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ORIGINAL ARTICLE

Effects of the AT_1 receptor blocker losartan and the calcium channel blocker benidipine on the accumulation of lipids in the kidney of a rat model of metabolic syndrome

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Unfavorable lipid accumulation may occur in the kidneys in the presence of metabolic syndrome and diabetes. The aim of this study was to investigate whether excess lipids would accumulate in the kidneys of Otsuka Long-Evans Tokushima Fatty (OLETF) rats, an animal model of metabolic syndrome. From 34 weeks of age, OLETF rats were treated orally with a calcium channel blocker, benidipine (3 mg kg^{-1} per day), or an AT1 receptor blocker, losartan (25 mg kg^{-1} per day), for 8 weeks. Blood pressure was slightly but significantly higher in the untreated OLETF rats (149 ± 4 mm Hg) than in Long-Evans Tokushima Otsuka (LETO) rats (136 ± 2 mm Hg), and both losartan (135 ± 3 mm Hg) and benidipine (138 ± 3 mm Hg) reduced blood pressure in OLETF rats to a level comparable to that in LETO rats. Tissue content of triglycerides (TG) was greater in OLETF rats than in LETO rats (6.24 ± 3.77 and 2.85 ± 1.32 μg mg^{-1} · tissue, respectively), and both losartan and benidipine reduced these values. Histological analysis showed lipid droplets in tubular cells in which increased dihydroethidium fluorescence was present. Expression of peroxisome proliferator-activated receptor-α, PGC-1α and uncoupling protein-2 was found to be higher in OLETF rats than in LETO rats; however, the expression of these genes was not altered by treatment with either antihypertensive drug. In contrast, both losartan and benidipine increased the amount of total and phosphorylated forms of AMP kinase and the expression of carnitine palmitoyltransferase-1 (CPT-1). In conclusion, treatment of OLETF rats with losartan and benidipine reduced the tissue content of TG, decreased the production of superoxide and regulated the expression of genes related to fatty acid oxidation such as AMP-activated protein kinase and CPT-1 in the kidneys.
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Keywords: calcium channel blocker; diabetes; kidney; lipotoxicity; renin angiotensin system

INTRODUCTION

Unfavorable lipid accumulation in the kidneys may occur in animal models of diabetes,[1–3] aging,[4] diet-induced obesity[5,6] and nephrectomy.[7] Although the precise mechanism by which lipid content is increased in the kidneys is still not fully elucidated, it may include upregulation of lipogenic gene expression in the kidneys[1,7,8] and uptake of filtered albumin-bound fatty acids by renal tubular cells when increased urinary excretion of albumin is present.[9] Transfer of lipogenic genes induced deposition of lipids and upregulation of fibrosis-related gene expression in the kidneys of diabetic animals, whereas lipogenic gene knockdown had the opposite effect,[1,8] suggesting that the accumulation of excessive lipid in the kidneys is one factor in the pathophysiological process of diabetic nephropathy.[6]

We reported earlier that administration of angiotensin II upregulates the expression of lipogenic genes and increases lipid content in the kidneys,[10] suggesting that activation of the renin angiotensin system may have a role in lipid accumulation in the kidney. It has been reported that lipid content is increased in the liver[11] and pancreas[12] of OLETF rats. In this study, therefore, we investigated whether excessive lipid accumulation occurs in the kidneys of Otsuka Long-Evans Tokushima Fatty (OLETF) rats, which exhibit features of metabolic syndrome,[13] and if present, whether angiotensin II receptor blockers and calcium channel blockers could exert similar effects on renal lipid content in OLETF rats. We used benidipine as the calcium channel blocker because this drug has been reported to reduce the extent of proteinuria in OLETF rats.[14]

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METHODS

Animals

The experiments were performed in accordance with the guidelines for animal experimentation approved by the Animal Center for Biomedical Research, Faculty of Medicine, University of Tokyo. Male OLETF and age-matched Long-Evans Tokushima Otsuka (LETO) rats, a genetic control, were obtained from the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan) and maintained under constant temperature and lighting conditions with free access to food and water. At 34 weeks of age, the OLETF rats were given 3 mg kg^{-1} benidipine or 25 mg kg^{-1} losartan per day orally, which was continued for 8 weeks. One day before sacrifice, the rats were kept in a metabolic cage, and urine was collected for 24 h under fasting conditions. Systolic blood pressure and heart rate were measured in conscious rats by tail-cuff plethysmography (BP-98A, Softron, Tokyo, Japan).

