-GAATTTGAACTTTAACCCGAGG-3′; PPRE3-mutant, 5′-GAATTAAAACTTAAACCCGAGG-3′. Probe was end labeled with [γ-^{32}P]ATP. Purified probe (~30,000 cpm) and proteins were used in 20 μl binding reactions containing reaction buffer [10 mM Tris (pH 7.5), 50 mM KCl, 2.5 mM $MgCl_2$, 0.05 mM EDTA, 0.1% (vol/vol) Triton X-100, 8.5% (vol/vol) glycerol, 1 μg of polydeoxyinosinic deoxycytidylic acid, 1 mM dithiothreitol, and 0.1% (wt/vol) nonfat dry milk]. Samples were loaded onto a nondenaturing 4% polyacrylamide gel. The gels were dried and autoradiographed.

ChIP Analysis

ChIP assays were performed as described previously (68). Primers used were as follows: PPRE1-f, 5′-CAGCTAGTCACATGCCTCCA-3′; PPRE1-r, 5′-TCAGCAGGTAAAAGCCCTCA-3′; PPRE2-f, 5′-CCTGCCATTTATGTGGTGCT-3′; PPRE2-r, 5′-AACAAAACGGGGGAACAAAG-3′; PPRE3-f, 5′-TGCAGGTGAGGCTGAGCTAT-3′; PPRE3-r, 5′-GGAGGAGGCTGAAGCAGAAG-3′.

Animals and Treatments

All experiments were approved by the Seoul National University Animal Experiment Ethics Committee. Male C57BL/6J, *ob/ob*, and *db/db* mice were housed in colony cages under 12-h light, 12-h dark cycles. For rosiglitazone treatment, the mice received an oral gavage of the drug (5 mg/kg body weight) (Calbiochem, La Jolla, CA) daily for 10 d. After the final administration, the animals were fasted for 4 h, and the plasma glucose levels were tested to confirm the hypoglycemic effects (glucose level <200 mg/dl) of rosiglitazone. The animals were killed by cervical dislocation 1 d later. For LPS injection, the mice were ip injected with the vehicle (PBS) or with 5 μg (for 24 h) or 50 μg (for 4 h) LPS.

Human Serum Samples

Human serum samples provided by Samsung Medical Center were analyzed for GPx3 protein expression and GPx activity. The procedure for obtaining human serum samples was approved by the Samsung Medical Center Institutional Review Board (IRB file no. 2006-03-053), and written informed consent was obtained from the volunteers.

Measurement of Glucose Uptake

Insulin-stimulated glucose uptake in the 3T3-L1 adipocytes was determined by measuring the [^{14}C]2-deoxyglucose uptake as described previously (51). In short, adenovirus-infected 3T3-L1 adipocytes were incubated in low- (5.5 mM) or high-glucose (25 mM) DMEM containing 0.1% BSA for 24 h at 37 C. The cells were stimulated with 100 nM insulin for 1 h at 37 C or were left untreated. Glucose uptake was initiated by treatment with [^{14}C]2-deoxy-*d*-glucose at a final concentration of 3 μmol/liter in HEPES-buffered saline [140 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 20 mM HEPES (pH 7.4)] for 10 min. The reaction was terminated by separating the cells from the HEPES-buffered saline and [^{14}C]2-deoxy-*d*-glucose. After three washings in ice-cold PBS, the cells were extracted with 0.1% sodium dodecyl sulfate and subjected to scintillation counting to determine their ^{14}C radioactivity. The protein concentrations were determined using a bicinchoninic acid assay kit (Pierce Chemical Co., Rockford, IL), and the radioactivities were normalized by determining each protein concentration.

GPx3 Neutralization

Mouse primary adipocytes were prepared by collagen digestion. After washing three times with DMEM supplemented

with 0.2% BSA, cells were treated with either the IgG fraction of a polyclonal rabbit antibody to mouse GPx3 (1 μg/ml) or with equivalent mounts of normal rabbit IgG as a control for 24 h.

Measurement of GPx3 Concentration

GPx3 protein concentration was measured using ELISA according to the manufacturer's protocol (Adipogen, Seoul, Korea).

Statistical Analysis

All the results are presented as mean ± SEM. Statistical significance was assessed by Student's *t* test. Differences were considered statistically significant at $P < 0.05$.

Acknowledgments

We thank Joseph Loscalzo for providing the human GPx3 expression vector and the GPx3 promoter luciferase vector.

Received January 18, 2008. Accepted June 10, 2008.

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This work was supported by grants from the Korea Science and Engineering Foundation funded by the Korean government (Ministry of Education, Science and Technology, MEST) (nos. R0A-2004-000-10359-0, R11-2005-009-01002-0, M10748000258-07N4800-25810). A.Y.K., J.W.C., K.S.P., and J.B.K. were supported by a BK21 Research Fellowship from the Ministry of Education and Human Resources Development.

Disclosure Statement: the authors have nothing to disclose.

