Deletion of angiotensin II type I receptor reduces hepatic steatosis

Short title: Role of AT1 receptor in hepatic steatosis

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List of Abbreviations

angiotensin II type 1 receptor, AT1R; angiotensin II type 1 receptor blocker, ARB; peroxisome

proliferator-activated receptor, PPAR; angiotensin, Ang; wild-type, WT; methionine-choline deficient, MCD; thiobarbituric acid-reactive substances, TBARS; triglyceride, TG; nonalcoholic fatty liver disease, NAFLD; nonalcoholic steatohepatitis, NASH; hepatic stellate cell, HSC; small interfering RNA, siRNA; free fatty acid, FFA; phosphate buffered saline, PBS; aspartate aminotransferase, AST; alanine aminotrasferase, ALT; apolipoprotein B, ApoB; acyl-CoA synthetase, ACSL.

Electronic word count: 3284 words for the main text and 197 words for the abstract

Number of figures/ tables: 6/1

Abstract

Background/Aims: A distinct subgroup of angiotensin II type 1 receptor (AT1R) blockers (ARBs) have been reported to suppress the development of hepatic steatosis. These effects were generally explained by selective peroxisome proliferator-activated receptor (PPAR)γ modulating properties of ARBs, independent of their AT1R blocking actions. Here, we provide genetic evidence of the direct role for AT1R in hepatic steatosis.

Methods: The effect of AT1R deletion on steatohepatitis was investigated in *AT1a*$^{-/-}$ mice. Furthermore, the influence of AT1R inhibition by telmisartan as well as gene silencing of AT1R by siRNA was assessed in an *in vitro* experiment using HepG2 cells.

Results: Compared to wild-type (WT), *AT1a*$^{-/-}$ mice fed methionine-choline deficient (MCD) diet resulted in negligible lipid accumulation in the liver with marked induction of PPARα mRNA. *In vitro* experiments also demonstrated reduced cellular lipid accumulation by telmisartan and AT1R knockdown following exposure of long chain fatty acids. This is presumably explained by the observation that the expression of PPARα and its target genes were significantly up-regulated in specific siRNA treated HepG2 cells.

Conclusions: Our data indicate, in addition to pharmacological effect of ARBs on PPARγ activation, a key biological role for AT1R in the regulation of hepatic lipid metabolism.

Keywords**:** lipid metabolism, renin-angiotensin system, nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, peroxisome proliferator-activated receptor α

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) encompasses a wide spectrum of liver pathology - from simple steatosis alone, through necroinflammatory disorder referred to as nonalcoholic steatohepatitis (NASH), to cirrhosis and liver cancer. Because of the high prevalence and the potential mortality, NAFLD/NASH has emerged as a serious public concern and therefore therapeutic strategies need to be established. Recent animal studies and clinical trials have demonstrated several pharmacological treatments as potential therapeutic targets, which include insulin sensitizers (e.g., thiazolidinediones, metoformin), lipid lowering agents (e.g., statins, fibrates), antioxidants, angiotensin (Ang) II type I receptor (AT1R) blockers (ARBs), etc [1-7]. Among these therapeutic options, ARBs are initially expected to suppress the development of hepatic fibrosis in NASH. Hepatic stellate cell (HSC), which is a major fibrogenic cell type in the liver and also contributes to hepatic inflammation through induction of cytokines, expresses AT1R, and blockade of Ang II signaling markedly attenuates hepatic inflammation and fibrosis in experimental models of chronic liver fibrosis [8,9]. In addition, clinical report has demonstrated that treatment with losartan improved hepatic necroinflammation and fibrosis in NASH patients [10].

In addition to anti-fibrotic/inflammatory effect, emerging data have suggested that ARBs improve glucose and lipid metabolism. In a large clinical trial, losartan substantially lowered the risk

for type 2 diabetes compared with other antihypertensive therapies [11]. Animal study using obese Zucker rat has also demonstrated that high dose of irbesartan improved insulin sensitivity [12]. More recently, it has been shown that telmisartan, unlike other ARBs improved the development of hepatic steatosis in animal models [7,13,14]. These metabolic effects of ARBs might be explained in part by the *in vitro* study, in which a distinct subtype of ARBs induced transcriptional activities of peroxisome proliferator-activated receptor (PPAR) γ, independent of their AT1R blocking actions [15,16]. However, it is noteworthy that PPAR γ-activating potency by ARBs appears rather modest as determined by transcription reporter assays, with median effective concentration (EC_{50}) of telmisartan, the most potent PPARγ activator among ARBs, 5.02 μmol/L compared to 0.2 μmol/L of pioglitazone, a full agonist of PPARγ [15,16]. Interestingly, treatment with olmesartan, which has no impact on PPARγ activation, has been proved to attenuate the development of hepatic steatosis, suggesting the possibility that the blockade of AT1R itself might contribute to hepatic lipid homeostasis [17]. To further investigate the direct role for AT1R in hepatic steatosis, we applied to different approaches: 1) animal model of steatohepatitis using mice lacking AT1aR (*AT1a*$^{-/-}$), which is the only Ang II receptor subtype expressed in rodent liver, and 2) *in vitro* cellular steatosis model in which gene silencing by RNA interference targeting AT1R was performed [18]. Our data demonstrated reduced lipid accumulation in the absence of AT1R with significant induction of PPARα. Apart from PPARγ modulating action of ARBs, these

data support the potential efficacy of AT1R blockade in the treatment of NAFLD/NASH.

2. Experimental Procedures

2.1. Animal

AT1a$^{-/-}$ mice were provided by Mitsubishi Tanabe Pharma (Osaka, Japan) and C57BL/6 mice were obtained from Charles River Laboratories (Yokohama, Japan) [19]. Both strains of the mice have the same genetic background. The mice were housed in a standard 12 h light/dark cycle facility, and fed either standard chow diet or methionine-choline deficient (MCD) diet (Oriental Yeast Co., Tokyo, Japan) for 8 weeks with free access to drinking water. Male mice at 6-8 weeks of age were used in this study, and all animal procedures were done according to the guideline of Institute of Laboratory Animal Science, Hiroshima University.

2.2. Histological examination

Liver samples were fixed in 4 % formaldehyde solution, embedded in paraffin, and cut into 5 μm-thick sections. Staining for hematoxylin and eosin (H-E) or Azan-Mallory was carried out with standard techniques.

2.3. Analytical techniques

Serum triglyceride (TG) concentration was determined enzymatically using a Triglyceride

E-test (Wako Chemicals, Osaka, Japan). To quantify hepatic TG content, hepatic lipid was extracted as previously described by Bligh and Dyer and subjected to the same procedure as serum assay followed by the standardization of protein concentrations [20]. Hepatic thiobarbituric acid-reactive substances (TBARS) levels were quantified using an OXI-TEK TBARS Assay Kit (Zeprometrix Corporation, New York) with protein standardization. Serum TBARS levels were assayed using the same kit. Beta-hydroxybutyrate was assayed using an assay kit (BioVision, Mountain View, CA). The activities of serum transaminases were determined enzymatically.

2.4. Cell culture and gene silencing by small interfering RNA (siRNA)

HepG2 cells (human hepatoma cell line) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. For gene silencing, two different sequences of small interfering RNA (siRNA) targeting human AT1R were purchased from Sigma-Aldrich (siRNA ID: SASI_Hs01_00206672, SASI_Hs02_00206672). Cells were transfected using Lipofectamine RNAiMAX (Invitrogen) in 6-well plates containing 2.5×10^5 cells in each well with 10 nM of siRNA duplex. siPerfect Negative Controls (Sigma-Aldrich) was utilized as a negative control siRNA. In the preliminary experiment, siRNA duplex of SASI_Hs02_00206672 effectively knocked down AT1R expression compared to SASI_Hs01_00206672, and this was used for the following experiments.

