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著者	Kurita Seiichiro, Takamura Toshinari, Ota Tsuguhito, Matsuzawa-Nagata Naoto, Kita Yuki, Uno Masafumi, Nabemoto Satoko, Ishikura Kazuhide, Misu Hirofumi, Ando Hitoshi, Zen Yoh, Nakanuma Yasuni, Kaneko Shuichi
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**Olmesartan ameliorates a dietary rat model of nonalcoholic steatohepatitis through its pleiotropic effects**

Seiichiro Kurita<sup>1</sup>, Toshinari Takamura<sup>1</sup>, Tsuguhito Ota<sup>1</sup>, Naoto Matsuzawa-Nagata<sup>1</sup>, Yuki Kita<sup>1</sup>, Masafumi Uno<sup>1</sup>, Satoko Nabemoto<sup>1</sup>, Kazuhide Ishikura<sup>1</sup>, Hirofumi Misu<sup>1</sup>, Hitoshi Ando<sup>1</sup>, Yoh Zen<sup>2</sup>, Yasuni Nakanuma<sup>2</sup>, and Shuichi Kaneko<sup>1</sup>

<sup>1</sup>Department of Disease Control and Homeostasis, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa, Japan

<sup>2</sup>Department of Human Pathology, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa, Japan

Contact information: Toshinari Takamura, MD, PhD

Department of Disease Control and Homeostasis, Kanazawa University Graduate School of Medical Science, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan  
Tel: +81 76 265 2233; Fax: +81 76 234 4250; E-mail: ttakamura@m-kanazawa.jp

## **Abstract**

Insulin resistance is a major pathological condition associated with obesity and metabolic syndrome. Insulin resistance and the renin-angiotensin system are intimately linked. We evaluated the role of the renin-angiotensin system in the pathogenesis of insulin resistance-associated, non-alcoholic steatohepatitis by using the angiotensin II type 1 receptor blocker olmesartan medoxomil in a diabetic rat model. The effects of olmesartan on methionine- and choline-deficient (MCD) diet-induced steatohepatitis were investigated in obese, diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats and control Long-Evans Tokushima Otsuka (LETO) rats. Components of the renin-angiotensin system were up-regulated in the livers of OLETF rats, compared with LETO rats. In OLETF, but not LETO, rats, oral administration of olmesartan for 8 weeks ameliorated insulin resistance. Moreover, olmesartan suppressed MCD diet-induced hepatic steatosis and the hepatic expression of lipogenic genes (sterol regulatory element-binding protein-1c and fatty acid synthase) in OLETF, but not LETO, rats. In both OLETF and LETO rats, olmesartan inhibited hepatic oxidative stress (4-hydroxy-2-nonenal-modified protein) and expression of NADPH oxidase. Olmesartan also inhibited hepatic fibrosis, stellate cell activation, and expression of fibrogenic genes (transforming growth factor- $\beta$ ,  $\alpha$ 1 [I] procollagen, plasminogen activator inhibitor-1) in both OLETF and LETO rats. In conclusion, pharmacological blockade of the angiotensin II type 1 receptor slows the development of steatohepatitis in the OLETF rat model. This angiotensin II type 1 receptor blocker may exert insulin resistance-associated effects against hepatic steatosis and inflammation as well as direct effects against the generation of reactive oxygen species and fibrogenesis.

**Key words:** angiotensin II type 1 receptor blocker, insulin resistance, NADPH oxidase, non-alcoholic steatohepatitis, oxidative stress, renin-angiotensin system.

## **1. Introduction**

Non-alcoholic steatohepatitis is a common liver disease and a leading cause of cryptogenic cirrhosis (Angulo, 2002; Brunt, 2001; Caldwell et al., 1999; Farrell and Larter, 2006; Reid, 2001). Non-alcoholic steatohepatitis is also strongly associated with obesity, type 2 diabetes, hypertension, and metabolic syndrome (DeFronzo and Ferrannini, 1991; Marceau et al., 1999; Reaven, 1988).

We recently reported a rat model of non-alcoholic steatohepatitis that exhibits insulin resistance, abundant visceral fat tissue, postprandial hyperglycemia, and hypertension. We have used this model to demonstrate that insulin resistance is an important therapeutic target in ameliorating steatohepatitis (Ota et al., 2007).

The renin-angiotensin system is intimately associated with obesity and metabolic syndrome. Much evidence suggests an interaction between the renin-angiotensin system and insulin action (Prasad and Quyyumi, 2004). Angiotensin II is a pro-oxidant cytokine, and angiotensin II-induced NADPH oxidase activation impairs insulin signaling in skeletal muscle cells (Wei et al., 2006). Angiotensin II type 1 receptor blockers have been shown to ameliorate insulin resistance in rodents (Okada et al., 2004).

The interaction between angiotensin II and the angiotensin II type 1 receptor also plays a pivotal role in liver fibrosis development, through a phenotypic transition of quiescent hepatic stellate cells into myofibroblastic hepatic stellate cells (Bataller et al., 2003a). Furthermore, all renin-angiotensin system components are up-regulated in hepatic stellate cells isolated from human cirrhotic livers and in culture-activated stellate cells that generate angiotensin II (Bataller et al., 2003b). These findings implicate the renin-angiotensin system in the activation of stellate cells and liver fibrogenesis, which are involved in the development of insulin resistance-associated steatohepatitis (Ota et al.,

2007).

Recently, Hirose *et al.* reported that an angiotensin II type 1 receptor blocker inhibited hepatic fibrosis in Wistar rats fed a methionine- and choline-deficient (MCD) diet (Hirose *et al.*, 2007). However, the steatohepatitis model used in that study did not exhibit insulin resistance (Rinella and Green, 2004), although insulin resistance is typically involved in the development of steatohepatitis in humans. Thus, the precise mechanism underlying the association between the improvement in steatohepatitis and the pharmacological blockade of angiotensin II type 1 receptor remains to be determined.

To address these issues, we investigated the effects of an angiotensin II type 1 receptor blocker, olmesartan, not only in a model of non-alcoholic steatohepatitis without insulin resistance but also in a model with insulin resistance that we recently established (Ota *et al.*, 2007). By comparing these models, we examined the role of the renin-angiotensin system in the development of non-alcoholic steatohepatitis and the complex mechanism underlying improvement after angiotensin II type 1 receptor blockade.

## **2. Materials and methods**

### *2.1. Animals and experimental design*

We used male Otsuka Long-Evans Tokushima Fatty (OLETF) rats (Otsuka Pharmaceutical, Tokushima, Japan) as an established animal model of obese type 2 diabetes (Kawano *et al.*, 1992) and male Long-Evans Tokushima Otsuka (LETO) rats (Otsuka Pharmaceutical) as control animals. LETO rats originated from the same colony as the OLETF rats; however, as a result of selective breeding, they do not develop diabetes. Four-week-old OLETF and LETO rats were housed under controlled

temperature (25°C), humidity, and lighting (12-h artificial light/dark cycle). The animals were given free access to standard laboratory rat chow and tap water.

At 24 weeks of age, OLETF rats were divided into two experimental groups and fed for 8 weeks as follows: (1) MCD diet (Oriental Yeast Co., Tokyo, Japan; n = 8) and (2) MCD diet mixed with 0.01% olmesartan medoxomil (donated by Daiichi Sankyo Company, Ltd., Tokyo, Japan), delivering a dose of 5 mg/kg per day (n = 5). Two groups of non-diabetic LETO rats were kept on the same regimen: (3) MCD diet (n = 8) and (4) MCD + olmesartan diet (n = 5).

During absorption in the gastrointestinal tract, orally administered olmesartan medoxomil is converted to olmesartan, the pharmacologically active metabolite. The medoxomil moiety is released as a diacetyl, which is rapidly cleared by further metabolism and excretion (Laeis et al., 2001).

The body weight and food intake of the individuals in each group were recorded weekly. Systolic and diastolic blood pressures were measured using tail-cuff plethysmography (BP-98A, Softron, Tokyo, Japan) in conscious pre-warmed rats, both before the start of olmesartan treatment and 8 weeks after.

All animal procedures were performed in accordance with the standards set forth in the Guidelines for the Care and Use of Laboratory Animals at the Takara-machi campus of Kanazawa University.