Measurement of lipid content in the serum and kidney

Serum levels of total cholesterol (TC), triglycerides (TG) and non-esterified fatty acid were measured by enzymatic methods (SRL, Tokyo, Japan). Contents of TG and TC in the kidney were measured from homogenate extracts by enzymatic colorimetric determination using the Triglyceride-E Test, the Cholesterol-E Test and the Free cholesterol-E Test, respectively (Wako Pure Chemicals, Osaka, Japan).

Histological analysis

Oil red O staining was performed on sections of unfixed, freshly frozen kidney samples (3 μm in thickness). For semi-quantification of lipid deposition, images of each specimen stained with oil red O were taken with an Olympus BX51 microscope and a DP12 digital camera system (Olympus, Tokyo, Japan). Five images taken in the cortical region of each sample were analyzed. The ratio of the areas of lipid deposition to the total tissue region area was calculated using Adobe Photoshop image analysis software (Adobe Systems, San Jose, CA, USA). *In situ* superoxide production was estimated using the oxidative fluorescent dye dihydroethidium (DHE) in unfixed frozen kidney specimens as described earlier.[10] Images were obtained from at least five fields in each section, and signal intensity was presented as a percentage of that in OLETO rats.

Western blot analysis

Western blot analysis was performed as described earlier.[15] Antibodies against total and phosphorylated forms of AMP-activated protein kinase (AMPK) (Cell Signaling Technology, Danvers, MA, USA), sterol regulatory element-binding protein (SREBP)-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and total and phosphorylated forms of acetyl-CoA carboxylase (ACC) (Cell Signaling Technology) were used at a dilution of 1/1000.

Real time RT-PCR

The mRNA expression of lipid metabolism-related genes was analyzed by real-time quantitative PCR performed using a LightCycler together with Hybri-Probe technology (Roche Diagnostics, Basel, Switzerland). The expression of target genes was normalized to the mRNA expression of the endogenous control, glyceraldehyde-3-phosphate dehydrogenase. The target genes were SREBP-1c, fatty acid synthase (FAS), 3-hydroxy-3-methylglutaryl coenzyme A reductase, peroxisome proliferator-activated receptor (PPAR)-γ, PPAR-α, PPAR-γ coactivator (PGC)-1α, CD36, carnitine palmitoyltransferase (CPT)-1 and uncoupling protein (UCP)-2. The forward and reverse primers used were described earlier.[16]

Statistical analysis

Data are expressed as mean ± s.e.m. ANOVA and Kruskal–Wallis analyses followed by a *post hoc* multiple comparison test were performed using the statistical analysis software Dr. SPSS II (SPSS Inc., Chicago, IL, USA). A value of $P<0.05$ was taken to be statistically significant.

RESULTS

Characteristics of the experimental animals

Body weight, blood pressure, heart rate and blood levels of lipids and glucose in each group have been described elsewhere.[17] Blood pressure was slightly but significantly higher in the untreated OLETF rats (149 ± 4 mm Hg, n=11, P=0.012) than in LETO rats (136 ± 2 mm Hg, n=11), and both losartan (135 ± 3 mm Hg, n=6) and benidipine (138 ± 3 mm Hg, n=11) reduced blood pressure in OLETF rats to a level comparable to that in LETO rats. Treatment of OLETF rats with either antihypertensive drug had no significant effect on circulating levels of triglyceride and glucose, which were higher in OLETF than in LETO rats. Compared with LETO rats, kidney weight was greater in the untreated OLETF rats and the OLETF rats treated with either losartan or benidipine, but no significant difference was observed in creatinine clearance among the groups examined (Figure 1). Urinary protein excretion was greater in OLETF rats than in LETO rats, and both losartan and benidipine reduced proteinuria to a similar extent.