After 24 h and 48 h of transfection, cells were subjected to gene quantification by real-time PCR and *in vitro* steatosis experiment, respectively.

2.5. In vitro model of cellular steatosis

Palmitic acid (C16:0) and oleic acid (C18:1) (Sigma, St. Louis, MO) were dissolved in isopropanol to obtain 20 or 40 mM stock mixture solution (2:1 oleate: palmitate), and the concentration of vehicle was 1 % in final incubations [21]. Telmisartan (provided by Boehringer Ingelheim, Germany) was resolved in DMSO to obtain 10 mM stock solution. To investigate the effect of telmisartan on cellular steatosis, cells were exposed to 200 or 400 μM of free fatty acids (FFAs) mixture with or without 2 h preincubation of 10 μM telmisartan. To assess the influence of AT1R knockdown on cellular steatosis, cells were treated with FFAs after 48 h of siRNA transfection. Following 24 h of incubation with FFAs, cells were subjected to determination of cellular lipid content by Nile Red assay and β-hydroxybutyrate levels in the media.

2.6. Nile Red assay

The lipid content in cultured cells was quantified fluorometrically using Nile Red, a vital lipophilic dye as previously described [22]. Briefly, cell monolayers were washed twice with

phosphate buffered saline (PBS) followed by fixation with 4 % formaldehyde solution for 15 min, and washed with PBS twice again. Cells were stained for 30 min with Nile Red solution at a final concentration of 200 μg/ml in PBS. Monolayers were washed thereafter with PBS and measured fluorometorically (excitation; 488 nm. emission; 550 nm.) [23].

2.7. Quantitative real-time PCR.

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Germany). cDNA was synthesized from 1μg of total RNA with GeneAmp Gold RNA PCR Core Kit (Applied Biosystems, Foster City, CA). To quantify AT1R mRNA expression in human heart and kidney, PCR Ready First Strand cDNA (BioChain, Hayward, CA) was utilized. Specific primers except AT1R from PrimerBank, a public resource for PCR primers (http://pga.mgh.harvard.edu/primerbank/, ID: 14043066) were designed using Primer3 (http://fokker.wi.mit.edu/primer3/input.htm) with nucleotide sequences from GenBank™ as listed in Table 1. Real-time PCR was carried out with Lightcycler 1.5 system using Lightcycler FastStart DNA Master plus SYBR Green I (Roche Applied Science). The relative expression levels were calculated with the formula $2^{-\Delta Ct}$, where ΔCt is the difference in threshold cycle (Ct) values between target gene and ribosomal protein S18 as a control.

2.8. Western blot analysis for AT1R

Fifty microgram of protein prepared from HepG2 cells using RIPA buffer supplemented with protease inhibitors (Roche Diagnostics), as well as Total Protein-Human Adult Normal Tissues (Biochain, Hayward, CA) were fractionated by SDS-PAGE and subjected to Western blot analysis using rabbit polyclonal antibodies against human AT1R (N-10) (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were visualized by enhanced chemiluminescence.

2.9. Statistical analysis

The data are expressed as the means±S.E. The statistical analysis was performed using Student's *t* test, and differences were considered statistically significant for a two-tailed $p<0.05$.

3. Results

3.1. Mice lacking AT1R are resistant to steatohepatitis

MCD diet has been reported to cause steatohepatitis which represents most of histological feature of human NASH [24,25]. As shown in Figure 1A, histological analysis of the livers demonstrated that MCD diet for 8 weeks resulted in apparent steatosis with inflammatory cell infiltration mainly in portal area in wild-type (WT) mice. However, in contrast to WT mice, *AT1a*$^{-/-}$ mice displayed no significant changes in the liver (Figure 1A, right panel). Azan-Mallory staining of the liver from WT mice revealed mild pericellular fibrosis, which was absent in *AT1a*$^{-/-}$ mice (Figure 1A, lower panels). In accordance with these histological observations, hepatic TG content increased by 3-fold in WT and remained no significant 1.5-fold increase in *AT1a*$^{-/-}$ mice following 8 weeks of MCD diet (Figure 1B, left panel). Serum TG level following MCD diet was reduced in both genetic groups with more drastic change in WT mice (Figure 1B, right panel). The substantial changes in hepatic TG content suggested the possibility that the expression of PPARα, a central player in hepatic lipid metabolism, might be influenced by AT1aR expression. This was assessed by quantitative real-time PCR. While MCD diet did not affect hepatic PPARα expression in both genetic strains, absence of AT1aR was associated with significant 3-fold increase in PPARα mRNA in chow diet-fed mice (Figure 1C, right panel). Since PPARα mediates hepatic expression of genes regulating lipid oxidation, we next assessed serum level of

β-hydroxybutyrate, an end product of hepatic fatty acid oxidation. Figure 1D demonstrates 3-fold increase in serum β-hydroxybutyrate in *AT1a*$^{-/-}$ mice compared to WT mice following MCD diet. This might be a potential explanation for reduced hepatic lipid accumulation in *AT1a*$^{-/-}$ mice.

3.2. Lack of AT1R ameliorates liver injury

As shown in Figure 2, there were no differences in aspartate aminotransferase (AST) and alanine aminotrasferase (ALT) levels between WT and *AT1a*$^{-/-}$ mice when fed chow diet. MCD diet caused significant increase in AST and ALT levels in WT mice, whereas there was no significant increase of these liver enzymes in *AT1a*$^{-/-}$ mice.

3.3. Influence of AT1R expression on oxidative stress

Since lipid peroxidation product plays an important role as a "2nd. hit" in the pathogenesis of NASH, we next examined serum and hepatic levels of TBARS [26]. Figure 3 demonstrates marked increase in TBARS levels in serum and liver of WT mice following MCD diet. In contrast, *AT1a*$^{-/-}$ mice showed no significant changes in TBARS levels, which presumably reflected the reduction of liver injury as demonstrated in AST and ALT levels.

3.4. Expression of AT1R in the liver and HepG2 cells

To further explore the protective effect of AT1R blockade in hepatic steatosis observed in animal model, we performed *in vitro* studies using HepG2 cells. We first ascertained AT1R expression in HepG2 cells comparing that of heart, kidney, and liver tissues, which are known to express functional AT1R. As displayed in Figure 4, Western blot analysis revealed AT1R protein expression in HepG2 cells, supporting the previous *in vivo* binding assay of Ang II that detected a homogeneous signal pattern throughout the liver parenchyma [27]. Although the quantities of loaded protein were same among samples, the band intensity in HepG2 appeared to be weaker compared to whole liver homogenate, which comprises nonparenchymal liver cells such as hepatic stellate cells and Kupffer cells. However, real-time PCR demonstrated similar level of AT1R in HepG2 as compared to kidney (Figure 4, right panel).