## *2.2 Blood sampling and analysis*

Blood samples were obtained from the tail vein of rats at 24 and 32 weeks of age, after a 12-h fast and under diethyl ether anesthesia. Blood glucose was determined by the glucose-oxidase method using Glucocard (Aventis Pharma, Tokyo, Japan). After

centrifugation, the serum was frozen at  $-80^{\circ}\text{C}$  for subsequent measurement of alanine aminotransferase (ALT), 8-isoprostane, triglycerides, free fatty acid, total cholesterol, high-density lipoprotein cholesterol (HDL-cholesterol), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) levels. Serum ALT was determined using a spectrophotometric assay kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. Circulating 8-isoprostane (Cayman Chemical, Ann Arbor, MI) and TNF- $\alpha$  (Biosource International, Camarillo, CA) were determined using ELISA kits according to the manufacturers' instructions. Serum free fatty acid levels were determined using the non-esterified fatty acid (NEFA) C-test (Wako, Osaka, Japan). Total cholesterol and HDL-cholesterol concentrations were quantified using a colorimetric assay as described previously (Ota et al., 2003).

### *2.3 Evaluation of insulin sensitivity*

After 8 weeks, the rats underwent an oral glucose tolerance test following a 12-h fast. Glucose (2 g/kg) was administered orally. Blood was drawn from the tail vein at 0, 30, 60, and 120 min for measurements of blood glucose concentration. An insulin tolerance test was performed following a 4-h fast. Rats were given an intraperitoneal injection of insulin (0.5 U/kg), and blood was drawn from the tail vein at 0, 15, 30, 45, and 60 min for measurements of blood glucose concentration. The area under the curve for the percentage basal glucose during the insulin tolerance test was estimated using the linear trapezoidal method.

### *2.4 Measurement of serum/liver triglycerides*

After 8 weeks, the rats were sacrificed and the liver weight, epididymal fat weight, mesenteric fat weight, and liver triglyceride content were measured. To quantify hepatic



triglycerides, livers were lysed in a buffer from a commercially available kit (TG E-test; Wako), and the lysate was sonicated. The triglyceride content of the homogenate and serum was determined using the same kit, according to the manufacturer's instructions.

### *2.5. Morphological analysis and immunohistochemistry*

Rat livers were fixed in 10% buffered formalin and embedded in paraffin. After staining with hematoxylin-eosin and Azan, the severity of hepatic histopathology was scored by a single pathologist, who was blind to the experimental design. Steatosis grade (inflammation) and stage (fibrosis) were semi-quantitatively evaluated according to the standard criteria for grading and staging non-alcoholic steatohepatitis, with minor modifications (Brunt et al., 1999). The degree of steatosis was scored based on the percentage of hepatocytes containing lipid droplets. Inflammation was scored as follow: 0, no hepatocyte injury or inflammation; 1, mild focal injury; 2, noticeable injury; and 3, severe zone 3 hepatocyte injury or inflammation. Fibrosis was scored as follows: 0, no fibrosis; 1, pericellular and perivenular fibrosis; 2, focal bridging fibrosis; 3, extensive bridging fibrosis with lobular distortion; and 4, cirrhosis.

Slides were immunostained with monoclonal mouse anti-human  $\alpha$ -smooth muscle actin (DAKO, Kyoto, Japan) or anti-rabbit transforming growth factor- $\beta$  (TGF- $\beta$ ; Santa Cruz Biotechnology, Santa Cruz, CA). The reactions were visualized by the immunoperoxidase technique using an Envision kit (DAKO). Peroxidase activity was identified by reaction with 3',3'-diaminobenzidine (DAB, Sigma). Two blinded observers independently evaluated each section. Cells with positive staining for anti- $\alpha$ -smooth muscle actin or anti-TGF- $\beta$  were expressed as a percentage of the field, using WinROOF software (version 5.7, Mitani Shoji Co., Ltd., Fukui, Japan).

## 2.6. Western blot analysis

Livers were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1% NP-40, and a protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL). Homogenized proteins (15 µg/lane) were separated by electrophoresis in 4-20% gradient SDS-polyacrylamide gels (Daiichi Pure Chemicals, Tokyo, Japan) at 130 V over 2 h. The separated proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) using a Transblot apparatus (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked in a buffer containing 5% nonfat milk, 50 mM Tris (pH 7.6), 150 mM NaCl, and 0.1% Tween 20 (TBS-T) for 1 h. They were then incubated for 1 h with a monoclonal anti-4-hydroxy-2-nonenal (4-HNE) antibody (NOF, Tokyo, Japan) diluted 1:200 in 5% bovine serum albumin (BSA) in TBS-T. Then the membranes were washed in TBS-T and incubated with a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA) at 1:2000 dilution in 5% BSA in TBS-T for 1 h. The 4-HNE-modified proteins were visualized using a luminescent image analyzer LAS-3000 (FUJIFILM, Tokyo, Japan).

## 2.7. Real-time quantitative polymerase chain reaction (PCR)

Total RNA was extracted from each liver using an RNeasy mini kit (Qiagen, Valencia, CA) as described previously (Takamura et al., 2004). Real-time quantitative PCR was performed for the angiotensin II types 1 and 2 receptors, angiotensinogen, sterol regulatory element-binding protein (SREBP)-1c, fatty acid synthase (FAS), acyl-CoA oxidase 1 (Acox 1), cytochrome P450 2e1 (CYP2E1), NADPH oxidase 1 (Nox 1), NADPH oxidase 4 (Nox 4), neutrophil cytosolic factor 1 (Ncf 1, also known as p47<sup>phox</sup>,

Noxo 2), glutathione peroxidase 1 (Gpx 1), TGF- $\beta$ , plasminogen activator inhibitor-1 (PAI-1), and  $\alpha$ 1(I) procollagen (collagen I) mRNAs, using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA).

Primers and TaqMan probes for angiotensin II type 1 receptor, angiotensin II type 2 receptor, angiotensinogen, FAS, Acox 1, CYP2E1, Nox 1, Nox 4, Ncf 1, Gpx 1, PAI-1, and collagen I were from Applied Biosystems. Primers and TaqMan probes for SREBP-1c and TGF- $\beta$  were designed using Primer Express software (version 1.5, Applied Biosystems). The primers used were 5'-GGGCAGCTCTGTACTCCTTCAA-3' (forward) and 5'-GCTAAGCTGTCCCGCAGGTA-3' (reverse) for SREBP-1c, and 5'-TTCCTGGCGTTACCTTGGT-3' (forward) and 5'-GCCACTGCCGGACAAC-3' (reverse) for TGF- $\beta$ . The TaqMan probes used were 5'-AGCCAGCCTGGCCATCTGTGAGA-3' for SREBP-1c and 5'-TACGCCTGAGTGGCTGTCTTTTGA-3' for TGF- $\beta$ . To control for variation in the amount of DNA available for PCR in the different samples, gene expression of the target sequence was normalized relative to an endogenous control, 18S ribosomal RNA (18S rRNA TaqMan control reagent kit; Applied Biosystems). The PCR conditions were one cycle at 50°C for 2 min and 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 58°C for 1 min.

## 2.8. Statistical analysis

All results are expressed as means  $\pm$  S.E.M. Means were compared by one-way analysis of variance (ANOVA). The Bonferroni multiple comparisons procedure was used to determine differences between pairs. Between-group differences for continuous variables were assessed using univariate analysis with Student's *t*-tests. Values of  $P < 0.05$  were

deemed statistically significant. All calculations were performed using SPSS software (version 11.0 for Windows, SPSS Inc., Chicago, IL).

### **3. Results**

#### *3.1. Metabolic parameters and insulin sensitivity*

As previously described (Ota et al., 2007), OLETF rats at 24 weeks of age showed obesity, especially visceral fat obesity, hyperglycemia, hyperinsulinemia, and hypertension, compared with LETO rats (data not shown). The expression of angiotensinogen and angiotensin II type 1 receptor mRNA in the livers of OLETF rats was up-regulated compared with that in LETO rats (Fig. 1). In contrast, angiotensin II type 2 receptor gene expression was not observed in the liver (data not shown). The angiotensin II type 2 receptor is expressed at high levels in the developing fetus (Matsubara, 1998). In the adult, its expression is restricted to the adrenals, uterus, ovary, heart, and specialized nuclei in the brain (Cox et al., 1993; Matsubara, 1998; Shanmugam et al., 1995). We recently reported that hepatic expression of the angiotensin II type 2 receptor was not detectable by real-time PCR analysis in fatty liver disease in mice and humans (Takeshita et al., 2008).