Accumulation of lipids in the kidney

The content of TG in the kidney was significantly greater in untreated OLETF rats than in LETO rats, and both losartan and benidipine treatment reduced renal TG content in the OLETF rats (Figure 2a). The content of TC in the kidney was not significantly different between LETO and untreated OLETF rats; however, both antihyper-

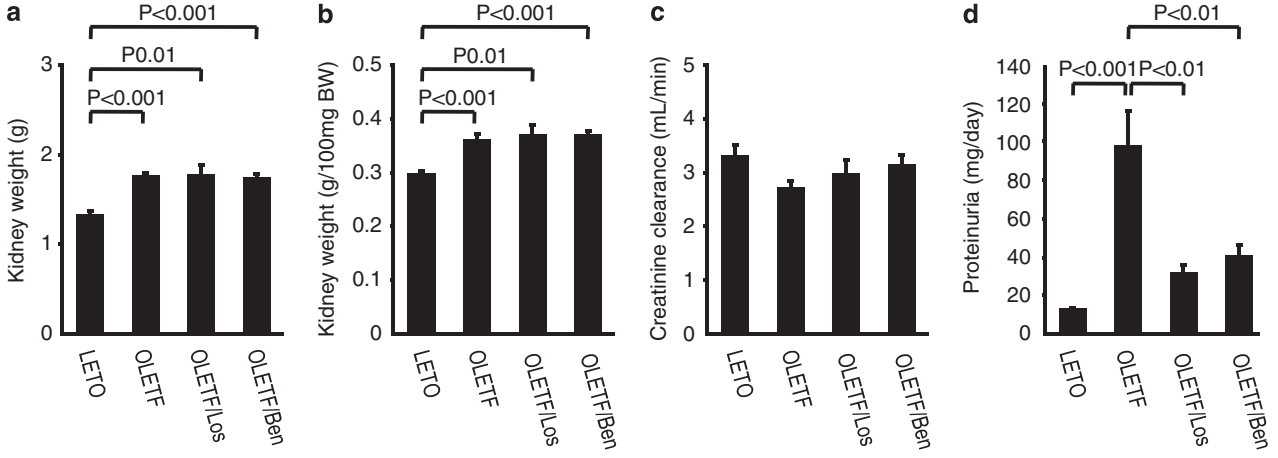

Figure 1 Kidney weight, creatinine clearance and proteinuria in LETO rats and untreated and antihypertensive drug-treated OLETF rats. Absolute values of kidney weight (**a**) and kidney weight expressed per 100 g body weight (**b**). Creatinine clearance (**c**) and daily excretion of urinary protein (**d**). Summary of data from four to six rats in each group.

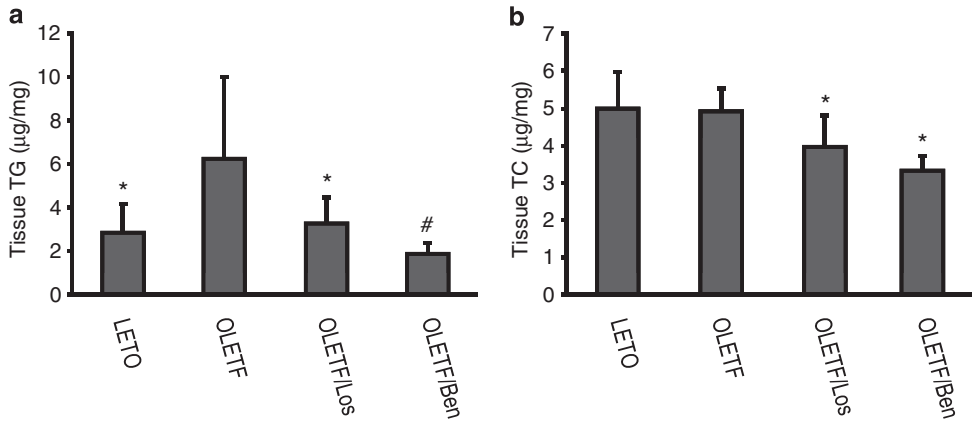

Figure 2 Tissue lipid content in the kidneys of LETO rats and untreated and antihypertensive drug-treated OLETF rats. Content of triglycerides (TG) (**a**) and total cholesterol (TC) (**b**) in the kidneys of LETO (n=4), OLETF (n=8), OLETF/Los (n=8) and OLETF/Ben (n=8) groups is shown.