3.5. AT1R blockade reduces lipid accumulation in hepatocellular in vitro model

To examine whether AT1R expression in HepG2 was functional, we utilized *in vitro* model of steatosis in which HepG2 cells were treated with FFAs mixture with or without 10 μM of telmisartan. As shown in Figure 5A, Nile Red assay quantified 4-5-fold increase in cellular lipid accumulation when cells were treated with FFAs for 24 h in the absence of telmisartan. These changes were markedly

attenuated by the presence of telmisartan by 60-70 %. To exclude the possibility that these anti-steatotic effects might be contributable to pharmacological property of telmisartan, we investigated the influence of AT1R knockdown using siRNA in the same experimental model. As shown in Figure 5B, transfection of HepG2 cells with siRNA targeting AT1R successfully depleted its mRNA expression by 80% when compared to control siRNA. This siRNA knockdown of AT1R resulted in significant 15-30 % decrease in cellular lipid deposition following FFAs exposure (Figure 5B, right panel). Compared to the treatment of telmisartan, the influence of AT1R knockdown appeared modest. This might be because siRNA suppression of AT1R was not sufficient to elicit complete inhibition of the AT1R signaling pathway. In connection with the observed ketogenesis in *AT1a*$^{-/-}$ mice, we examined β-hydroxybutyrate levels in the culture media. Under the condition of 200 μM of FFAs overload, the treatment of telmisartan as well as AT1R knockdown significantly increased β-hydroxybutyrate levels (Figure 5C).

3.6. AT1R knockdown induces PPARa and its target genes

The observation that hepatic PPARα expression was up-regulated in *AT1a*$^{-/-}$ mice suggests the possible link between AT1R signaling and PPARα expression. In this setting, our working hypothesis is that AT1R down-regulation may in turn influence the hepatocellular expressions of PPARα and its

target genes. As shown in Figure 6A, transient AT1R silencing by siRNA resulted in significant up-regulation of *PPARα* by 41%. In addition, PPARα target genes, acyl-CoA synthetase 1 (*ACSL1)* and apolipoprotein B (*Apo B) 100* were also induced by 27 and 21 %, respectively. These data exclude the possibility that PPARα up-regulation in *AT1a*$^{-/-}$ mice might be resulted from chronic adaptation to the absence of AT1R. To exclude the possible contribution of the other PPAR isoforms in hepatocellular steatosis, the expression of PPARγ and PPARβ(δ) were assessed by real-time PCR, which resulted in no significant changes following AT1R depletion (Figure 5B).

4. Discussion

The present study indicates a major role for AT1R in hepatic steatosis by providing a precise genetic approach. Homozygous disruption of AT1R in mice resulted in marked reduction of hepatic lipid accumulation as determined morphologically and by hepatic TG content. Recent studies have demonstrated that ARBs improve insulin resistance and hyperinsulinemia. Because the improvement of insulin resistance leads to reduction of FFAs flux to the liver together with increased uptake and storage of FFAs in adipose tissue, ARBs are expected to reduce hepatic steatosis. However, the effect of ARBs on hepatic steatosis is still controversial [7,14,17,28-30]. These conflicting findings might be explained by differences in selective PPARγ modulating properties of ARBs [15,16], or attributed to differences in the dose and length of ARBs supplementation as well as potential differences in the mechanisms of steatosis in the different experimental models. Our data reinforces the theory that blockade of AT1R, unrelated to pharmacological properties of ARBs, ameliorates the development of hepatic steatosis.

The pathophysiology of NASH remains poorly understood, but is often described as “two-hit process” consisting of hepatic TG accumulation (the 1st. step) and development of oxidative stress and proinflammatory cytokines (the 2nd. step), which leads to hepatocyte injury, inflammation, and fibrosis

[26]. Previous reports have demonstrated that pharmacological blockade or gene deletion of renin-angiotensin system (RAS) significantly attenuates hepatic fibrosis in experimental animal models [31,32]. Moreover, Ang II, the effecter peptide for AT1R, has been reported to stimulates an array of fibrogenic actions in activated HSCs through the mediation of reactive oxygen species [33]. More recent data has revealed that angiotensin converting enzyme (ACE) deficient (*ACE*$^{-/-}$) mice which exhibit reduced levels of Ang II by 70 % in plasma and by 85-97 % in tissues [34] showed pronounced increase in hepatic gene expression related to lipolysis and fatty acid oxidation, suggesting close link between RAS and hepatic lipid metabolism [35]. Taken together with our finding of the anti-steatotic effect, the blockade of AT1R appears to contribute to wide range of steps and phases in the pathogenesis of NASH.

The up-regulation of PPARα in the liver of *AT1a*$^{-/-}$ mice is intriguing because PPARα plays a central role in hepatic lipid homeostasis. It is well known that many of the genes encoding enzymes involved in the mitochondrial and peroxisomal fatty acid β-oxidation pathways are regulated by PPARα. To exclude the possibility that adaptation to persistent AT1R absence may induce PPARα expression in mice, we performed *in vitro* siRNA experiments, which confirmed induction of PPARα and its target genes by transient AT1R knockdown. In support of these observations, previous experiments have demonstrated telmisartan-mediated induction of PPARα [36]. Whereas induction of PPARα was observed in *AT1a*$^{-/-}$ mice, the present study lacks the direct evidence that suppressive effect of AT1R

blockade on hepatic steatosis is mediated by PPARα. In this connection, we examined β-hydroxybutyrate levels since it is documented that PPARα activation leads to stimulation of ketogenesis [37,38]. This yielded marked increase in serum β-hydroxybutyrate in *AT1a*$^{-/-}$ mice. In addition, our *in vitro* experiments demonstrated significant increase in β-hydroxybutyrate in the culture media in response to AT1R blockade. These findings support the detected changes in gene expressions of PPARα and ACSL1 following AT1R deletion. Although it remains controversial whether the blockade of AT1R directly stimulates PPARα, up-regulation of PPARα likely increases the sensitivity to agonist and thus clinical usage of ARBs together with PPARα ligand such as fibrates might have synergistic effects on hepatic lipid metabolism.

A potential limitation of the present study is the use of MCD dietary animal model. Although this model develops steatohepatitis morphologically similar to human NASH with increase in oxidative stress and has been widely used for the study of NASH, absence of insulin resistance has been reported [24,25,39,40]. Since insulin resistance is considered to be pivotal in the development of NASH, this model might not entirely reflect the natural course and the etiological background of human NASH [41,42]. Some other potential dietary models include ad libitum feeding of the high fat diet, which develops obesity and insulin resistance but not noticeable steatohepatitis [43]. Additionally, intragastric overfeeding of high fat diet induces NASH pathology, but some of the biological changes observed in

the liver did not mimic human NASH [44]. Altogether, the study of pathophysiological process of NASH is limited by the lack of suitable experimental animal models [45]. Interestingly, the effect of olmesartan has been investigated in a genetically diabetic rat fed MCD diet [30]. In contrast to our findings, olmesartan reduced hepatic steatosis only in diabetic but not in control rats. In the present study, MCD diet caused increase in hepatic TG content associated with decrease in serum TG level, suggesting that impaired TG secretion from liver might contribute to hepatic steatosis. These changes were blunted in the absence of AT1R, implying improvement of TG secretion in *AT1a*$^{-/-}$ mice. This might be in part explained by the observation of *in vitro* experiment that AT1 knockdown induced Apo B which is requisite for the formation of very low density lipoprotein (VLDL).

In the present study, human hepatoma cell line HepG2 instead of rodent primary hepatocytes was utilized for *in vitro* model of cellular steatosis. This was based on the previous report that human primary hepatocytes and HepG2 cells reached similar levels of intracellular lipid accumulation close to that determined in hepatocytes from human steatosis liver [23]. In addition, the observed up-regulation of hepatic PPARα in *AT1a*$^{-/-}$ mice led to the speculation that PPARα might play a role in the anti-steatotic effect of AT1 blockade. Because hepatic expression of PPARα and the sensitivity to it notably differ among species [46], human-derived cell line was applied.