The metabolic parameters of rats fed the MCD diet for 8 weeks are shown in Table 1. The MCD diet caused weight loss but did not affect the physical appearance or behavior of the rats, as described previously (George et al., 2003). The MCD diet decreased serum lipid levels in OLETF rats (data not shown). It has been reported that the MCD diet decreases serum lipid levels in obese diabetic mice, probably as a result of increased uptake of fatty acid and reduced secretion of very low-density lipoprotein by the liver (Rinella et al., 2008).

Olmesartan administration did not affect the health of the rats; food intake and body weight remained the same. However, olmesartan significantly decreased the systolic and diastolic blood pressure and liver weight in both OLETF and LETO rats, although circulating fasting glucose and lipid levels were not affected (Table 1). Additionally, olmesartan significantly decreased fasting serum insulin levels in OLETF, but not in LETO, rats.

We have previously described insulin resistance as an important factor in the pathology and development of steatohepatitis (Ota et al., 2007). Here, we conducted oral glucose tolerance and insulin tolerance tests at 8 weeks to evaluate the effect of olmesartan on insulin sensitivity (Fig. 2). After 60 min of glucose administration, blood glucose levels were significantly reduced in olmesartan-treated OLETF rats, whereas blood glucose levels were unaffected by this treatment throughout the test period in LETO rats (Fig. 2A). Additionally, as demonstrated by the area under the curve, the hypoglycemic effect of insulin was significantly enhanced in OLETF rats treated with olmesartan ( $P < 0.05$ ); in contrast, olmesartan treatment had no effect on this parameter in LETO rats (Fig. 2B). Olmesartan ameliorated insulin resistance in OLETF, but not in LETO, rats.

### 3.2. MCD diet-induced steatohepatitis

Photomicrographs of liver sections obtained from OLETF and LETO rats fed the MCD or MCD + olmesartan diet for 8 weeks are shown in Figs. 3A and B. Our quantitative analysis of the severity of steatosis, inflammation, and fibrosis is shown in Fig. 3C. The MCD diet caused marked macrovesicular steatosis, with focal lymphocytic infiltration and hepatocellular drop-outs, in LETO rats, whereas it caused intense lobular inflammation and perivenular and pericellular fibrosis, prominently in zone 3, in OLETF

rat livers (Fig. 3A, B, C). Olmesartan treatment attenuated fibrogenesis in LETO rats on the MCD diet. In contrast, in OLETF rat livers, olmesartan treatment significantly improved not only fibrogenesis but also steatosis and inflammation induced by the MCD diet.

Consistent with the histological assessment of steatosis, olmesartan treatment significantly decreased the hepatic triglyceride content and serum ALT level in OLETF, but not LETO, rats (Fig. 4A, B).

### *3.3. Effect of olmesartan treatment on lipogenic gene expression*

To address the molecular basis of olmesartan's actions in steatosis, we assessed the expression levels of key genes involved in fatty acid synthesis, by quantitative real-time PCR. Hepatic expression of SREBP-1c, a transcriptional regulator of fatty acid synthesis in the liver (Ide et al., 2004; Shimano et al., 1996), and its downstream target gene, FAS, were coordinately up-regulated in OLETF rats, compared with LETO rats (Fig. 5A, B). In OLETF rats, olmesartan treatment suppressed the hepatic expression of SREBP-1c and FAS by 52 and 72%, respectively. However, olmesartan treatment had no effect on these parameters in LETO rats.

### *3.4. Effect of olmesartan treatment on oxidative stress*

Given that reactive oxygen species may be involved in the development of steatohepatitis (Garcia-Ruiz et al., 1995) and angiotensin II type 1 receptor blockers suppress hepatic reactive oxygen species generation in experimental models of liver fibrosis (Bataller et al., 2005; Bataller et al., 2003c), we investigated the effect of olmesartan on 4-HNE-modified hepatic proteins and circulating 8-isoprostane levels,

which are markers for cumulative oxidative stress. The levels of hepatic proteins modified with 4-HNE, a major aldehyde end-product of membrane lipid peroxidation, decreased after olmesartan treatment in both OLETF and LETO rats (Fig. 6A). Circulating 8-isoprostane levels also decreased after olmesartan treatment in OLETF rats (Fig. 6B). These results suggest that olmesartan treatment suppressed MCD diet-induced reactive oxygen species generation.

Oxidative stress is determined by the balance between reactive oxygen species production and antioxidant enzyme activity (Nordberg and Arner, 2001). We investigated several genes thought to be involved in reactive oxygen species generation. Hepatic expression of the NADPH oxidase complex component Ncf 1, also known as p47<sup>phox</sup>/Nox 2, was up-regulated during the course of MCD diet-induced steatohepatitis (Fig. 6C). However, expression levels of genes encoding peroxisomal Acox 1 and CYP2E1 were unaffected.

We next examined the effect of olmesartan treatment on the expression of NADPH oxidase complex components, which include membrane-bound cytochrome b558 (Nox 1-5 and p22<sup>phox</sup>) and the cytosolic subunit Ncf 1 (De Minicis et al., 2006). We examined the expression of Nox 1, which is involved in angiotensin II-mediated hypertension (Matsuno et al., 2005); Nox 4, which is important for the activation of mouse hepatic stellate cells (De Minicis et al., 2006); and Ncf 1, which plays a key role in the activation of NADPH oxidase (Wei et al., 2006) (Fig. 6D, E, F). Olmesartan treatment down-regulated hepatic expression of Nox 1, Nox 4, and Ncf 1 in OLETF rats; the expression of Nox 1 and Ncf 1 was also down-regulated in LETO rats. In contrast, olmesartan treatment up-regulated the expression of Gpx 1, an antioxidant enzyme, in OLETF rats (Fig. 6G).

### *3.5. Effect of olmesartan treatment on the circulating TNF- $\alpha$ level*

TNF- $\alpha$ , an inflammatory cytokine produced in adipose tissue and macrophages, aggravates steatohepatitis (Crespo et al., 2001) and insulin resistance (Houstis et al., 2006) through the generation of reactive oxygen species. Serum TNF- $\alpha$  was significantly elevated in OLETF rats compared with LETO rats (Fig. 7). Olmesartan treatment significantly ( $P < 0.05$ ) decreased the serum TNF- $\alpha$  level in OLETF rats, while the TNF- $\alpha$  level was unchanged in LETO rats. Thus, olmesartan may improve steatohepatitis and insulin resistance, in part, by reducing the TNF- $\alpha$  level in OLETF rats.

### *3.6. Effect of olmesartan treatment on hepatic fibrosis*

Angiotensin II stimuli such as oxidative stress and inflammatory cytokines also activate hepatic stellate cells, which are major sources of collagen and other extracellular matrix proteins, in liver fibrosis (Friedman, 2000; Leonarduzzi et al., 1997). We performed an immunohistochemical analysis of  $\alpha$ -smooth muscle actin, an activated hepatic stellate cell marker, at 8 weeks. Representative photomicrographs of liver sections stained with an anti- $\alpha$ -smooth muscle actin antibody are shown in Fig. 8A. We morphometrically quantified the area of the liver section that was positive for  $\alpha$ -smooth muscle actin in the four groups. The number of activated stellate cells increased to a greater degree in OLETF rats than in LETO rats. Development of  $\alpha$ -smooth muscle actin-positive cells was completely suppressed by olmesartan treatment in both OLETF and LETO rats receiving the MCD diet.

In parallel with the activation of hepatic stellate cells, hepatic expression of TGF- $\beta$ , a key inducer of fibrogenesis, increased to a greater degree in OLETF rats than in LETO



rats (Fig. 8B). Olmesartan treatment completely suppressed hepatic TGF- $\beta$  expression in both groups. These results are consistent with the histological observation that the MCD diet induced more severe fibrosis in OLETF rats than in LETO rats and that olmesartan ameliorated liver fibrosis in both rat types.

To assess the mechanism underlying olmesartan-mediated attenuation of hepatic fibrosis, we examined the hepatic gene expression of TGF- $\beta$  and its target genes, collagen I and PAI-1 (Fig. 8C, D, E). In OLETF rats, olmesartan treatment inhibited the up-regulation of hepatic TGF- $\beta$ , collagen I, and PAI-1 by 62, 68, and 61%, respectively. Olmesartan also inhibited the up-regulation of TGF- $\beta$ , collagen I, and PAI-1 in LETO rats, by 72, 55, and 70%, respectively.