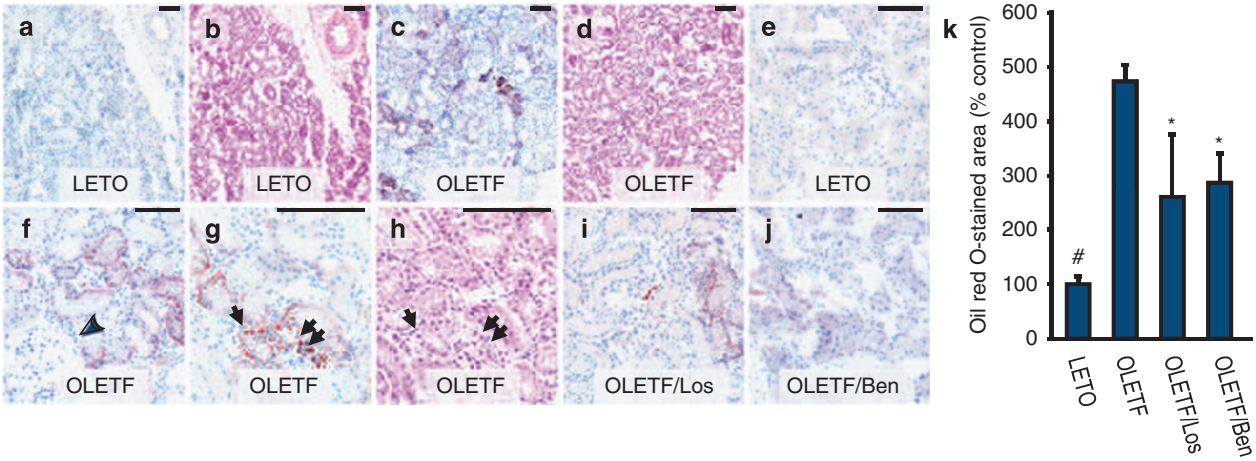

Figure 3 Accumulation of lipids in the kidney. (**a**, **b**, **e**) Kidney sections from LETO rats. (**c**, **d**, **f**–**h**) Kidney sections from untreated OLETF rats. (**i**, **j**) Kidney sections from OLETF rats treated with losartan (**i**) and benidipine (**j**). (**a**, **c**, **e**–**g**, **i**, **j**) Oil red O staining. (**b**, **d**, **h**) Hematoxylin and eosin staining; (**a**, **b**), (**c**, **d**) and (**g**, **h**) are serial sections. Lipid droplets were observed in the tubular (**c**, **g**), but not glomerular (**f**, arrowhead), regions of kidney from untreated OLETF rats. In some hematoxylin and eosin-stained specimens, lipid droplets could be identified by unstained small vesicles (**g**, **h**, arrows). The extent of lipid accumulation in the tubular cells was diminished by treatment of OLETF rats with either losartan (**i**) or benidipine (**j**). Scale bars indicate 100 μm. (**k**) Semi-quantification of the oil red O-stained area. Summary of data from five to seven experiments in each group. Kruskal–Wallis analyses followed by a *post hoc* multiple comparison test were performed. $^{*}P<0.05$ and $^{\#}P<0.01$ versus untreated OLETF rats.

tensive drugs reduced renal TC content in OLETF rats (Figure 2b). Histological analysis showed that only a trace amount of oil red O-positive lipid droplets was present in the kidneys of LETO rats (Figure 3). By contrast, increased lipid droplets were observed in the tubular and interstitial regions of untreated OLETF rats. Some lipid droplets appeared as small cavities on hematoxylin and eosin-stained frozen specimens (Figures 3g and h, arrows). Areas of oil red O-positive deposits were significantly reduced by the treatment of OLEFT rats with losartan or benidipine (Figures 3i–k). The correlation coefficients between tissue TG content and the extent of proteinuria and between the oil red O stained area and the extent of proteinuria were 0.40 ($P<0.05$, n=33) and 0.65 ($P<0.001$, n=36), respectively.

Localization of superoxide

Fluorescent signals on DHE staining were greater in untreated OLETF rats than in LETO rats and were reduced by treatment with either losartan or benidipine (Figure 4). In untreated OLETF rat kidney, DHE signals were found to be increased in tubular epithelial (Figures 4e–j, arrowheads) and vascular wall cells (Figures 4e–g, arrows), the former of which contained lipid droplets.