In conclusion, mice lacking AT1R are resistant to the development of hepatic steatosis with

up-regulation of PPARα in MCD diet-induced steatohepatitis model. Accordingly, the levels of TBARS, a marker of oxidative stress, as well as aminotransferases, indicators of liver damage were significantly attenuated in *AT1a*$^{-/-}$ mice. In addition, *in vitro* experiment of hepatocellular steatosis revealed that blockade of AT1R by telmisartan and specific siRNA knockdown markedly decreased cellular lipid accumulation. Up-regulation of PPARα was also confirmed by transient AT1R knockdown. These data provide strong evidence that, in addition to pharmacological effect of ARBs on PPARγ activation, AT1R signaling is involved in hepatic lipid metabolism.

References

[1] Belfort R, Harrison SA, Brown K, Darland C, Finch J, Hardies J, et al. A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. N Engl J Med 2006;355:2297-2307.

[2] Promrat K, Lutchman G, Uwaifo GI, Freedman RJ, Soza A, Heller T, et al. A pilot study of pioglitazone treatment for nonalcoholic steatohepatitis. Hepatology 2004;39:188-196.

[3] Marchesini G, Brizi M, Bianchi G, Tomassetti S, Zoli M, Melchionda N. Metformin in non-alcoholic steatohepatitis. Lancet 2001;358:893-894.

[4] Antonopoulos S, Mikros S, Mylonopoulou M, Kokkoris S, Giannoulis G. Rosuvastatin as a novel treatment of non-alcoholic fatty liver disease in hyperlipidemic patients. Atherosclerosis 2006;184:233-234.

[5] Basaranoglu M, Acbay O, Sonsuz A. A controlled trial of gemfibrozil in the treatment of patients with nonalcoholic steatohepatitis. J Hepatol 1999;31:384.

[6] Jin H, Yamamoto N, Uchida K, Terai S, Sakaida I. Telmisartan prevents hepatic fibrosis and enzyme-altered lesions in liver cirrhosis rat induced by a choline-deficient L-amino acid-defined diet. Biochem Biophys Res Commun 2007;364:801-807.

[7] Fujita K, Yoneda M, Wada K, Mawatari H, Takahashi H, Kirikoshi H, et al. Telmisartan, an

angiotensin II type 1 receptor blocker, controls progress of nonalcoholic steatohepatitis in rats. Dig Dis Sci 2007;52:3455-3464.

[8] Bataller R, Gines P, Nicolas JM, Gorbig MN, Garcia-Ramallo E, Gasull X, et al. Angiotensin II induces contraction and proliferation of human hepatic stellate cells. Gastroenterology 2000;118:1149-1156.

[9] Kanno K, Tazuma S, Nishioka T, Hyogo H, Chayama K. Angiotensin II participates in hepatic inflammation and fibrosis through MCP-1 expression. Dig Dis Sci 2005;50:942-948.

[10] Yokohama S, Yoneda M, Haneda M, Okamoto S, Okada M, Aso K, et al. Therapeutic efficacy of an angiotensin II receptor antagonist in patients with nonalcoholic steatohepatitis. Hepatology 2004;40:1222-1225.

[11] Dahlof B, Devereux RB, Kjeldsen SE, Julius S, Beevers G, de Faire U, et al. Cardiovascular morbidity and mortality in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. Lancet 2002;359:995-1003.

[12] Henriksen EJ, Jacob S, Kinnick TR, Teachey MK, Krekler M. Selective angiotensin II receptor receptor antagonism reduces insulin resistance in obese Zucker rats. Hypertension 2001;38:884-890.

[13] Sugimoto K, Kazdova L, Qi NR, Hyakukoku M, Kren V, Simakova M, et al. Telmisartan increases

fatty acid oxidation in skeletal muscle through a peroxisome proliferator-activated receptor-gamma dependent pathway. J Hypertens 2008;26:1209-1215.

[14] Ibanez P, Solis N, Pizarro M, Aguayo G, Duarte I, Miquel JF, et al. Effect of losartan on early liver fibrosis development in a rat model of nonalcoholic steatohepatitis. J Gastroenterol Hepatol 2007;22:846-851.

[15] Benson SC, Pershadsingh HA, Ho CI, Chittiboyina A, Desai P, Pravenec M, et al. Identification of telmisartan as a unique angiotensin II receptor antagonist with selective PPARgamma-modulating activity. Hypertension 2004;43:993-1002.

[16] Schupp M, Janke J, Clasen R, Unger T, Kintscher U. Angiotensin type 1 receptor blockers induce peroxisome proliferator-activated receptor-gamma activity. Circulation 2004;109:2054-2057.

[17] Hirose A, Ono M, Saibara T, Nozaki Y, Masuda K, Yoshioka A, et al. Angiotensin II type 1 receptor blocker inhibits fibrosis in rat nonalcoholic steatohepatitis. Hepatology 2007;45:1375-1381.

[18] Burson JM, Aguilera G, Gross KW, Sigmund CD. Differential expression of angiotensin receptor 1A and 1B in mouse. Am J Physiol 1994;267:E260-267.

[19] Sugaya T, Nishimatsu S, Tanimoto K, Takimoto E, Yamagishi T, Imamura K, et al. Angiotensin II type 1a receptor-deficient mice with hypotension and hyperreninemia. J Biol Chem

1995;270:18719-18722.

[20] Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911-917.

[21] Malhi H, Bronk SF, Werneburg NW, Gores GJ. Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. J Biol Chem 2006;281:12093-12101.

[22] Greenspan P, Fowler SD. Spectrofluorometric studies of the lipid probe, nile red. J Lipid Res 1985;26:781-789.

[23] Gomez-Lechon MJ, Donato MT, Martinez-Romero A, Jimenez N, Castell JV, O'Connor JE. A human hepatocellular in vitro model to investigate steatosis. Chem Biol Interact 2007;165:106-116.

[24] Tomita K, Oike Y, Teratani T, Taguchi T, Noguchi M, Suzuki T, et al. Hepatic AdipoR2 signaling plays a protective role against progression of nonalcoholic steatohepatitis in mice. Hepatology 2008;48:458-473.

[25] Igolnikov AC, Green RM. Mice heterozygous for the Mdr2 gene demonstrate decreased PEMT activity and diminished steatohepatitis on the MCD diet. J Hepatol 2006;44:586-592.

[26] Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology 1998;114:842-845.

[27] Paizis G, Cooper ME, Schembri JM, Tikellis C, Burrell LM, Angus PW. Up-regulation of

components of the renin-angiotensin system in the bile duct-ligated rat liver. Gastroenterology 2002;123:1667-1676.

[28] Ran J, Hirano T, Adachi M. Angiotensin II infusion increases hepatic triglyceride production via its type 2 receptor in rats. J Hypertens 2005;23:1525-1530.

[29] Sugimoto K, Qi NR, Kazdova L, Pravenec M, Ogihara T, Kurtz TW. Telmisartan but not valsartan increases caloric expenditure and protects against weight gain and hepatic steatosis. Hypertension 2006;47:1003-1009.

[30] Kurita S, Takamura T, Ota T, Matsuzawa-Nagata N, Kita Y, Uno M, et al. Olmesartan ameliorates a dietary rat model of non-alcoholic steatohepatitis through its pleiotropic effects. Eur J Pharmacol 2008;588:316-324.

[31] Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Nakatani T, et al. Angiotensin-II type 1 receptor interaction is a major regulator for liver fibrosis development in rats. Hepatology 2001;34:745-750.

[32] Kanno K, Tazuma S, Chayama K. AT1A-deficient mice show less severe progression of liver fibrosis induced by CCl(4). Biochem Biophys Res Commun 2003;308:177-183.

[33] Bataller R, Schwabe RF, Choi YH, Yang L, Paik YH, Lindquist J, et al. NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis. J Clin Invest

2003;112:1383-1394.