#### **4. Discussion**

In the present study, genes for renin-angiotensin system components such as angiotensinogen and the angiotensin II type 1 receptor were up-regulated in the livers of OLETF rats compared with LETO rats, suggesting that the renin-angiotensin system is locally activated in the liver of rats with obesity-related diabetes. Activation of the renin-angiotensin system has been implicated in insulin resistance, partly via an interaction with insulin signaling (Shiuchi et al., 2004; Velloso et al., 1996). We have recently shown that insulin resistance accelerated the pathogenesis of experimental steatohepatitis in OLETF, but not in LETO, rats (Ota et al., 2007). Here, we investigated the effects of the angiotensin II type 1 receptor blocker olmesartan in OLETF and LETO rats. An inhibitory effect of olmesartan on the development of steatosis and inflammation was observed in OLETF rats, but not in LETO rats, suggesting that olmesartan prevented the development of steatohepatitis by its effects on insulin resistance in the liver of the

obese, diabetic rats. The olmesartan-induced reduction of serum TNF- $\alpha$  may also contribute to the improvement in insulin resistance (Houstis et al., 2006).

It has been suggested that angiotensin II type 1 receptor blockers that have with PPAR $\gamma$  activity, such as telmisartan, beneficially affect insulin sensitivity (Schupp et al., 2004). However, that is not the case with olmesartan. The use of ramipril, an angiotensin-converting enzyme inhibitor, has recently been reported to increase regression to normoglycemia in persons with impaired fasting glucose levels or impaired glucose tolerance (Bosch et al., 2006). Togashi et al. (Houstis et al., 2006; Togashi et al., 2000) showed that another angiotensin-converting enzyme inhibitor, temocapril, and olmesartan produced a reduction in the TNF- $\alpha$  level that correlated with insulin resistance in rat skeletal muscle. They also demonstrated that angiotensin II increased TNF- $\alpha$  secretion from cultured muscle tissue. These findings suggest that blockade of the renin-angiotensin system may be an attractive target in the treatment of insulin resistance.

SREBP-1c is a master gene in the regulation of hepatic lipogenesis, via the control of lipogenic genes such as FAS (Shimano et al., 1996). It also plays a central role in the development of high-fat diet-induced insulin resistance in the liver, by down-regulation of insulin receptor substrate 2 (Ide et al., 2004). Our results demonstrated that the genes for lipogenic SREBP-1c and FAS were up-regulated in concert with the hepatic triglyceride content in OLETF rats. This suggests that SREBP-1c-mediated *de novo* synthesis of fatty acids is increased in the insulin-resistant state and plays a significant role in the development of hepatic steatosis in this model. The olmesartan-induced reduction of hepatic SREBP-1c and FAS mRNA levels may contribute to the improvement in hepatic steatosis and possibly insulin resistance (Ide et al., 2004) in OLETF rats.

Activated hepatic stellate cells contain a non-phagocyte type of NADPH oxidase that is stimulated by pro-inflammatory and fibrogenic mediators such as angiotensin II. In contrast, Kupffer cells contain phagocyte-type NADPH oxidase that is activated by bacterial products and TNF- $\alpha$ . In chronic liver disease, activation of both types of NADPH oxidases leads to increased reactive oxygen species generation, resulting in inflammation and fibrogenesis (De Minicis et al., 2006). Our results suggest that local activation of the renin-angiotensin system and an increased level of serum of TNF- $\alpha$  contribute to the activation of non-phagocytic and phagocytic types of NADPH oxidase, respectively. Olmesartan treatment down-regulated hepatic expression of NADPH oxidase via the pharmacological blockade of angiotensin II type 1 receptors and decreased serum TNF- $\alpha$ . Additionally, olmesartan treatment up-regulated the hepatic expression of the antioxidant enzyme Gpx 1 in OLETF rats. These findings are consistent with previous results showing that the angiotensin II type 1 receptor blocker irbesartan reversed high-glucose-induced impairment of Gpx activity and mRNA expression in adolescents with diabetic angiopathy (Chiarelli et al., 2005). These pleiotropic effects of olmesartan collectively decreased 4-HNE-modified proteins and attenuated the development of hepatic fibrosis in both OLETF and LETO rats.

In the liver, circulating or tissue angiotensin II–angiotensin II type 1 receptor interactions have been implicated in the pathophysiology of fibrosis. Indeed, it has been demonstrated that an increase in systemic angiotensin II, via infusion of angiotensin II into bile duct-ligated rats, augmented hepatic fibrosis and promoted inflammation and oxidative stress (Bataller et al., 2005). It has also been reported that renin-angiotensin system blockers protect against experimental liver fibrosis caused by CCl<sub>4</sub> administration (Kanno et al., 2003) and bile duct ligation (Kurikawa et al., 2003; Yang et al., 2005). In

our study, olmesartan inhibited key pathogenic events induced by the MCD diet, including the accumulation of activated stellate cells and fibrogenic TGF- $\beta$ , PAI-1, and collagen I, thereby preventing the development of liver fibrosis in both the presence (OLETF) and absence (LETO) of insulin resistance. Thus, in addition to improving insulin resistance as described above, angiotensin II type 1 receptor blockers may also be involved in the attenuation of hepatic fibrosis.

During the preparation of this manuscript, Hirose et al. (Hirose et al., 2007) reported that olmesartan inhibited hepatic fibrosis in Wistar rats fed an MCD diet. However, they used a model of steatohepatitis without insulin resistance (Rinella and Green, 2004). In the present study, we used not only a model of non-alcoholic steatohepatitis without insulin resistance but also a model *with* insulin resistance, which we recently established (Ota et al., 2007). This is important because insulin resistance is usually involved in the development of non-alcoholic steatohepatitis in humans. By comparing these models, we showed for the first time that the pharmacological blockade of angiotensin II type 1 receptor ameliorated the pathology of steatohepatitis, by improving insulin resistance-associated lipid metabolism and inflammation and by direct inhibition of reactive oxygen species generation and fibrogenesis.

A pilot study has shown that the angiotensin II type 1 receptor blocker losartan reduced the severity of hepatic histological defects in patients with non-alcoholic steatohepatitis (Yokohama et al., 2004). Thus, olmesartan may also be an effective clinical treatment for non-alcoholic steatohepatitis, particularly in patients with the complication of insulin resistance. Prospective randomized clinical trials are needed to investigate the beneficial effects of renin-angiotensin system inhibitors in obese and/or diabetic patients with non-alcoholic steatohepatitis.

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## Figure legends

Fig. 1. Activation of the renin-angiotensin system in the liver of the insulin-resistant Otsuka Long-Evans Tokushima Fatty (OLETF) rat. Hepatic expression of (A) angiotensinogen and (B) angiotensin II type 1 receptor was up-regulated in OLETF rats, compared with that in Long-Evans Tokushima Otsuka (LETO) rats. Gene expression was assessed at 24 weeks of age using real-time quantitative polymerase chain reaction (PCR). The results were normalized against 18S ribosomal RNA (rRNA) mRNA expression. Values are the mean  $\pm$  S.E.M. (n = 8). \* $P$  < 0.01.

Fig. 2. Olmesartan decreased post-challenge glucose and insulin sensitivity in OLETF rats fed the MCD diet. Blood glucose levels during the (A) oral glucose tolerance test and (B) insulin tolerance test in OLETF rats and LETO rats fed the MCD diet or MCD+olmesartan diet for 8 weeks. Values are means  $\pm$  S.E.M. (n = 4). Values are expressed relative to the basal blood glucose level (100%) in B. \* $P$  < 0.05, versus OLETF rats fed the MCD diet;  $^{\dagger}P$  < 0.05, versus LETO rats fed the MCD diet.