REFERENCES

1. Scherer PE 2006 Adipose tissue: from lipid storage compartment to endocrine organ. Diabetes 55:1537–1545
2. Trujillo ME, Scherer PE 2006 Adipose tissue-derived factors: impact on health and disease. Endocr Rev 27: 762–778
3. Keaney Jr JF, Larson MG, Vasan RS, Wilson PW, Lipinska I, Corey D, Massaro JM, Sutherland P, Vita JA, Benjamin EJ 2003 Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham Study. Arterioscler Thromb Vasc Biol 23:434–439
4. Fujita K, Nishizawa H, Funahashi T, Shimomura I, Shimabukuro M 2006 Systemic oxidative stress is associated with visceral fat accumulation and the metabolic syndrome. Circ J 70:1437–1442
5. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I 2004 Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest 114:1752–1761
6. Mates JM, Sanchez-Jimenez F 1999 Antioxidant enzymes and their implications in pathophysiologic processes. Front Biosci 4:D339–D345
7. Simonian NA, Coyle JT 1996 Oxidative stress in neurodegenerative diseases. Annu Rev Pharmacol Toxicol 36: 83–106
8. Stocker R, Keaney Jr JF 2004 Role of oxidative modifications in atherosclerosis. Physiol Rev 84:1381–1478
9. Grattagliano I, Palmieri VO, Portincasa P, Moschetta A, Palasciano G 2008 Oxidative stress-induced risk factors associated with the metabolic syndrome: a unifying hypothesis. J Nutr Biochem 19:491–504
10. Leopold JA, Loscalzo J 2005 Oxidative enzymopathies and vascular disease. Arterioscler Thromb Vasc Biol 25: 1332–1340
11. Roskams T, Yang SQ, Koteish A, Durnez A, DeVos R, Huang X, Achten R, Verslype C, Diehl AM 2003 Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease. Am J Pathol 163:1301–1311
12. Upchurch Jr GR, Ramdev N, Walsh MT, Loscalzo J 1998 Prothrombotic consequences of the oxidation of fibrinogen and their inhibition by aspirin. J Thromb Thrombolysis 5:9–14
13. Vadseth C, Souza JM, Thomson L, Seagraves A, Nagaswami C, Scheiner T, Torbet J, Vilaire G, Bennett JS, Murciano JC, Muzykantov V, Penn MS, Hazen SL, Weisel JW, Ischiropoulos H 2004 Pro-thrombotic state induced by post-translational modification of fibrinogen by reactive nitrogen species. J Biol Chem 279:8820–8826
14. Matsuoka T, Kajimoto Y, Watada H, Kaneto H, Kishimoto M, Umayahara Y, Fujitani Y, Kamada T, Kawamori R, Yamasaki Y 1997 Glycation-dependent, reactive oxygen species-mediated suppression of the insulin gene promoter activity in HIT cells. J Clin Invest 99:144–150
15. Maddux BA, See W, Lawrence Jr JC, Goldfine AL, Goldfine ID, Evans JL 2001 Protection against oxidative stress-induced insulin resistance in rat L6 muscle cells by mircomolar concentrations of α-lipoic acid. Diabetes 50:404–410
16. Rudich A, Tirosh A, Potashnik R, Hemi R, Kanety H, Bashan N 1998 Prolonged oxidative stress impairs insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. Diabetes 47:1562–1569
17. Houstis N, Rosen ED, Lander ES 2006 Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 440:944–948
18. Lin Y, Berg AH, Iyengar P, Lam TK, Giacca A, Combs TP, Rajala MW, Du X, Rollman B, Li W, Hawkins M, Barzilai N, Rhodes CJ, Fantus IG, Brownlee M, Scherer PE 2005 The hyperglycemia-induced inflammatory response in adipocytes: the role of reactive oxygen species. J Biol Chem 280:4617–4626
19. Cohen G, Hochstein P 1963 Glutathione peroxidase: the primary agent for the elimination of hydrogen peroxide in erythrocytes. Biochemistry 2:1420–1428
20. Izawa S, Inoue Y, Kimura A 1996 Importance of catalase in the adaptive response to hydrogen peroxide: analysis of acatalasaemic *Saccharomyces cerevisiae*. Biochem J 320:61–67
21. Drevet JR 2006 The antioxidant glutathione peroxidase family and spermatozoa: a complex story. Mol Cell Endocrinol 250:70–79
22. Maddipati KR, Marnett LJ 1987 Characterization of the major hydroperoxide-reducing activity of human plasma. Purification and properties of a selenium-dependent glutathione peroxidase. J Biol Chem 262:17398–17403
23. Takahashi K, Avissar N, Whitin J, Cohen H 1987 Purification and characterization of human plasma glutathione peroxidase: a selenoglycoprotein distinct from the known cellular enzyme. Arch Biochem Biophys 256: 677–686
24. Maeda K, Okubo K, Shimomura I, Mizuno K, Matsuzawa Y, Matsubara K 1997 Analysis of an expression profile of genes in the human adipose tissue. Gene 190:227–235
25. Urs S, Smith C, Campbell B, Saxton AM, Taylor J, Zhang B, Snoddy J, Jones Voy B, Moustaid-Moussa N 2004 Gene expression profiling in human preadipocytes and adipocytes by microarray analysis. J Nutr 134:762–770