[34] Campbell DJ, Alexiou T, Xiao HD, Fuchs S, McKinley MJ, Corvol P, et al. Effect of reduced angiotensin-converting enzyme gene expression and angiotensin-converting enzyme inhibition on angiotensin and bradykinin peptide levels in mice. Hypertension 2004;43:854-859.

[35] Jayasooriya AP, Mathai ML, Walker LL, Begg DP, Denton DA, Cameron-Smith D, et al. Mice lacking angiotensin-converting enzyme have increased energy expenditure, with reduced fat mass and improved glucose clearance. Proc Natl Acad Sci U S A 2008;105:6531-6536.

[36] Clemenz M, Frost N, Schupp M, Caron S, Foryst-Ludwig A, Bohm C, et al. Liver-specific peroxisome proliferator-activated receptor alpha target gene regulation by the angiotensin type 1 receptor blocker telmisartan. Diabetes 2008;57:1405-1413.

[37] Mandard S, Müller M, Kersten S. Peroxisome proliferator-activated receptor alpha target genes. Cell Mol Life Sci 2004;61:393-416.

[38] Luci S, Giemsa B, Kluge H, Eder K. Clofibrate causes an upregulation of PPAR-{alpha} target genes but does not alter expression of SREBP target genes in liver and adipose tissue of pigs. Am J Physiol Regul Integr Comp Physiol 2007;293:R70-7.

[39] Rinella ME, Green RM. The methionine-choline deficient dietary model of steatohepatitis does not exhibit insulin resistance. J Hepatol 2004;40:47-51.

[40] Vetelainen R, van Vliet A, van Gulik TM. Essential pathogenic and metabolic differences in steatosis induced by choline or methione-choline deficient diets in a rat model. J Gastroenterol Hepatol 2007;22:1526-1533.

[41] Pagano G, Pacini G, Musso G, Gambino R, Mecca F, Depetris N, et al. Nonalcoholic steatohepatitis, insulin resistance, and metabolic syndrome: further evidence for an etiologic association. Hepatology 2002;35:367-72.

[42] Sanyal AJ, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, et al. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. Gastroenterology 2001;120:1183-92.

[43] Collins S, Martin TL, Surwit RS, Robidoux J. Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics. Physiol Behav 2004;81:243-248.

[44] Deng QG, She H, Cheng JH, French SW, Koop DR, Xiong S, et al. Steatohepatitis induced by intragastric overfeeding in mice. Hepatology 2005;42:905-914.

[45] Koteish A, Mae Diehl A. Animal models of steatohepatitis. Best Pract Res Clin Gastroenterol 2002;16:679-690.

[46] Gonzalez FJ, Peters JM, Cattley RC. Mechanism of action of the nongenotoxic peroxisome proliferators: role of the peroxisome proliferator-activator receptor alpha. J Natl Cancer Inst

1998;90:1702-9.

Figure Legends

Fig. 1. The absence of AT1aR expression attenuates the development of MCD diet-induced hepatic steatosis in mice. A. Liver sections from MCD diet-fed WT (A, C) and *AT1a*$^{-/-}$ mice (B, D) were processed for Haematoxylin & Eosin (HE) (upper panels) or Azan-Mallory staining (lower panels). Hepatic steatosis as well as fibrosis are evident only in WT mice (original magnification ×100). B. TG concentrations in serum (left panel) and liver (right panel) obtained from WT (open bars) and *AT1a*$^{-/-}$ (closed bars) mice (n=6/each group) were determined after feeding either normal chow or MCD diet. C. Hepatic PPARα mRNA expression was quantified (n=5/each group) by quantitative real-time PCR. D. The serum level of β-hydroxybutyrate in WT (n=5) and *AT1a*$^{-/-}$ (n=6) mice fed MCD diet was determined. *p<0.05, chow-fed vs. MCD diet-fed mice. †p<0.05, WT vs. *AT1a*$^{-/-}$ mice.

Fig. 2. Diminished hepatic injury following 8 weeks of MCD diet in the absence of AT1aR. AST and ALT levels were determined in WT (open bars) and *AT1a*$^{-/-}$ (closed bars) mice fed either normal chow or MCD diet for 8 weeks (n=6/group). *p<0.05, chow-fed vs. MCD diet-fed mice. †p<0.05, WT vs. *AT1a*$^{-/-}$ mice.

Fig. 3. Influence of AT1aR expression on TBARS levels in serum and liver. Thiobarbituric

acid-reactive substances (TBARS) levels were assayed in serum (left panel) and liver (right panel) from WT (open bars) and *AT1a*$^{-/-}$ (closed bars) mice after feeding either normal chow or MCD diet (n=6/each group). *p<0.05, chow-fed vs. MCD diet-fed mice. $^{\dagger}p$<0.05, WT vs. *AT1a*$^{-/-}$ mice.

Fig. 4. Expressions of AT1R in the liver and HepG2 cells. Western blots of AT1R protein expression in homogenates from human liver and HepG2 cells by comparison with heart and kidney as positive tissues for AT1R expression (50KDa) (left panel). Quantitative real-time PCR analysis for AT1R in HepG2 (right panel). Results represent from at least 3 independent experiments.

Fig. 5. AT1R blockade attenuates cellular steatosis following fatty acids overload. A. Influence of telmisartan on intracellular steatosis induced by free fatty acids (FFAs) overload (2:1 oleate: palmitate) (n=6/each group). B. Effect of siRNA targeting AT1R on *AT1* mRNA expression (left panel) and influence of AT1 knockdown (KD) on cellular steatosis induced by FFAs overload (2:1 oleate: palmitate, right panel) (n=6/each group). C. Influence of AT1R blockade on β-hydroxybutyrate levels in media (n=6/each group). **p<0.005, vs. control siRNA. *p<0.05, vs. non-treated groups. $^{\dagger}p$<0.05, AT1R blockade (either by telmisartan or AT1 knockdown) vs. control (either vehicle or control siRNA) HepG2.

Fig. 6. Influence of AT1R knockdown on the expression of PPARα target genes (A) and the other PPAR isoforms (B). Real-time PCR quantified the expression of genes regulated by PPARα as well as of the other PPAR isoforms, PPARγ and PPARβ(δ) in HepG2 cells transfected with either siRNA targeting AT1R (n=6) or negative control siRNA (n=6). *p<0.05, vs. control siRNA. ** p<0.005, vs. control siRNA. N.S., not statistically significant.

Acknowledgement

The authors thank Dr. Naseem Gaur for helpful discussion. This work was supported by a Grant-in-aid from the Ministry of Health, Labor and Welfare of Japan to ST. This work was carried out in part at the Analysis Center of Life Science, Hiroshima University.

Table 1. Primer used for quantitative real-time PCR

Gene	Forward	Reverse	GenBank accession num.
mPPARα	tgcaaacttggacttgaacg	tgatgtcacagaacggcttc	BC016892
AT1	atccaagatgattgtcccaaagc	gcccatagtggcaaagtcagtaa	
PPARα	cagtggagcattgaacatcg	gttgtgtgacatcccgacag	NM_001001928
ApoB100	agccttgctgaagaaaacca	atgcccctcttgatgttcag	M14162
ASCL1	ccagaagggcttcaagactg	gccttctctggcttgtcaac	NM_001995
RPS18	atagcctttgccatcactgc	ggacctggctgtattttcca	NM_022551

mPPARα, mouse peroxisome proliferator-activated receptor α; AT1, angiotensin II receptor type I; PPARα, human PPARα; ApoB100, apolipoprotein B-100; ACSL1, acyl-CoA synthetase long-chain family member 1; RPS18, ribosomal protein S18

Fig. 1.