Fig. 3. Olmesartan treatment slowed the pathological progression of diet-induced steatohepatitis. Representative photomicrographs show the effects of the MCD diet (white bars, n = 8) and the MCD+olmesartan diet (hatched bars; n = 5) on the liver histology in OLETF and LETO rats. Rats were fed the diets for 8 weeks. Paraffin-embedded sections were stained with (A) hematoxylin-eosin or (B) Azan. Bar, 200  $\mu$ m. Original magnification,  $\times$ 100. (C) Blind observers scored the hematoxylin-eosin stained sections for the severity of steatosis and grade (inflammation); azan-stained

samples were scored for stage (fibrosis). The scoring criteria are described in the Materials and Methods. Values are means  $\pm$  S.E.M. \* $P < 0.05$ .  $\dagger P < 0.05$  versus LETO rats fed the MCD diet.

Fig. 4. Olmesartan improved insulin resistance-accelerated hepatic fat accumulation and liver injury in diet-induced non-alcoholic steatohepatitis. LETO and OLETF rats were fed the MCD (white bars,  $n = 8$ ) or MCD+olmesartan diet (hatched bars,  $n = 5$ ) for 8 weeks. (A) Hepatic triglyceride content, and (B) serum alanine aminotransferase (ALT) levels. Values are the mean  $\pm$  S.E.M. \* $P < 0.05$  versus the MCD diet.  $\dagger P < 0.05$  versus LETO rats fed the MCD diet.

Fig. 5. Olmesartan suppressed hepatic expression of lipogenic genes in OLETF rats fed the MCD diet. LETO rats and OLETF rats were fed the MCD (white bars,  $n = 8$ ) or MCD+olmesartan diet (hatched bars,  $n = 5$ ) for 8 weeks. Real-time quantitative PCR was used to measure hepatic expression of (A) sterol regulatory element-binding protein (SREBP)-1c and (B) fatty acid synthase (FAS) in LETO and OLETF rats fed the MCD diet. Results were normalized against 18S rRNA. Values are means  $\pm$  S.E.M. \* $P < 0.05$ .  $\dagger P < 0.05$  versus LETO rats fed the MCD diet.

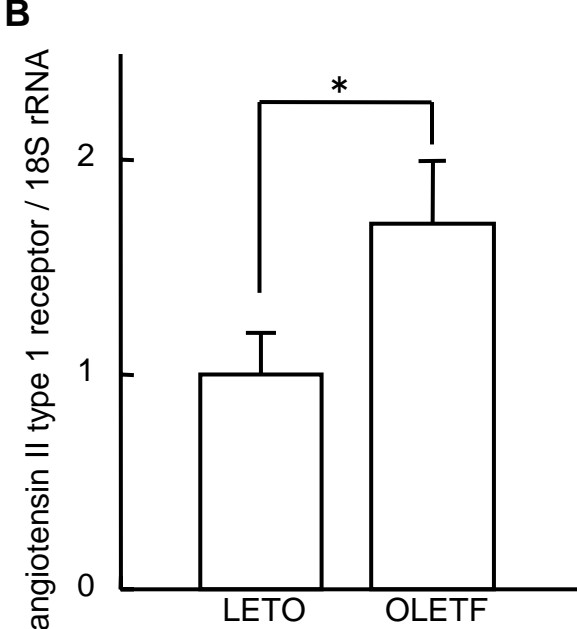
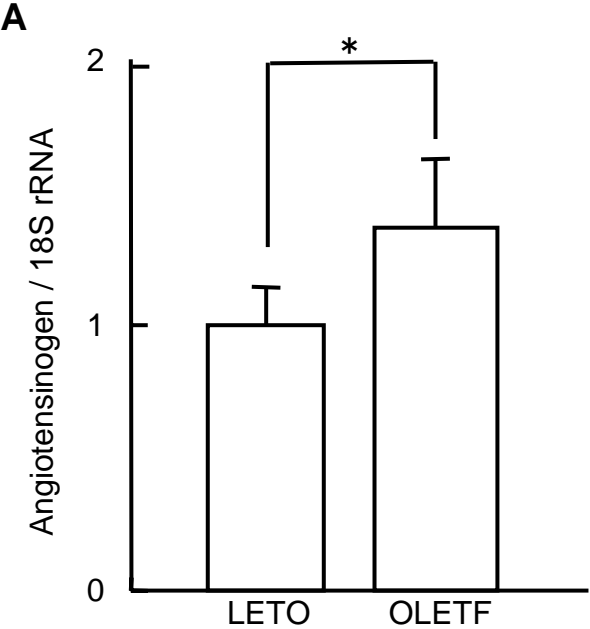
Fig. 6. Olmesartan inhibited oxidative stress and hepatic expression of NADPH oxidase. LETO and OLETF rats were fed the MCD (white bars,  $n = 8$ ) or MCD+olmesartan diet (hatched bars,  $n = 5$ ) for 8 weeks. (A) Western blots of 4-hydroxy-2-nonenal-modified hepatic proteins in LETO and OLETF rats. Protein loads were normalized to 15  $\mu$ g/sample. (B) Serum 8-isoprostane. (C) Time course of hepatic mRNA expression

for neutrophil cytosolic factor 1 (Ncf 1, also known as p47<sup>phox</sup>/Noxo 2), acyl-CoA oxidase 1 (Acox 1), and cytochrome P450 2e1 (CYP2E1) in OLETF rats fed the MCD diet. Real-time quantitative PCR was used to measure the hepatic expression of (D) Ncf 1, (E) NADPH oxidase 1 (Nox 1), (F) NADPH oxidase 4 (Nox 4), and (G) glutathione peroxidase 1 (Gpx 1). Results were normalized against 18S rRNA. Values are means  $\pm$  S.E.M. \* $P < 0.05$ .  $^{\dagger}P < 0.05$ , versus LETO rats fed the MCD diet.