26. Yamasaki T, Tahara K, Takano S, Inoue-Murayama M, Rose MT, Minashima T, Aso H, Ito S 2006 Mechanism of plasma glutathione peroxidase production in bovine adipocytes. Cell Tissue Res 326:139–147
27. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF 1995 A novel serum protein similar to C1q, produced exclusively in adipocytes. J Biol Chem 270:26746–26749
28. Chu FF, Esworthy RS, Doroshow JH, Doan K, Liu XF 1992 Expression of plasma glutathione peroxidase in human liver in addition to kidney, heart, lung, and breast in humans and rodents. Blood 79:3233–3238
29. Asayama K, Nakane T, Dobashi K, Kodera K, Hayashibe H, Uchida N, Nakazawa S 2001 Effect of obesity and troglitazone on expression of two glutathione peroxidases: cellular and extracellular types in serum, kidney and adipose tissue. Free Radic Res 34:337–347
30. Hotamisligil GS 2006 Inflammation and metabolic disorders. Nature 444:860–867
31. Zhang B, Berger J, Hu E, Szalkowski D, White-Carrington S, Spiegelman BM, Moller DE 1996 Negative regulation of peroxisome proliferator-activated receptor-γ gene expression contributes to the antiadipogenic effects of tumor necrosis factor-α. Mol Endocrinol 10:1457–1466
32. Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, Kuriyama H, Hotta K, Nakamura T, Shimomura I, Matsuzawa Y 2001 PPARγ ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. Diabetes 50:2094–2099
33. Murdoch C, Muthana M, Lewis CE 2005 Hypoxia regulates macrophage functions in inflammation. J Immunol 175:6257–6263
34. Fleischmann E, Kurz A, Niedermayr M, Schebesta K, Kimberger O, Sessler DI, Kabon B, Prager G 2005 Tissue oxygenation in obese and non-obese patients during laparoscopy. Obes Surg 15:813–819
35. Kabon B, Nagele A, Reddy D, Eagon C, Fleshman JW, Sessler DI, Kurz A 2004 Obesity decreases perioperative tissue oxygenation. Anesthesiology 100:274–280
36. Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K, Furukawa S, Tochino Y, Komuro R, Matsuda M, Shimomura I 2007 Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. Diabetes 56:901–911
37. Kim KH, Song MJ, Chung J, Park H, Kim JB 2005 Hypoxia inhibits adipocyte differentiation in a HDAC-independent manner. Biochem Biophys Res Commun 333: 1178–1184
38. Wang B, Wood IS, Trayhurn P 2007 Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. Pflugers Arch 455:479–492
39. Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT 1998 Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc Natl Acad Sci USA 95:11715–11720
40. An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, Neckers LM 1998 Stabilization of wild-type p53 by hypoxia-inducible factor 1α. Nature 392:405–408
41. Jiang BH, Zheng JZ, Leung SW, Roe R, Semenza GL 1997 Transactivation and inhibitory domains of hypoxia-inducible factor 1α. Modulation of transcriptional activity by oxygen tension. J Biol Chem 272:19253–19260
42. Kelly AS, Bank AJ 2007 The cardiovascular effects of the thiazolidinediones: a review of the clinical data. J Diabetes Complications 21:326–334
43. Berger J, Bailey P, Biswas C, Cullinan CA, Doebber TW, Hayes NS, Saperstein R, Smith RG, Leibowitz MD 1996 Thiazolidinediones produce a conformational change in peroxisomal proliferator-activated receptor-γ: binding and activation correlate with antidiabetic actions in db/db mice. Endocrinology 137:4189–4195
44. Nolan JJ, Ludvik B, Beerdsen P, Joyce M, Olefsky J 1994 Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. N Engl J Med 331:1188–1193
45. Saitoh Y, Chun-Ping C, Noma K, Ueno H, Mizuta M, Nakazato M 2007 Pioglitazone attenuates fatty acid-induced oxidative stress and apoptosis in pancreatic β-cells. Diabetes Obes Metab 10:564–573
46. Sommer M, Wolf G 2007 Rosiglitazone increases PPARγ in renal tubular epithelial cells and protects against damage by hydrogen peroxide. Am J Nephrol 27:425–434
47. Jung TW, Lee JY, Shim WS, Kang ES, Kim SK, Ahn CW, Lee HC, Cha BS 2007 Rosiglitazone protects human neuroblastoma SH-SY5Y cells against MPP+ induced cytotoxicity via inhibition of mitochondrial dysfunction and ROS production. J Neurol Sci 253:53–60
48. Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM 1994 mPPAR γ2: tissue-specific regulator of an adipocyte enhancer. Genes Dev 8:1224–1234
49. Lin Y, Rajala MW, Berger JP, Moller DE, Barzilai N, Scherer PE 2001 Hyperglycemia-induced production of acute phase reactants in adipose tissue. J Biol Chem 276:42077–42083
50. Gauss KA, Nelson-Overton LK, Siemsen DW, Gao Y, Deleo FR, Quinn MT 2007 Role of NF-κB in transcriptional regulation of the phagocyte NADPH oxidase by tumor necrosis factor-α. J Leukoc Biol 82:729–741
51. Park J, Rho HK, Kim KH, Choe SS, Lee YS, Kim JB 2005 Overexpression of glucose-6-phosphate dehydrogenase is associated with lipid dysregulation and insulin resistance in obesity. Mol Cell Biol 25:5146–5157
52. Park J, Choe SS, Choi AH, Kim KH, Yoon MJ, Suganami T, Ogawa Y, Kim JB 2006 Increase in glucose-6-phosphate dehydrogenase in adipocytes stimulates oxidative stress and inflammatory signals. Diabetes 55:2939–2949
53. Freedman JE, Loscalzo J, Benoit SE, Valeri CR, Barnard MR, Michelson AD 1996 Decreased platelet inhibition by nitric oxide in two brothers with a history of arterial thrombosis. J Clin Invest 97:979–987
54. Kenet G, Freedman J, Shenkman B, Regina E, Brok-Simoni F, Holzman F, Vavva F, Brand N, Michelson A, Trolliet M, Loscalzo J, Inbal A 1999 Plasma glutathione peroxidase deficiency and platelet insensitivity to nitric oxide in children with familial stroke. Arterioscler Thromb Vasc Biol 19:2017–2023
55. Rosen P, Nawroth PP, King G, Moller W, Tritschler HJ, Packer L 2001 The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society. Diabetes Metab Res Rev 17: 189–212
56. Packer L, Kraemer K, Rimbach G 2001 Molecular aspects of lipoic acid in the prevention of diabetes complications. Nutrition 17:888–895
57. McClung JP, Roneker CA, Mu W, Lisk DJ, Langlais P, Liu F, Lei XG 2004 Development of insulin resistance and obesity in mice overexpressing cellular glutathione peroxidase. Proc Natl Acad Sci USA 101:8852–8857
58. Mahadev K, Zilbering A, Zhu L, Goldstein BJ 2001 Insulin-stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1b in vivo and enhances the early insulin action cascade. J Biol Chem 276:21938–21942
59. Avissar N, Ornt DB, Yagil Y, Horowitz S, Watkins RH, Kerl EA, Takahashi K, Palmer IS, Cohen HJ 1994 Human kidney proximal tubules are the main source of plasma glutathione peroxidase. Am J Physiol 266:C367–C375
60. Yoshimura S, Suemizu H, Nomoto Y, Sakai H, Katsuoka Y, Kawamura N, Moriuchi T 1996 Plasma glutathione peroxidase deficiency caused by renal dysfunction. Nephron 73:207–211