Regulation of genes related to lipid metabolism

The expression of mature SREBP-1 protein did not significantly differ among the four groups examined (Figure 5). Compared with LETO rats, expression of both total and phosphorylated forms of AMPK α was increased in OLETF rats treated with either losartan or benidipine, although it was not increased in untreated OLETF rats. The expression of total ACC protein was unaffected by losartan or benidipine in OLETF rats; however, treatment with either antihypertensive drug significantly increased the amount of the phosphorylated form of ACC.

Among the genes tested, mRNA expression of SREBP-1c, FAS, 3-hydroxy-3-methylglutaryl coenzyme A reductase, PPAR-γ, LDL-r and CD36 did not significantly differ between untreated OLETF and

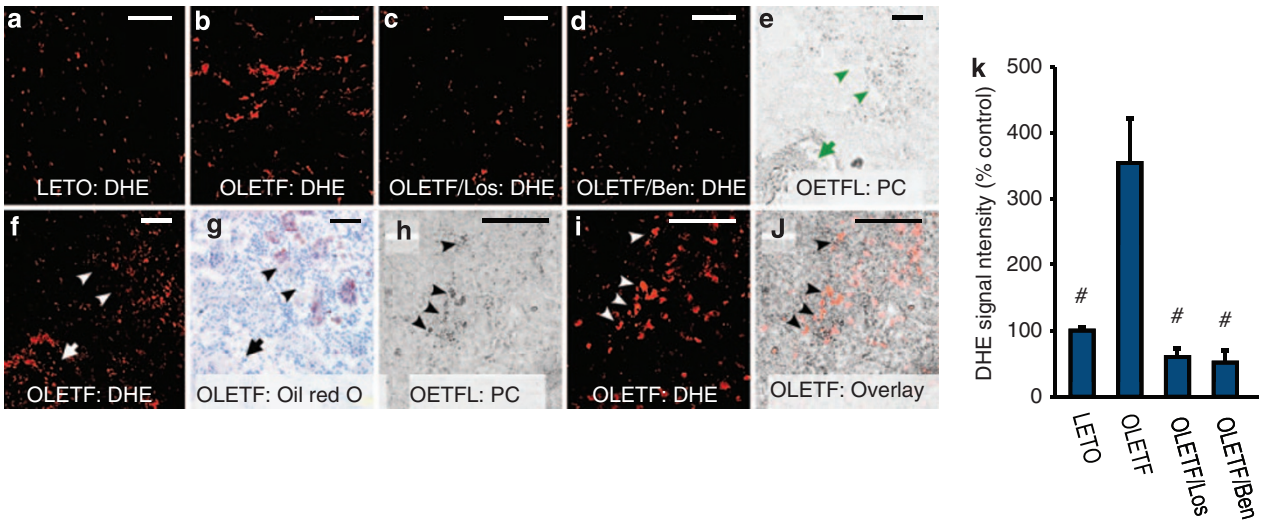

Figure 4 Localization of lipid droplets and superoxide in kidney sections. (**a**) Kidney sections from LETO rats. (**b**, **e**–**j**) Kidney sections from untreated OLETF rats. (**c**, **d**) Kidney sections from OLETF rats treated with losartan (**c**) and benidipine (**d**). (**a**–**d**, **f**, **i**) Dihydroethidium (DHE) staining. (**e**, **h**) Phase contrast (PC) microscopic images. (**j**) PC microscopic image overlaid with DHE stained images. (**g**) Oil red O staining; (**e**, **f**) and (**h**–**j**) are the same section; (**e**–**g**) are serial sections. DHE signals were more intense in OLETF kidneys (**b**) than in LETO kidneys (**a**), and both losartan (**c**) and benidipine (**d**) reduced DHE signal intensity. Granular droplets could be observed in the tubular regions, presumably lipid droplets, by PC imaging (**e**, arrowheads) of the unstained specimens, and DHE-stained superoxide was increased in these regions and in vascular wall cells (arrow, **e**, **f**). Oil red O staining (**h**) of the serial specimen confirmed that granular materials were lipid droplets. Higher magnification imaging demonstrated that DHE signals were increased in cells with granular droplets. Scale bars indicate 100 μm. (**k**) Semi-quantification of the DHE signals. Summary of data from five to seven experiments in each group. Kruskal–Wallis analyses followed by a *post hoc* multiple comparison test were performed. $^{\#}P<0.01$ versus untreated OLETF rats.