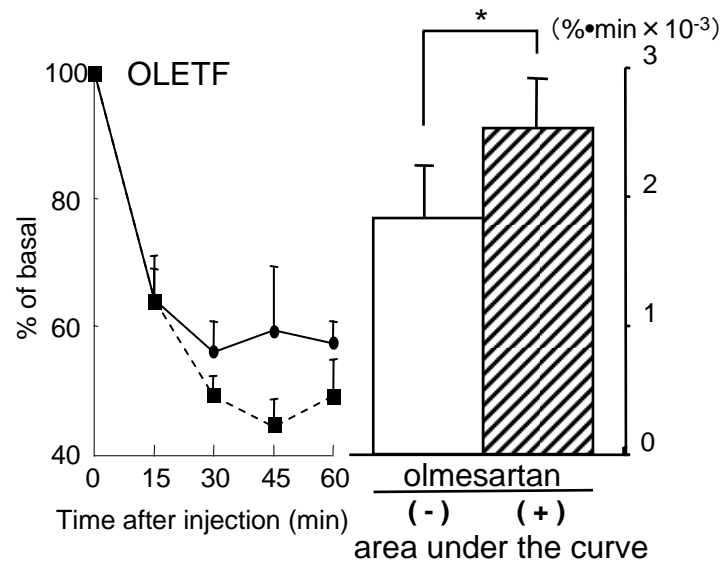
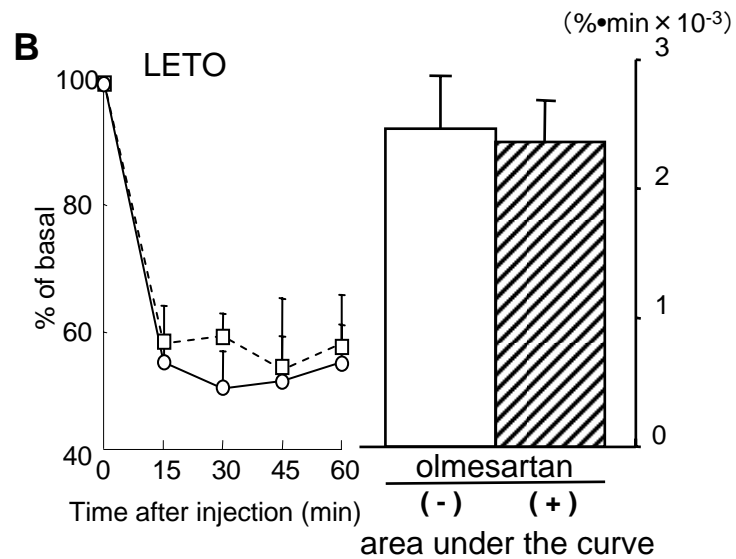
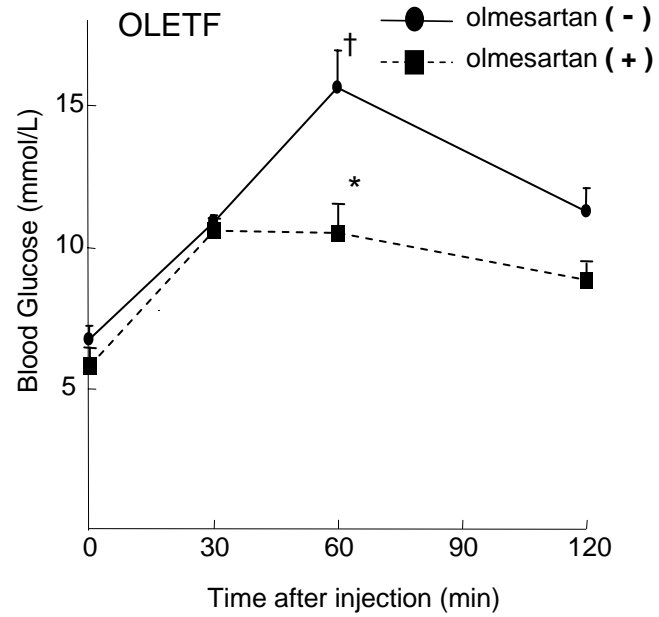
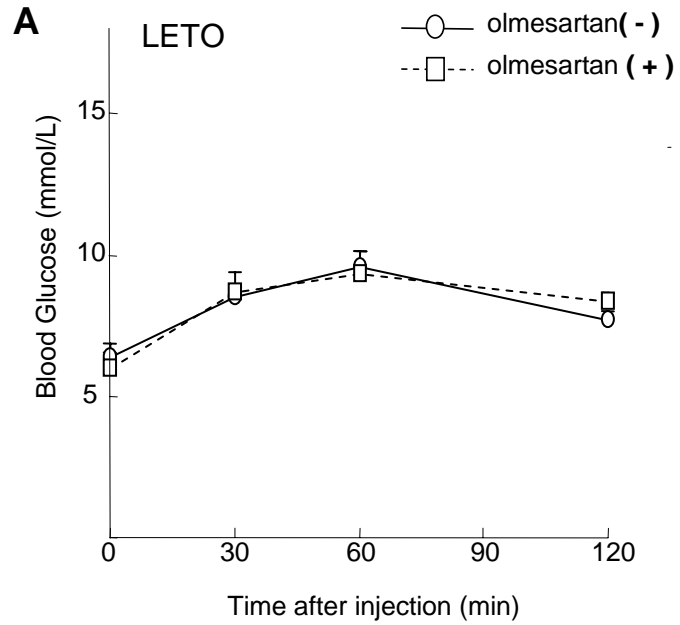
Fig. 7. Olmesartan suppressed the circulating tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) level in OLETF rats fed the MCD diet. LETO and OLETF rats were fed the MCD (white bars, n = 8) or MCD+olmesartan diet (hatched bars, n = 5) for 8 weeks. Serum TNF- $\alpha$  levels were measured using an ELISA kit according to the manufacturer's instructions. Values are means  $\pm$  S.E.M. \* $P < 0.05$ .  $^{\dagger}P < 0.05$  versus LETO rats.

Fig. 8. Olmesartan inhibited hepatic fibrosis. LETO and OLETF rats were fed the MCD (white bars, n = 8) or MCD+olmesartan diet (hatched bars, n = 5) for 8 weeks. Sections were immunohistochemically stained with (A) anti- $\alpha$ -smooth muscle actin and (B) anti-TGF- $\beta$  antibody and were counterstained with hematoxylin. Positive staining for  $\alpha$ -smooth muscle actin or TGF- $\beta$  is indicated by arrows. Bar, 200  $\mu$ m. Original magnification,  $\times 100$ . Real-time quantitative PCR was used to measure the hepatic expression of (C) transforming growth factor- $\beta$  (TGF- $\beta$ ), (D)  $\alpha 1(I)$  procollagen (collagen I), and (E) plasminogen activator-1 (PAI-1). Results were normalized against 18S rRNA. Values are means  $\pm$  S.E.M. \* $P < 0.05$ .  $^{\dagger}P < 0.05$  versus LETO rats fed the MCD diet.

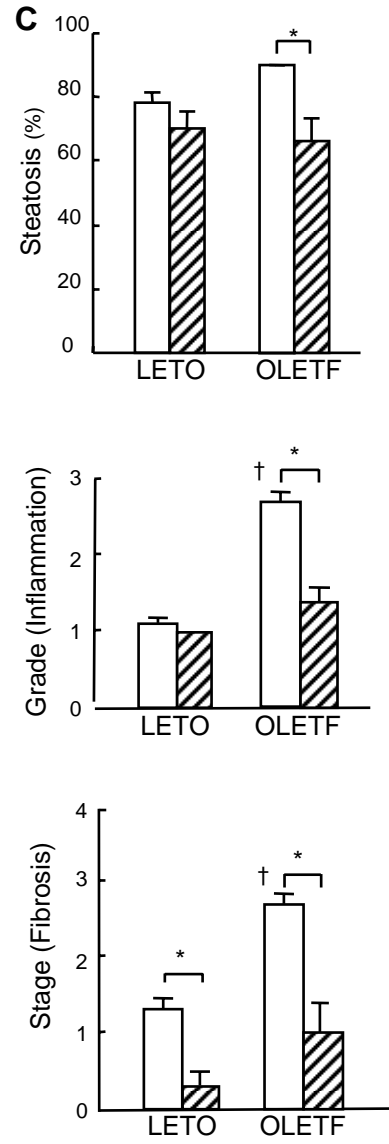
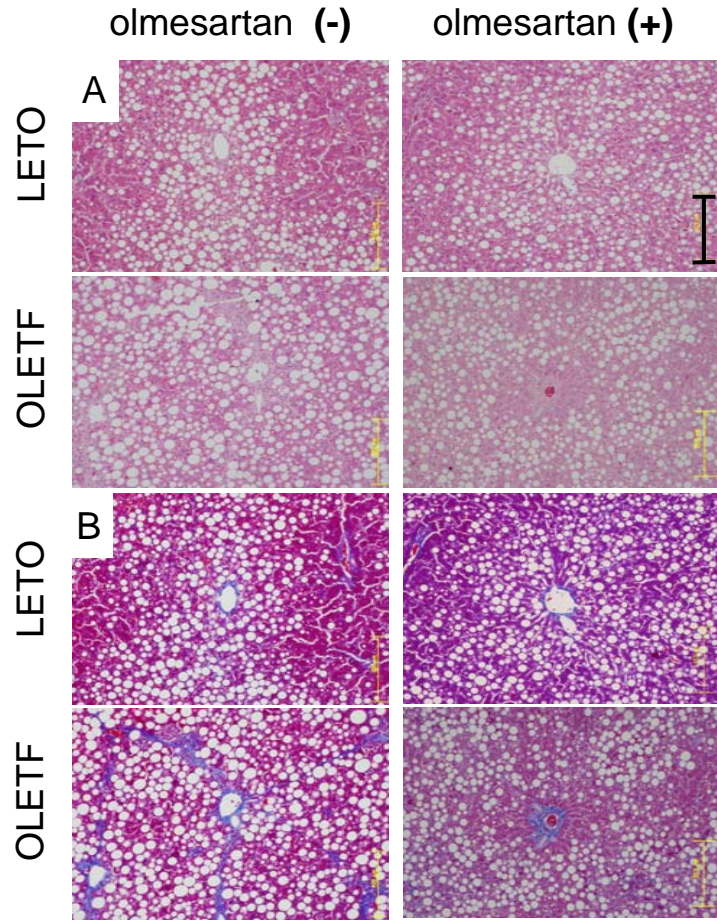
Figure 1