61. Whitin JC, Tham DM, Bhamre S, Ornt DB, Scandling JD, Tune BM, Salvatierra O, Avissar N, Cohen HJ 1998 Plasma glutathione peroxidase and its relationship to renal proximal tubule function. Mol Genet Metab 65: 238–245
62. Bierl C, Voetsch B, Jin RC, Handy DE, Loscalzo J 2004 Determinants of human plasma glutathione peroxidase (GPx-3) expression. J Biol Chem 279:26839–26845
63. Kapur S, Marcotte B, Marette A 1999 Mechanism of adipose tissue iNOS induction in endotoxemia. Am J Physiol 276:E635–E641
64. Green SP, Hamilton JA, Uhlinger DJ, Phillips WA 1994 Expression of p47-phox and p67-phox proteins in murine bone marrow-derived macrophages: enhancement by lipopolysaccharide and tumor necrosis factor α but not colony stimulating factor 1. J Leukoc Biol 55:530–535
65. Da Ros R, Assaloni R, Ceriello A 2004 The preventive anti-oxidant action of thiazolidinediones: a new therapeutic prospect in diabetes and insulin resistance. Diabet Med 21:1249–1252
66. Noguchi N, Sakai H, Kato Y, Tsuchiya J, Yamamoto Y, Niki E, Horikoshi H, Kodama T 1996 Inhibition of oxidation of low density lipoprotein by troglitazone. Atherosclerosis 123:227–234
67. Petruschke T, Hauner H 1993 Tumor necrosis factor-α prevents the differentiation of human adipocyte precursor cells and causes delipidation of newly developed fat cells. J Clin Endocrinol Metab 76:742–747
68. Lee YS, Sohn DH, Han D, Lee HW, Seong RH, Kim JB 2007 Chromatin remodeling complex interacts with ADD1/SREBP1c to mediate insulin-dependent regulation of gene expression. Mol Cell Biol 27:438–452
69. Lee YS, Lee HH, Park J, Yoo EJ, Glackin CA, Choi YI, Jeon SH, Seong RH, Park SD, Kim JB 2003 Twist2, a novel ADD1/SREBP1c interacting protein, represses the transcriptional activity of ADD1/SREBP1c. Nucleic Acids Res 31:7165–7174