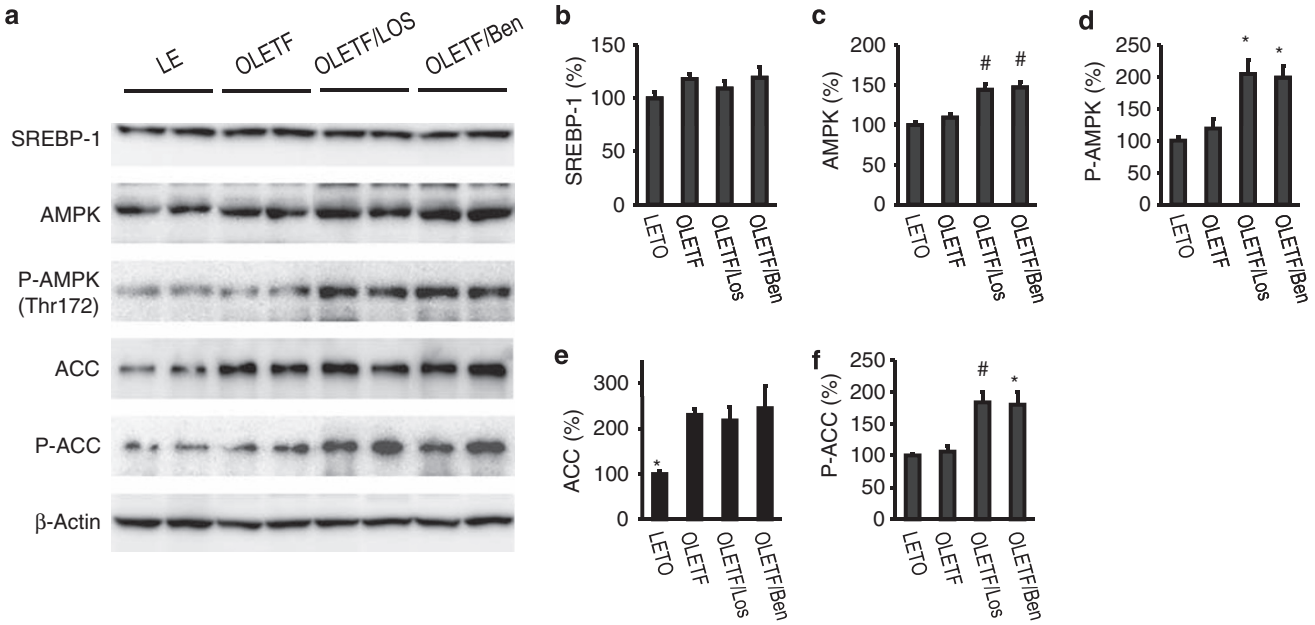

Figure 5 Western blot analysis. Western blot of sterol regulatory element-binding protein-1 (SREPB-1), AMP-activated protein kinase α (AMPK), the phosphorylated (activated) form of AMPK α (P-AMPK), acetyl-CoA carboxylase (ACC) and phosphorylated ACC. (**a**–**f**) Summary of data from four to six experiments in each group. $^{*}P<0.01$ and $^{\#}P<0.05$ versus untreated OLETF rats by Dunnett's *post hoc* analysis.

LETO rats (Figure 6). By contrast, mRNA expression of PPAR-α and UCP-2 was greater, whereas that of PGC-1α was lower, in untreated, losartan-treated and benidipine-treated OLETF rats than in LETO rats. Although the mRNA expression of CPT-1 did not differ between untreated OLETF and LETO rats, treatment of OLETF rats with either losartan or benidipine significantly increased CPT-1 expression.

DISCUSSION

In this study, we found that TG and TC contents were increased in the kidneys of untreated OLETF rats compared with LETO rats. In the kidneys of OLETF rats, oil red O-positive lipid droplets were observed mainly in tubular epithelial cells, in which increased superoxide was present. Treatment of OLETF rats with either losartan or benidipine,