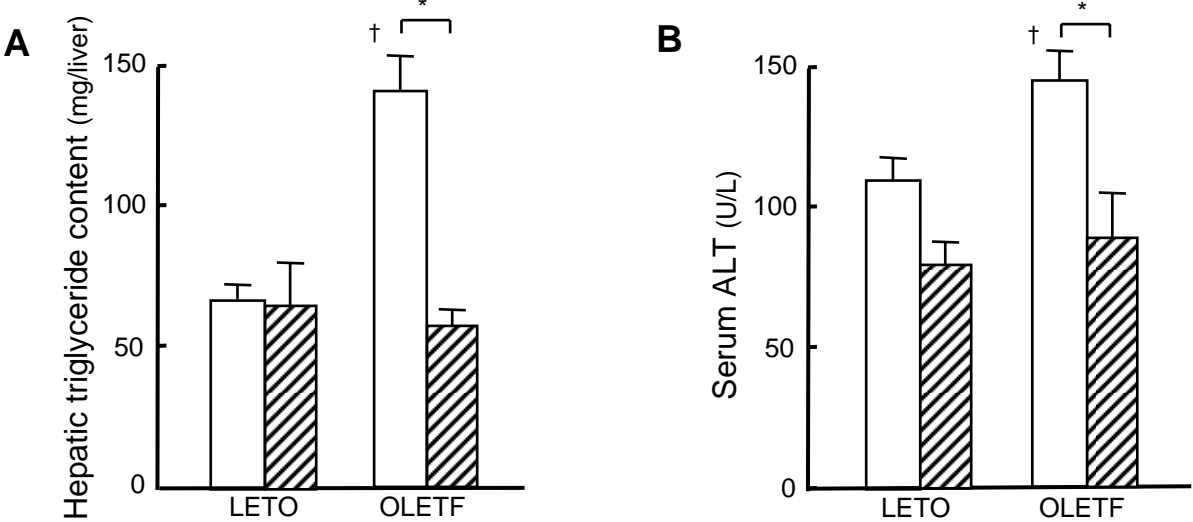
**Figure 2**



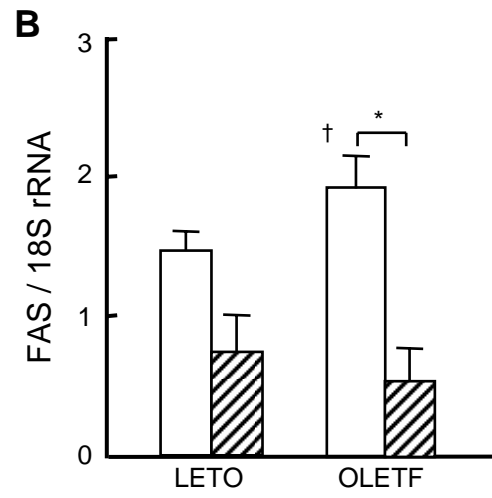
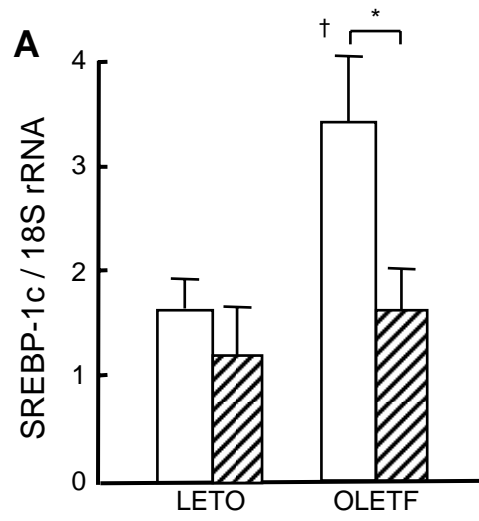
**Figure 3**