Molecular Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Obesity Research & Clinical Practice (2009) xxx, xxx–xxx

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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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Received 25 August 2008; received in revised form 21 October 2008; accepted 7 November 2008

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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1871-403X/$ – see front matter
doi:10.1016/j.orcp.2008.11.002

Please cite this article in press as: Kobayashi N, et al. 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. Obes Res Clin Pract (2009), doi:10.1016/j.orcp.2008.11.002

KEYWORDS
Glucocorticoid;
Obesity;
Diabetes;
11β-Hydroxysteroid dehydrogenase;
PPARγ

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

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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

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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 $\times$ 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 $\pm$ 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

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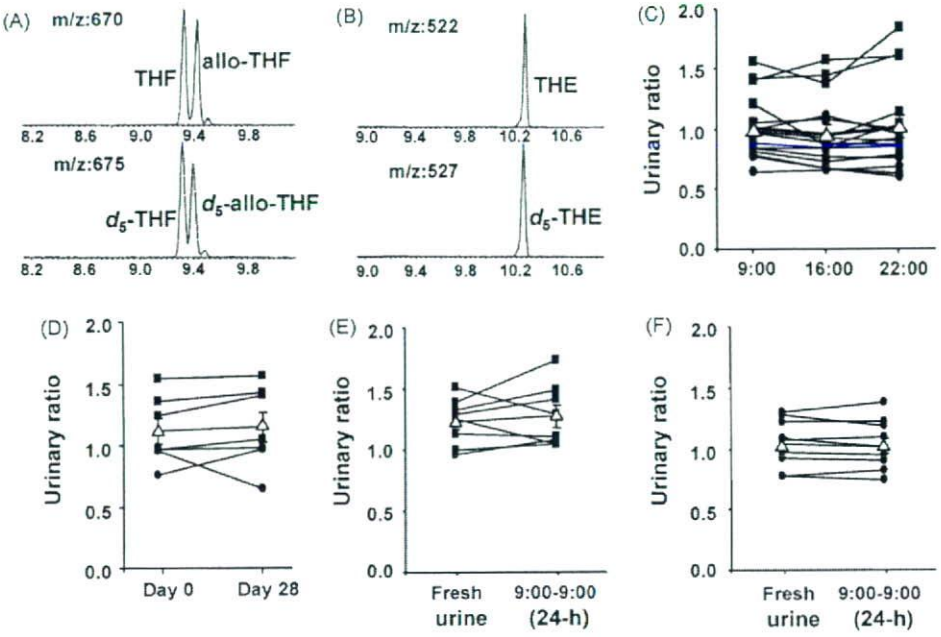

Figure 1 (A and B) Fresh urine samples (1 ml) were used to analyze the intracellular end products of glucocorticoid metabolism, namely, THF, allo-THF, and THE by gas chromatography and mass spectrometry [8–11]. Internal standards [1,2,3,4,5-$^2H_5$] tetrahydrocortisol (THF-d_5) [1,2,3,4,5-$^2H_5$] allo-tetrahydrocortisol (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,

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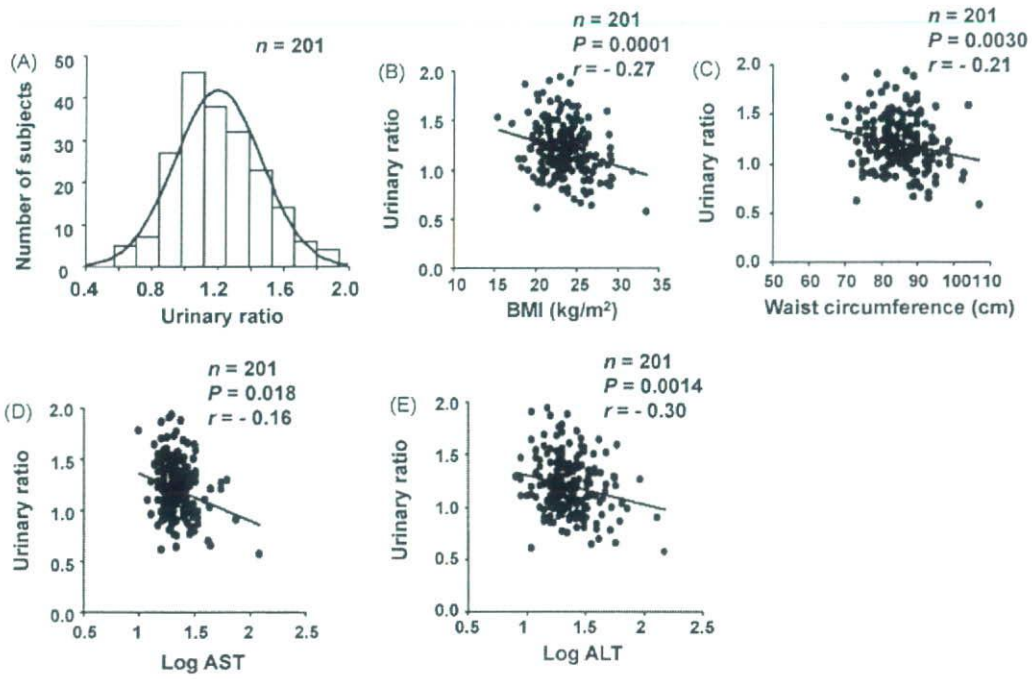