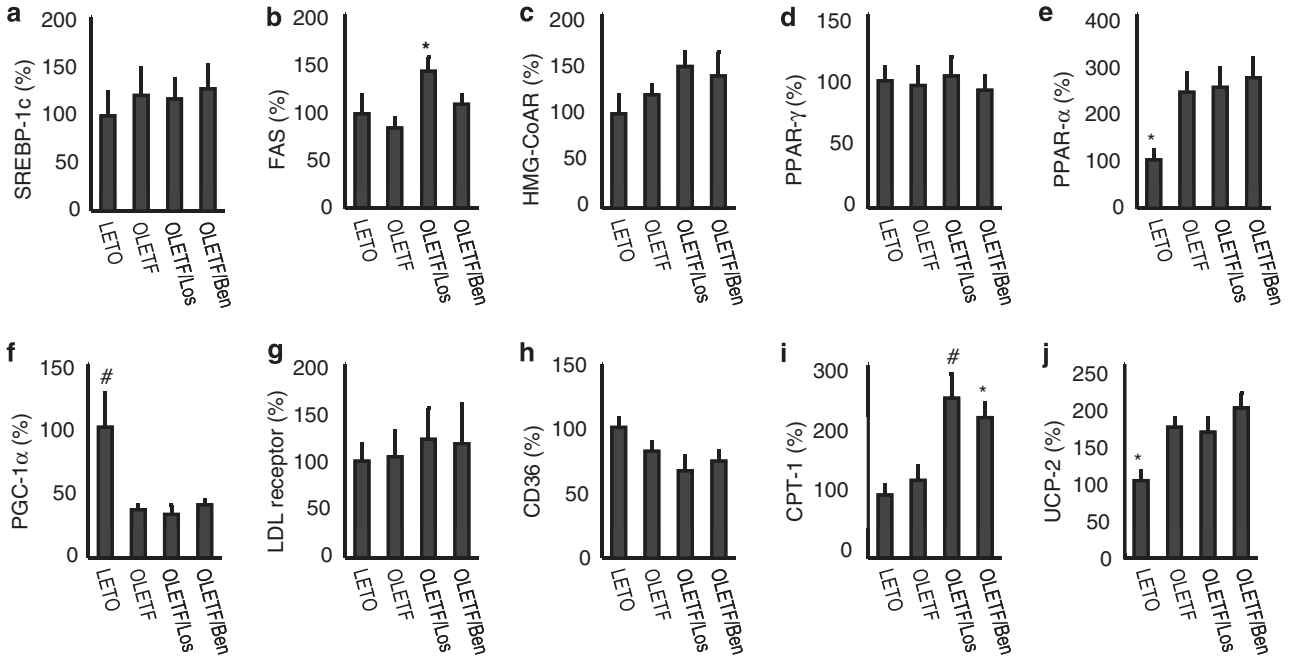

Figure 6 mRNA expression and regulation of lipid metabolism-related genes. (**a**) Sterol regulatory element-binding protein-1c (SREBP-1c). (**b**) Fatty acid synthase (FAS). (**c**) 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR). (**d**) Peroxisome proliferator-activated receptor (PPAR)-γ. (**e**) PPAR-α. (**f**) PPAR-γ coactivator (PGC)-1α. (**g**) LDL receptor. (**h**) CD36. (**i**) Carnitine palmitoyltransferase (CPT)-1. (**j**) Uncoupling protein (UCP)-2. Summary of data from six to ten experiments in each group $^{*}P<0.01$ and $^{\#}P<0.05$ versus untreated OLETF rats by Dunnett's *post hoc* analysis.

both of which lowered blood pressure to a similar extent, reduced tissue TG content, oil red O-positive deposits, superoxide signals and urinary protein excretion in the kidneys to a similar extent.

The mechanisms underlying lipid accumulation in the kidney in various animal models and in humans have not been fully elucidated; however, the presumed mechanisms include the regulation of genes related to the uptake, biosynthesis, catabolism and efflux of lipids[1,3,4,10,18] and the uptake of fatty acids carried on filtered albumin by the renal tubules.[7,9] What is the possible mechanism underlying the reduction in the renal lipid content of OLETF rats caused by both losartan and benidipine? We reported earlier that long-term administration of angiotensin II upregulates the expression of SREBP-1 and FAS and increases the renal content of lipids, which was suppressed by losartan. This effect of losartan may, at least in part, be independent of its depressor effect.[19] Therefore, it is possible that benidipine may also reduce renal lipid content through a mechanism independent of its depressor effect. To our knowledge, however, no earlier studies have shown that a calcium channel blocker can regulate the expression of certain lipogenic gene and/or reduce tissue lipid content. Toblli *et al.*[20] reported that renin angiotensin system inhibition, but not calcium channel blockade, suppressed lipid deposition in the heart[20] and liver[21] in obese Zucker rats.