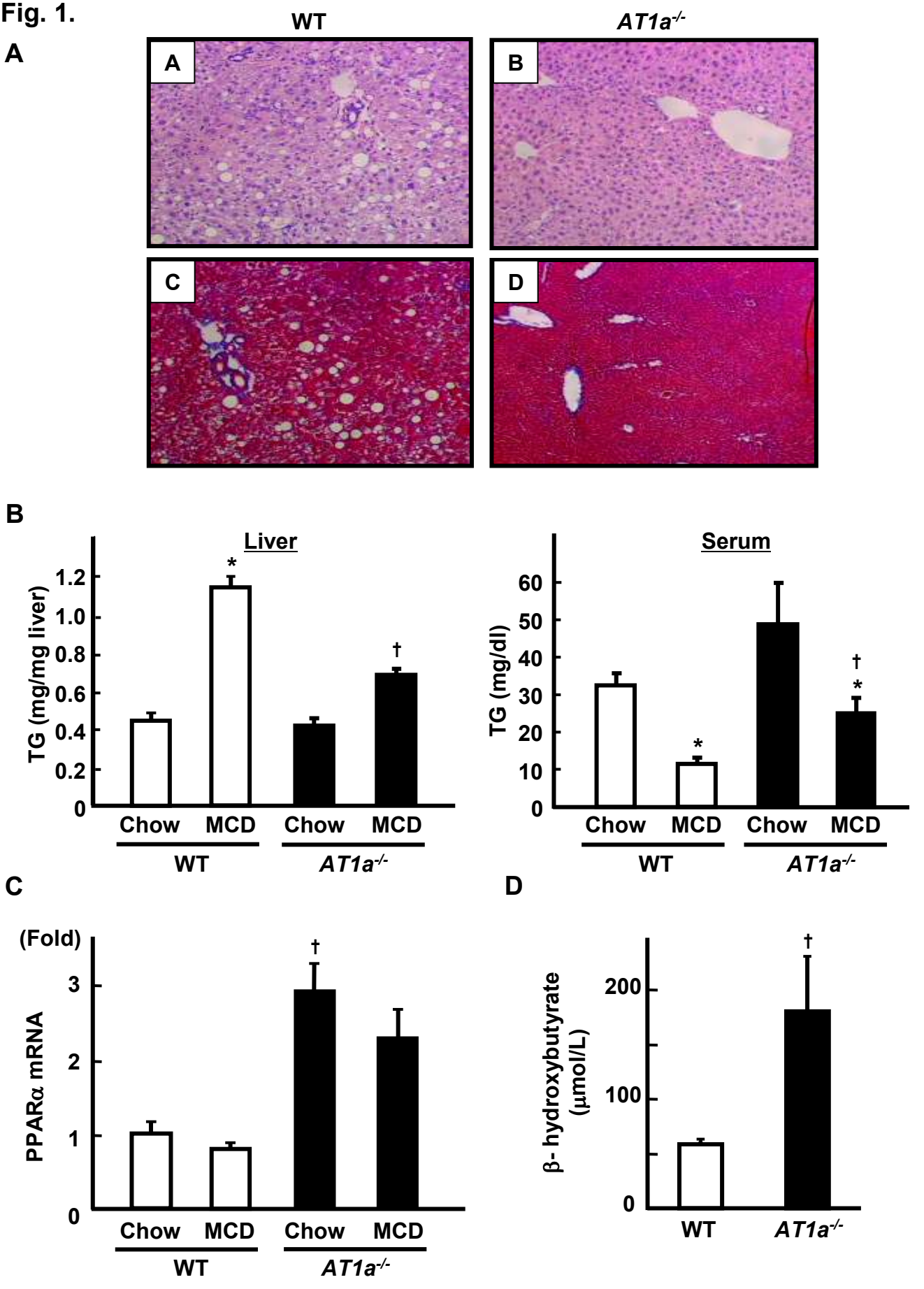

A
WT
AT1a-/-
A
B
C
D
B
Liver
TG (mg/mg liver)
1.2
1.0
0.8
0.6
0.4
0.2
0
Chow
MCD
Chow
MCD
WT
AT1a-/-
Serum
TG (mg/dl)
60
50
40
30
20
10
0
Chow
MCD
Chow
MCD
WT
AT1a-/-
C
(Fold)
PPARα mRNA
3
2
1
0
Chow
MCD
Chow
MCD
WT
AT1a-/-
D
β- hydroxybutyrate (μmol/L)
200
100
0
WT
AT1a-/-

Fig. 2.

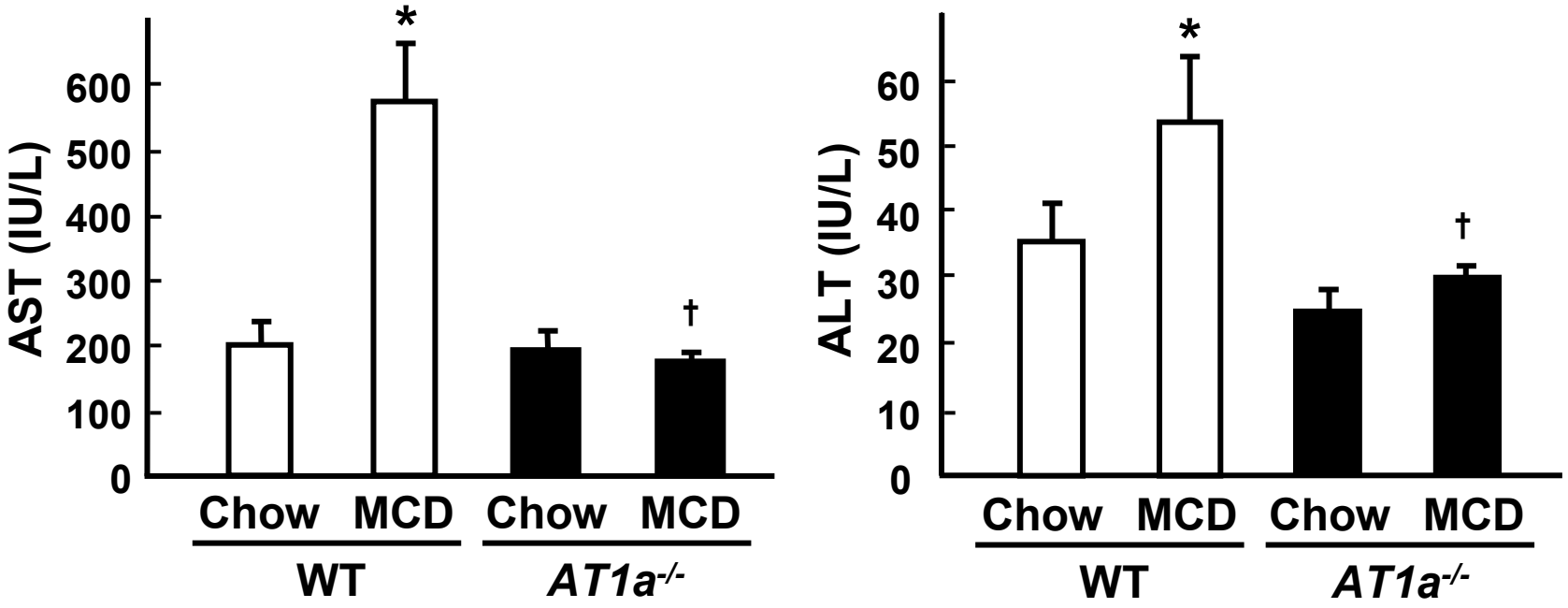

AST (IU/L)
600
500
400
300
200
100
0
*
†
Chow
MCD
Chow
MCD
WT
AT1a-/-
ALT (IU/L)
60
50
40
30
20
10
0
*
†
Chow
MCD
Chow
MCD
WT
AT1a-/-

Fig. 3.