Figure 2 (A) Urinary ratio showed a normal distribution curve. Slight but significant inverse correlations were observed between the urinary ratio and BMI ($P < 0.001$) (B), waist circumference ($P < 0.01$) (C), log AST ($P < 0.05$) (D), and log ALT ($P < 0.01$) (E) in 201 Japanese males.

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 ± 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 ± 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 ± 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 ± 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 ± 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 ± 0.2) was much greater compared to that in group X (delta HbA1c = 0.05 ± 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 ± 0.05; $P = 0.0014$) (Fig. 4D). Accordingly, the decrease in the urinary ratio in group Y (delta urinary

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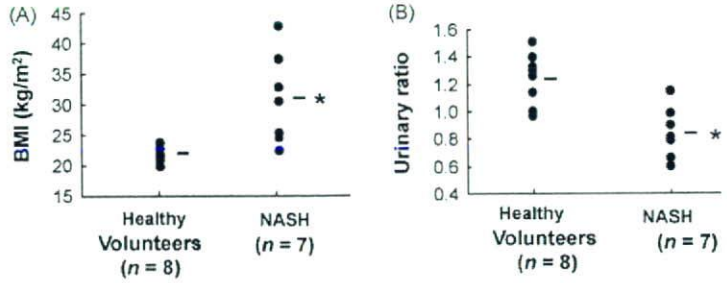

Figure 3 Values of BMI and the urinary ratio in each subject were shown as black circles. (A) The average value of BMI was significantly higher in patients with NASH than in healthy volunteers ($^{*}P < 0.01$). (B) The average value of the urinary ratio in NASH patients was significantly lower than that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

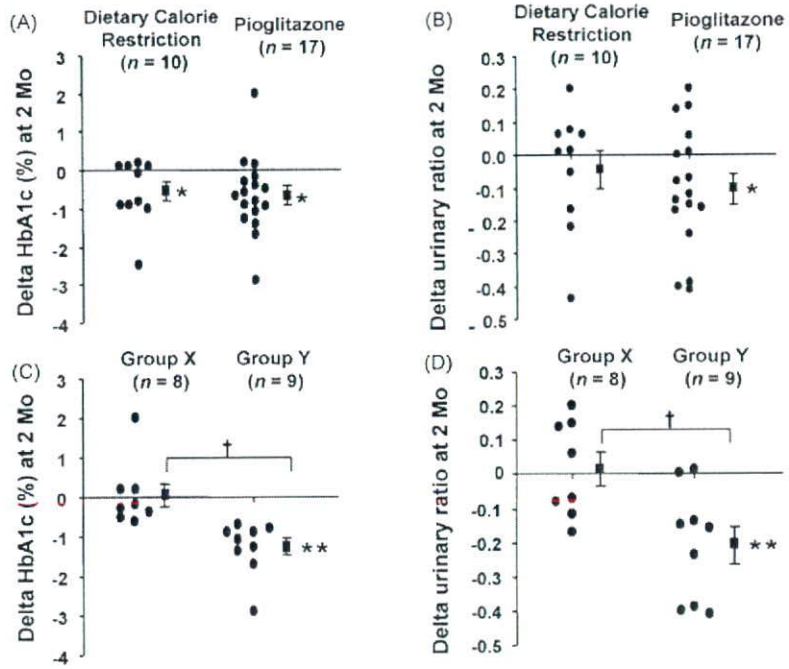

Figure 4 Urinary ratio from patients with type 2 diabetes who were treated with mild calorie restriction ($n = 10$, 3 males and 7 females; BMI, $30.7 \pm 1.3\,kg/m^2$; HbA1c, $8.0 \pm 0.3\%$) or pioglitazone ($n = 17$, 6 males and 11 females; BMI, $29.1 \pm 1.2\,kg/m^2$; HbA1c, $8.2 \pm 0.3\%$). (A) 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.

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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

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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-

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Table 2 Changes in the urinary ratio in diseases/treatments.