In this study, the expression of SREBP-1 and FAS was not upregulated in the kidneys of OLETF rats compared with LETO rats, and neither losartan nor benidipine reduced the expression of these genes in the kidneys of OLETF rats. Therefore, it can be stated that the mechanism by which lipid content increased in the kidneys of OLETF rats may be, at least in part, different from that in angiotensin II-infused rats. This could be the reason why losartan was not more effective than benidipine in terms of reducing lipid content in the kidney.

As enhanced urinary albumin excretion may lead to the subsequent tubular absorption of lipid-bound albumin,[7,9] another possibility is that both losartan and benidipine reduced renal lipid content by their anti-proteinuric effect. Several studies have reported that benidipine may have a greater anti-proteinuric effect than other CCBs such as amlodipine,[22] and the renoprotective effect of benidipine may be, in part, mediated by the preservation of an essential cofactor of nitric oxide synthase, (6R)-5,6,7,8–tetrahydrobiopterin.[14] As both losartan and benidipine reduced the extent of proteinuria, both drugs might reduce the transport of the albumin-bound fatty acid to tubular cells. The close relationship between the extent of lipid deposition and proteinuria observed in this study may support this notion. Furthermore, the reduction of blood pressure *per se* might have a role in the modulation of lipid content in the kidney.

In this study, tubular cells that contained lipid droplets were positive for DHE signals (Figure 4). A spatial relationship between lipid droplets and superoxide was also observed in the kidneys and heart of angiotensin II-induced hypertensive animals,[10,16] indicating that these two phenomena have a relationship[23] under conditions of hypertension and metabolic syndrome, although a causal and resultant relationship has not yet been determined. Taking these observations into account, future studies should examine whether other antihypertensive drugs such as calcium channel blockers of other subclasses, anti-oxidative agents[24] and other drugs that may have an anti-proteinuric effect are effective in reducing lipid content in the kidneys of OLETF rats.

In this study, we also found several differences between OLETF and LETO rats in terms of the expression of lipid regulatory genes. The expression of PPAR-α and UCP-2 (mRNA) and ACC (protein) was higher, whereas that of PGC-1α (mRNA) was lower, in untreated OLETF rats than in LETO rats. Several earlier studies have shown

altered expression of these genes under conditions of diabetes or metabolic syndrome in the kidney or other organs. Proctor *et al.* reported that PPAR-α expression is decreased in the kidneys of diabetic animals compared to their non-diabetic counterparts, which results in decreased fatty acid oxidation.[2] In addition, the expression of UCP-2 was found to be increased in diabetic kidneys.[25] The mRNA expression of UCP-2 was also found to be upregulated in the liver, skeletal muscles, heart and aorta in OLETF rats compared to LETO rats.[26,27] Downregulation of PGC-1α mRNA expression in skeletal muscles was also reported in diabetes,[28] which might have a role in reducing mitochondrial function, leading to muscular lipotoxicity.[29]

Although regulation of the expression of these genes may have played a role in the increased lipid accumulation in OLETF rat kidneys, treatment with either losartan or benidipine did not significantly alter the mRNA expression of PPAR-α, PGC-1α or UCP-2 in OLETF rats. Compared with untreated OLETF rats, OLETF rats treated with either losartan or benidipine showed increased phosphorylation of AMPK (activated form) and ACC (inactivated form) and increased expression of CPT-1 mRNA, which may result in increased β-oxidation.[11] Therefore, it is possible that changes in the expression of these genes may have a role in the anti-steatotic effects of losartan and benidipine.

In summary, lipid content in the kidney was increased in untreated OLETF rats compared with LETO rats. Oil red O-stainable lipid droplets were primarily found in tubular epithelial cells, which also showed increased superoxide production. Treatment of OLETF rats with either losartan or benidipine, both of which suppressed proteinuria, reduced tissue TG content and modulated the expression of several lipid regulatory genes such as the total and phosphorylated forms of AMPK and CPT-1. These data collectively suggest that losartan and benidipine are both effective in suppressing proteinuria and normalizing lipid homeostasis in the kidneys of a rat model of metabolic syndrome. The underlying mechanisms by which these antihypertensive agents reduced lipid content in the kidney should be investigated in future studies.

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