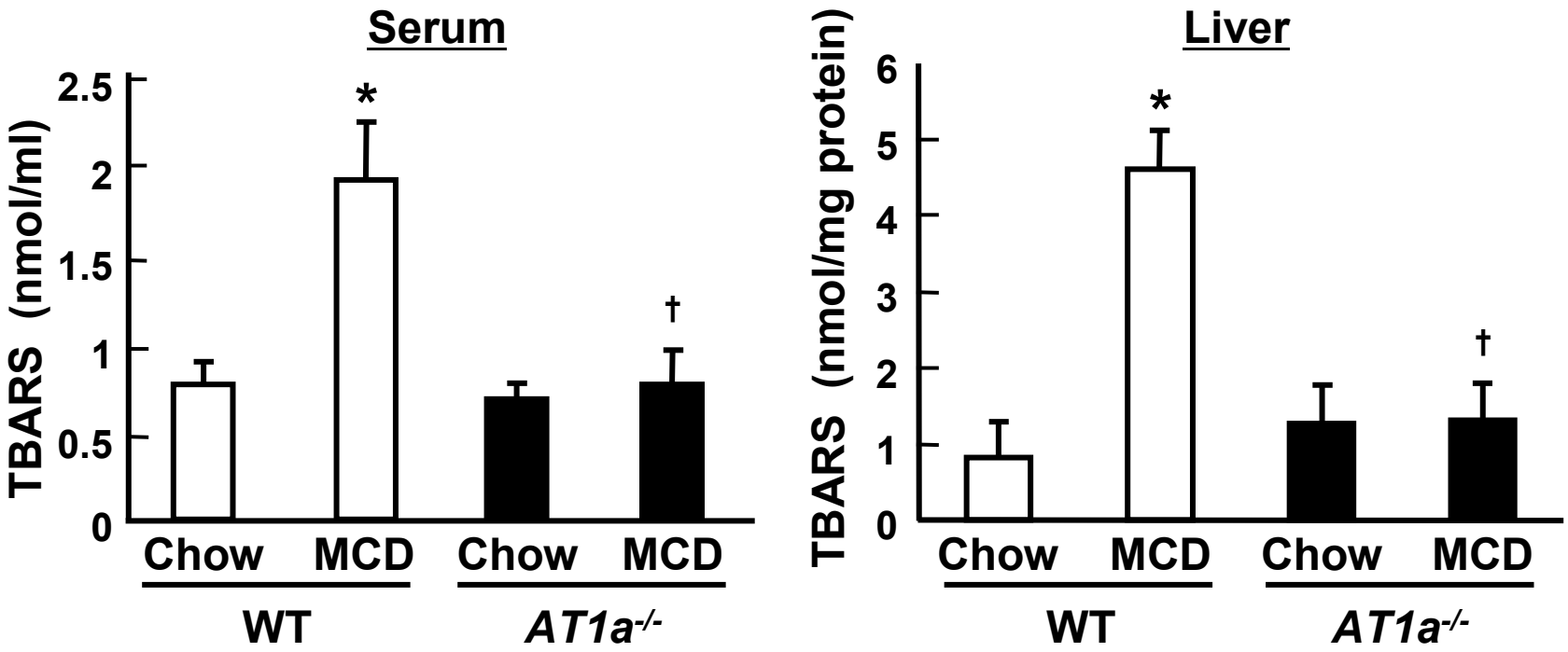
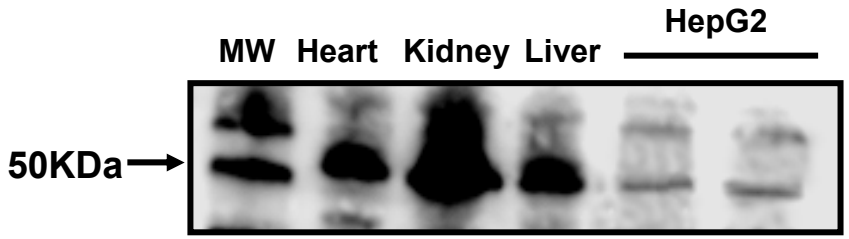

Serum
TBARS (nmol/ml)
2.5
2
1.5
1
0.5
0
*
†
Chow
MCD
Chow
MCD
WT
AT1a-/-
Liver
TBARS (nmol/mg protein)
6
5
4
3
2
1
0
*
†
Chow
MCD
Chow
MCD
WT
AT1a-/-

Fig. 4.


MW
Heart
Kidney
Liver
HepG2
50KDa

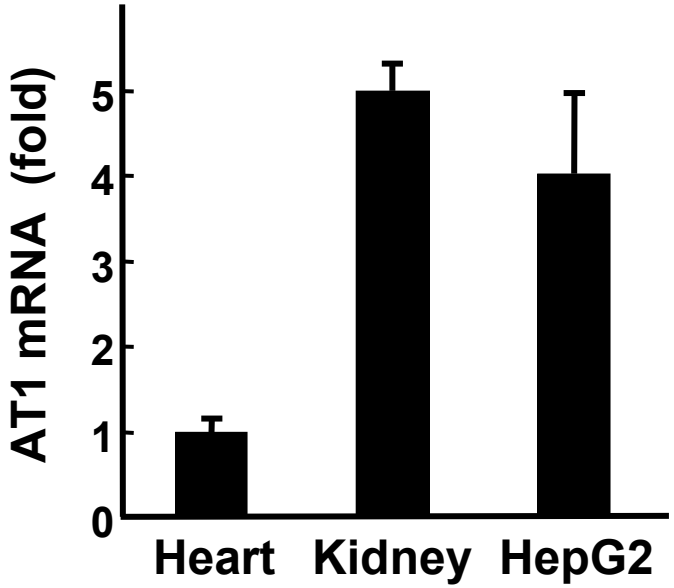

AT1 mRNA (fold)
0
1
2
3
4
5
Heart
Kidney
HepG2

Fig. 5.