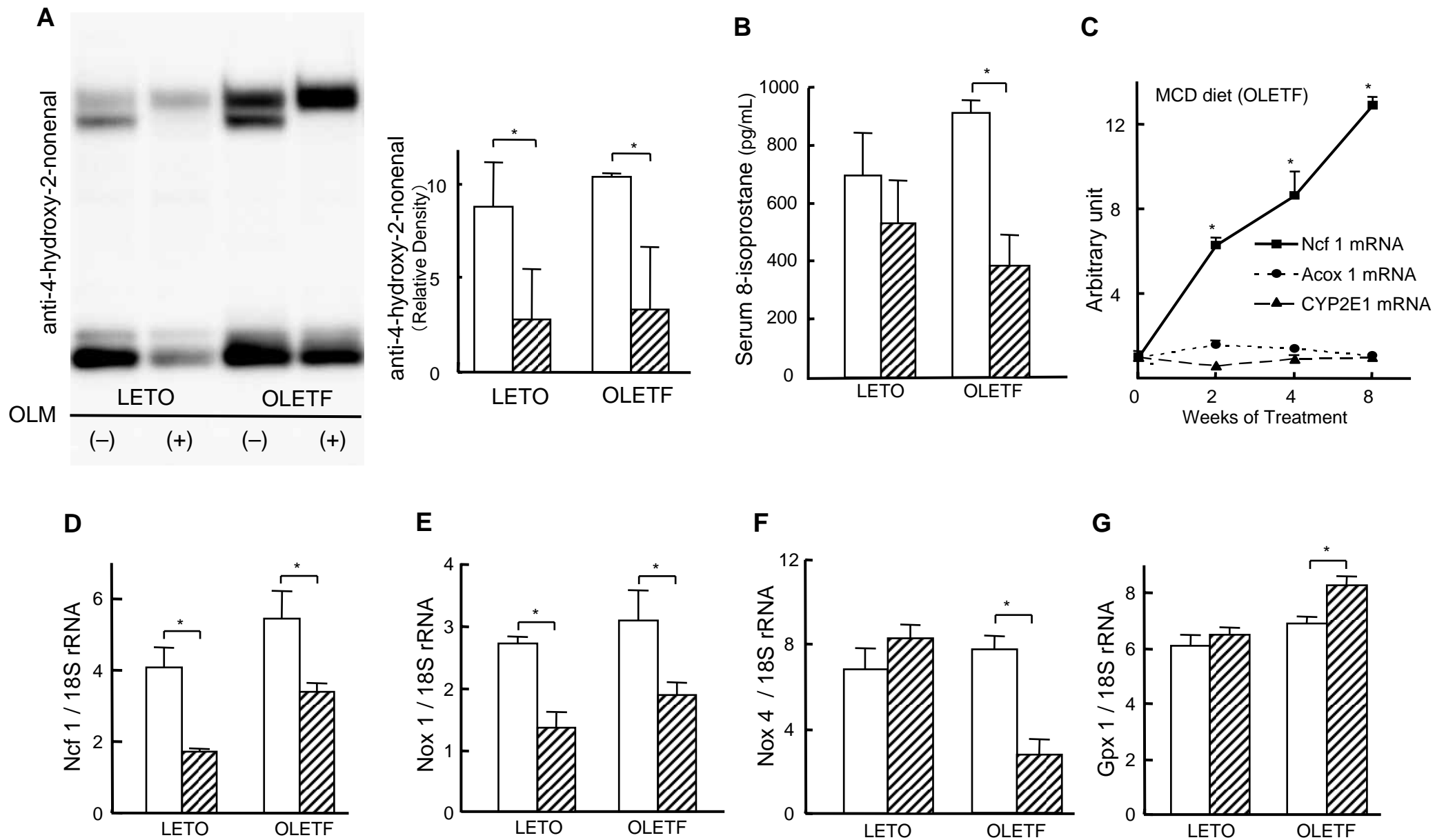
**Figure 4**



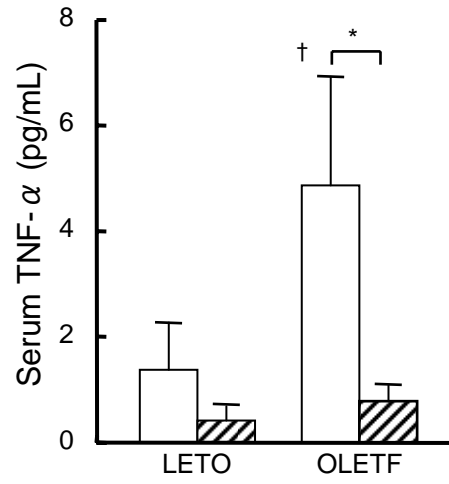
**Figure 5**



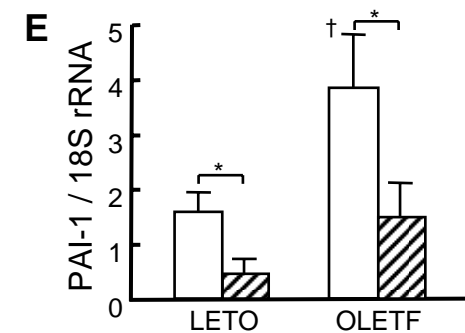
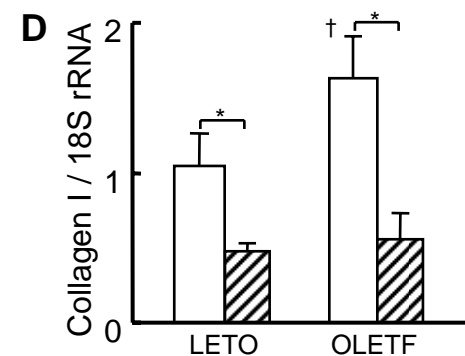
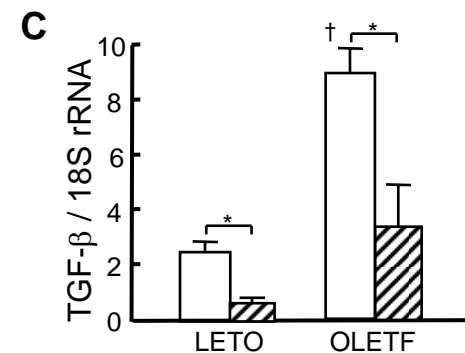
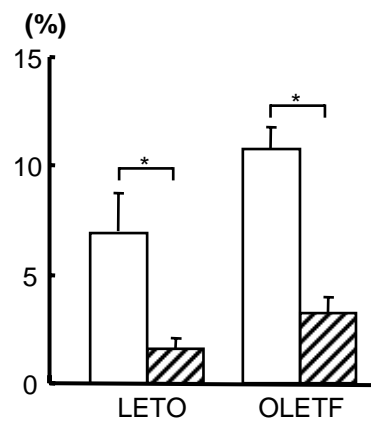
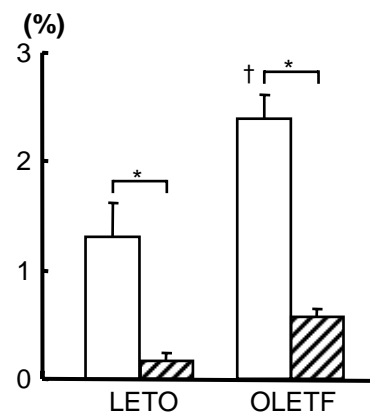
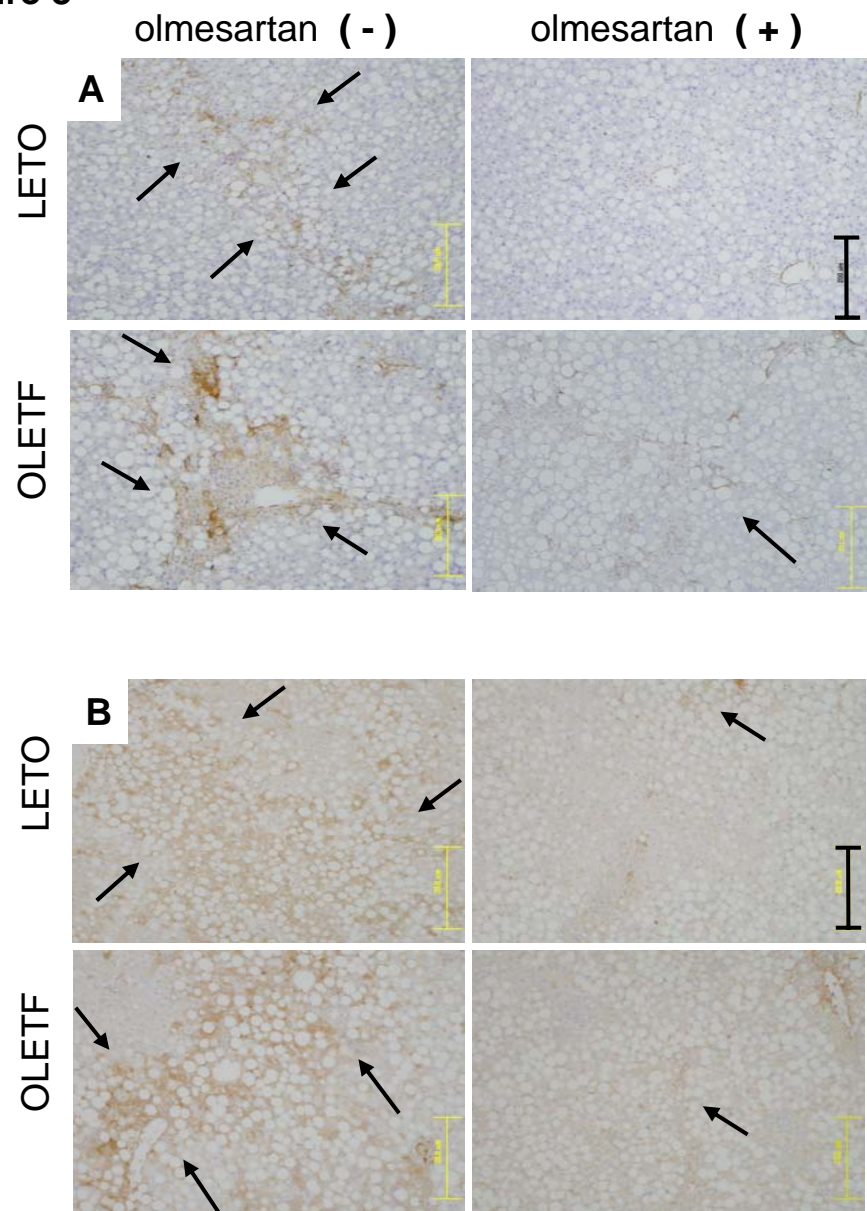


**Figure 6**

**Figure 7**



**Figure 8**



**TABLE 1**

Effects of olmesartan medoxomil treatment on metabolic parameters in rats fed a methionine- and choline-deficient (MCD) diet for 8 weeks.

	LETO		OLETF	
	olmesartan (-)	olmesartan (+)	olmesartan (-)	olmesartan (+)
Body weight (g)	382 ± 6	372 ± 9	404 ± 6	385 ± 13
Liver weight (g)	9.6 ± 0.3	8.4 ± 0.3 <sup>a</sup>	14.0 ± 0.5 <sup>b</sup>	12.6 ± 0.8 <sup>a</sup>
Epididymal fat pad weight (g)	3.0 ± 0.3	2.9 ± 0.4	3.5 ± 0.2 <sup>b</sup>	4.0 ± 0.5
Mesenteric fat weight (g)	3.1 ± 0.1	3.6 ± 0.4	7.1 ± 0.7 <sup>b</sup>	6.9 ± 0.9
Systolic blood pressure (mmHg)	109 ± 1	74 ± 3 <sup>a</sup>	139 ± 9 <sup>b</sup>	91 ± 2 <sup>a</sup>
Diastolic blood pressure (mmHg)	94 ± 3	61 ± 2 <sup>a</sup>	114 ± 7 <sup>b</sup>	81 ± 2 <sup>a</sup>
Fasting blood glucose (mmol/L)	5.9 ± 0.5	5.8 ± 0.3	6.5 ± 0.4	6.1 ± 0.4
<u>Fasting serum insulin (pmol/L)</u>	<u>172 ± 29</u>	<u>240 ± 141</u>	<u>497 ± 125<sup>b</sup></u>	<u>170 ± 66<sup>a</sup></u>
Serum triglyceride (mmol/L)	1.01 ± 0.08	1.00 ± 0.04	1.00 ± 0.04	1.17 ± 0.07
Serum free fatty acid (mEq/L)	0.77 ± 0.06	0.75 ± 0.07	0.79 ± 0.05	0.85 ± 0.06
Serum total cholesterol (mmol/L)	1.71 ± 0.16	1.37 ± 0.03	1.09 ± 0.15 <sup>b</sup>	0.87 ± 0.07
Serum HDL-cholesterol (mmol/L)	0.59 ± 0.02	0.54 ± 0.03	0.55 ± 0.08	0.45 ± 0.07

Data are the mean ± standard error (n = 5–8).

<sup>a</sup>  $P < 0.05$  versus untreated rats fed the MCD diet.

<sup>b</sup>  $P < 0.05$  versus LETO rats fed the MCD diet.

OLETF, Otsuka Long-Evans Tokushima Fatty; LETO, Long-Evans Tokushima Otsuka; HDL-cholesterol, high density lipoprotein cholesterol