Diseases/treatments	Change in urinary ratio
Obese subjects	↓
NASH	↓
Mild calorie restriction	→
Pioglitazone	
Responded group (group Y)	↓
Non-responded group (group X)	→

The present study demonstrated an inverse correlation between the urinary ratio and BMI in a large Japanese cohort. Together with that the urinary ratio was also decreased in patients with NASH, decrease in 11β-HSD1 activity in liver of obese individuals may contribute, at least partly, to the fall in the urinary ratio in obesity. Although further studies are warranted, the finding that decrement of the urinary ratio was exaggerated in patients who responded to pioglitazone may be a reflection of 11β-HSD1 inhibition mainly in adipose tissue.

strated that growth hormone supplementation in patients with adult growth hormone deficiency (AGHD) markedly lowered the urinary ratio, reflecting 11β-HSD1 inhibition mainly in adipose tissue [34].

In summary, the present study is the first to provide novel evidence that the urinary ratio in fresh urine reflects a facet of metabolic function in adipose tissue and liver (Table 2), thereby offering a unique method to evaluate the metabolic status and therapeutic effectiveness in human clinics.

Conflicts of interest

None of the authors have any conflict of interest.

Acknowledgments

We thank H. Chusho, N. Saito and H. Aono for their help and discussions. We are grateful to M. Nagamoto for experimental assistance. This work was supported in part by Grants-in-Aid for Scientific Research (B2) and (S2), Research on Measures for Intractable Diseases (Health and Labor Science Research Grant), Special Coordination Funds for Promoting Science and Technology (JST), Research Grant of National Cardiovascular Center, Takeda Medical Research Foundation, Smoking Research Foundation and Sankyo Research Foundation.

References

[1] Kotelevtsev Y, Holmes MC, Burchell A, Houston PM, Schmoll D, Jamieson P, et al. 11beta-Hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. Proc Natl Acad Sci USA 1997;94(26):14924–9.

[2] Masuzaki H, Paterson J, Shinyama H, Morton NM, Mullins JJ, Seckl JR, et al. A transgenic model of visceral obesity and the metabolic syndrome. Science 2001;294(5549):2166–70.

[3] Matsuzawa Y. Therapy insight: adipocytokines in metabolic syndrome and related cardiovascular disease. Nat Clin Pract Cardiovasc Med 2006;3(1):35–42.

[4] Stewart PM. Tissue-specific Cushing's syndrome, 11beta-hydroxysteroid dehydrogenases and the redefinition of corticosteroid hormone action. Eur J Endocrinol 2003;149(3):163–8.

[5] Seckl JR. 11beta-Hydroxysteroid dehydrogenases: changing glucocorticoid action. Curr Opin Pharmacol 2004;4(6):597–602.

[6] Tomlinson JW, Stewart PM. Mechanisms of disease: selective inhibition of 11beta-hydroxysteroid dehydrogenase type 1 as a novel treatment for the metabolic syndrome. Nat Clin Pract Endocrinol Metab 2005;1(2):92–9.

[7] Valsamakis G, Anwar A, Tomlinson JW, Shackleton CH, McTernan PG, Chetty R, et al. 11beta-Hydroxysteroid dehydrogenase type 1 activity in lean and obese males with type 2 diabetes mellitus. J Clin Endocrinol Metab 2004;89(9):4755–61.

[8] Andrew R, Phillips DI, Walker BR. Obesity and gender influence cortisol secretion and metabolism in man. J Clin Endocrinol Metab 1998;83(5):1806–9.

[9] Stewart PM, Boulton A, Kumar S, Clark PM, Shackleton CH. Cortisol metabolism in human obesity: impaired cortisone → cortisol conversion in subjects with central adiposity. J Clin Endocrinol Metab 1999;84(3):1022–7.

[10] Rask E, Olsson T, Soderberg S, Andrew R, Livingstone DE, Johnson O, et al. Tissue-specific dysregulation of cortisol metabolism in human obesity. J Clin Endocrinol Metab 2001;86(3):1418–21.

[11] Rask E, Walker BR, Soderberg S, Livingstone DE, Eliasson M, Johnson O, et al. Tissue-specific changes in peripheral cortisol metabolism in obese women: increased adipose 11beta-hydroxysteroid dehydrogenase type 1 activity. J Clin Endocrinol Metab 2002;87(7):3330–6.

[12] Draper N, Echwald SM, Lavery GG, Walker EA, Fraser R, Davies E, et al. Association studies between microsatellite markers within the gene encoding human 11beta-hydroxysteroid dehydrogenase type 1 and body mass index, waist to hip ratio, and glucocorticoid metabolism. J Clin Endocrinol Metab 2002;87(11):4984–90.

[13] Westerbacka J, Yki-Jarvinen H, Vehkavaara S, Hakkinen AM, Andrew R, Wake DJ, et al. Body fat distribution and cortisol metabolism in healthy men: enhanced 5beta-reductase and lower cortisol/cortisone metabolite ratios in men with fatty liver. J Clin Endocrinol Metab 2003;88(10):4924–31.