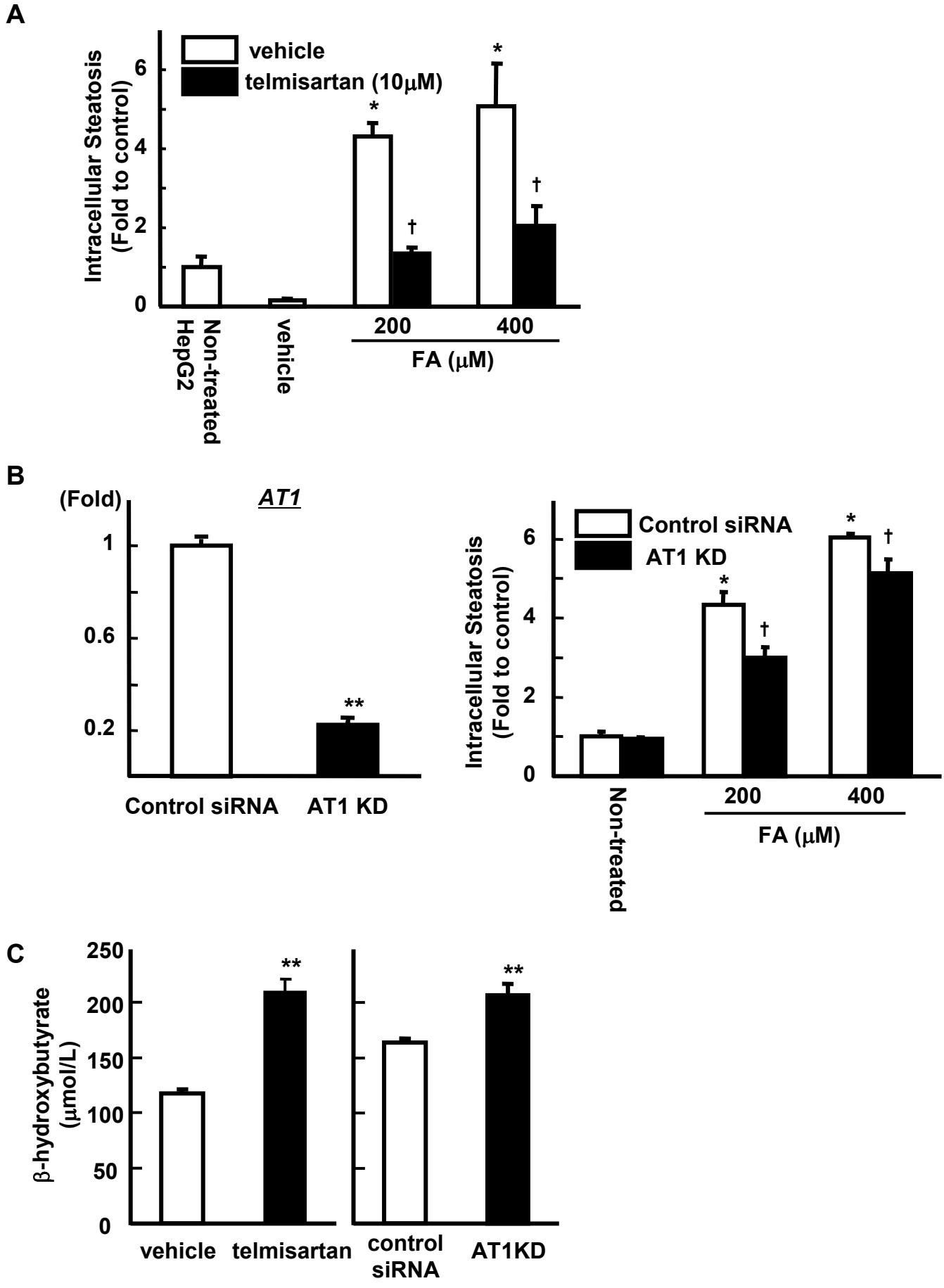
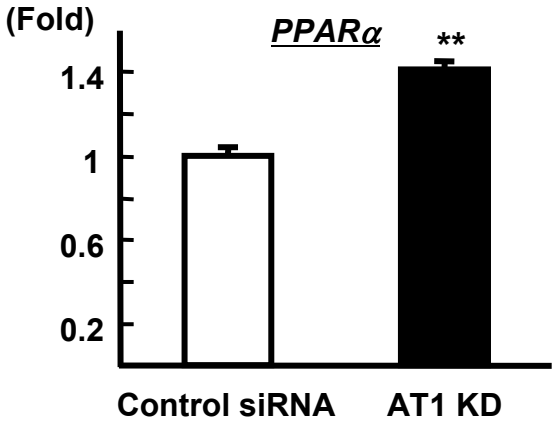
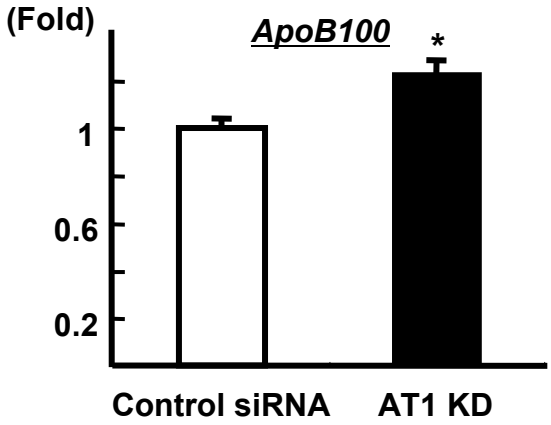
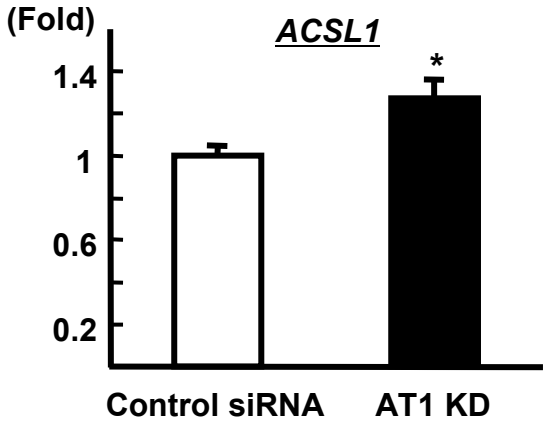

A
vehicle
telmisartan (10μM)
Intracellular Steatosis
(Fold to control)
0
2
4
6
*
†
*
†
HepG2
Non-treated
vehicle
200
400
FA (μM)
B
(Fold)
AT1
1
0.6
0.2
**
Control siRNA
AT1 KD
Control siRNA
AT1 KD
Intracellular Steatosis
(Fold to control)
0
2
4
6
*
†
*
†
Non-treated
200
400
FA (μM)
C
β-hydroxybutyrate
(μmol/L)
0
50
100
150
200
250
**
**
vehicle
telmisartan
control
siRNA
AT1KD

Fig. 6.

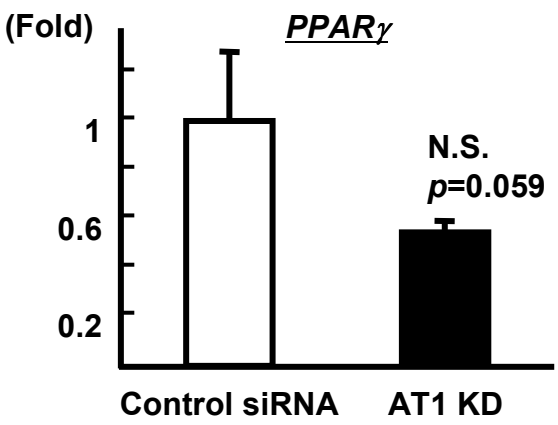
A

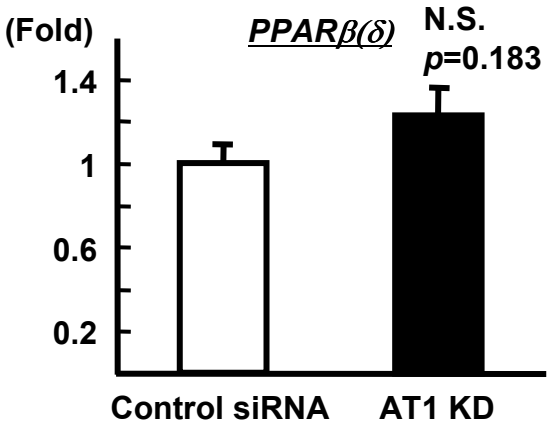

(Fold)
PPARα
**
1.4
1
0.6
0.2
Control siRNA
AT1 KD


(Fold)
ApoB100
*
1
0.6
0.2
Control siRNA
AT1 KD


(Fold)
ACSL1
*
1.4
1
0.6
0.2
Control siRNA
AT1 KD

B


(Fold)
PPARγ
N.S.
p=0.059
1
0.6
0.2
Control siRNA
AT1 KD


(Fold)
PPARβ(δ)
N.S.
p=0.183
1.4
1
0.6
0.2
Control siRNA
AT1 KD