[14] 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 2005;54(3):872–9.

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[15] Paterson JM, Morton NM, Fievet C, Kenyon CJ, Holmes MC, Staels B, et al. Metabolic syndrome without obesity: hepatic overexpression of 11beta-hydroxysteroid dehydrogenase type 1 in transgenic mice. Proc Natl Acad Sci USA 2004;101(18):7088–93.

[16] Ulick S, Tedde R, Mantero F. Pathogenesis of the type 2 variant of the syndrome of apparent mineralocorticoid excess. J Clin Endocrinol Metab 1990;70(1):200–6.

[17] Bujalska IJ, Kumar S, Stewart PM. Does central obesity reflect "Cushing's disease of the omentum"? Lancet 1997;349(9060):1210–3.

[18] 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 2002;87(6):2701–5.

[19] Lindsay RS, Wake DJ, Nair S, Bunt J, Livingstone DE, Permana PA, et al. Subcutaneous adipose 11 beta-hydroxysteroid dehydrogenase type 1 activity and messenger ribonucleic acid levels are associated with adiposity and insulinemia in Pima Indians and Caucasians. J Clin Endocrinol Metab 2003;88(6):2738–44.

[20] 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 2003;88(8):3983–8.

[21] Livingstone DE, Jones GC, Smith K, Jamieson PM, Andrew R, Kenyon CJ, et al. Understanding the role of glucocorticoids in obesity: tissue-specific alterations of corticosterone metabolism in obese Zucker rats. Endocrinology 2000;141(2):560–3.

[22] Andrews RC, Herlihy O, Livingstone DE, Andrew R, Walker BR. Abnormal cortisol metabolism and tissue sensitivity to cortisol in patients with glucose intolerance. J Clin Endocrinol Metab 2002;87(12):5587–93.

[23] Vogt B, Dick B, N'Gankam V, Frey FJ, Frey BM. Reduced 11beta-hydroxysteroid dehydrogenase activity in patients with the nephrotic syndrome. J Clin Endocrinol Metab 1999;84(2):811–4.

[24] Yoshizumi T, Nakamura T, Yamane M, Islam AH, Menju M, Yamasaki K, et al. Abdominal fat: standardized technique for measurement at CT. Radiology 1999;211(1):283–6.

[25] Brunt EM. Nonalcoholic steatohepatitis: definition and pathology. Semin Liver Dis 2001;21(1):3–16.

[26] Masuzaki H, Flier JS. Tissue-specific glucocorticoid reactivating enzyme, 11beta-hydroxysteroid dehydrogenase type 1 (11beta-HSD1)—a promising drug target for the treatment of metabolic syndrome. Curr Drug Targets Immune Endocr Metabol Disord 2003;3(4):255–62.

[27] Furuta T, Namekawa T, Shibasaki H, Kasuya Y. Simultaneous determination of tetrahydrocortisol and tetrahydrocortisone in human plasma and urine by stable isotope dilution mass spectrometry. J Chromatogr B Biomed Sci Appl 1998;706(2):181–90.

[28] Shackleton CH. Mass spectrometry in the diagnosis of steroid-related disorders and in hypertension research. J Steroid Biochem Mol Biol 1993;45(1–3):127–40.

[29] Eriksson S, Eriksson KF, Bondesson L. Nonalcoholic steatohepatitis in obesity: a reversible condition. Acta Med Scand 1986;220(1):83–8.

[30] Brunt EM. Nonalcoholic steatohepatitis. Semin Liver Dis 2004;24(1):3–20.

[31] Berger J, Tanen M, Elbrecht A, Hermanowski-Vosatka A, Moller DE, Wright SD, et al. Peroxisome proliferator-activated receptor-gamma ligands inhibit adipocyte 11beta-hydroxysteroid dehydrogenase type 1 expression and activity. J Biol Chem 2001;276(16):12629–35.

[32] Xu C, Wang LL, Liu HY, Ruan CM, Zhou XB, Cao YL, et al. A novel dual peroxisome proliferator-activated receptors alpha and gamma agonist with beneficial effects on insulin resistance and lipid metabolism. Biotechnol Lett 2006;28(12):863–8.

[33] Satoh N, Ogawa Y, Usui T, Tagami T, Kono S, Uesugi H, et al. Antiatherogenic effect of pioglitazone in type 2 diabetic patients irrespective of the responsiveness to its antidiabetic effect. Diabetes Care 2003;26(9):2493–9.

[34] Paulsen SK, Pedersen SB, Jorgensen JO, Fisker S, Christiansen JS, Flyvbjerg A, et al. Growth hormone (GH) substitution in GH-deficient patients inhibits 11beta-hydroxysteroid dehydrogenase type 1 messenger ribonucleic acid expression in adipose tissue. J Clin Endocrinol Metab 2006;91(3):1093–8.

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