Telmisartan, An Angiotensin II Type 1 Receptor Blocker, Controls Progress of Nonalcoholic Steatohepatitis in Rats

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Abstract The term nonalcoholic steatohepatitis (NASH) has recently been proposed to identify a fatty liver disease accompanied by diffuse fatty infiltration and inflammation. However, no drug therapy has been established for NASH as yet. In the present study, we demonstrate the effect of the angiotensin II type 1 receptor antagonist telmisartan on the development of NASH in a rat model. Telmisartan, but not the angiotensin receptor antagonist valsartan, markedly attenuated hepatic steatosis, inflammation, and fibrosis in these rats. The quantitative parameters of steatosis, inflammation, and fibrosis were also ameliorated by treatment with telmisartan. Compared with telmisartan, the peroxisome proliferator-activated receptor-γ agonist pioglitazone attenuated hepatic steatosis and fibrosis of the liver to a similar degree. However, telmisartan, but not pioglitazone, dramatically decreased both subcutaneous and visceral fat. In conclusion, these results indicated that telmisartan should be the drug of first choice for the treatment of patients with NASH.

Keywords Steatosis · Fibrosis · Inflammation · AT-II blockade · PPAR-γ activation

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver injury, and represents a spectrum of conditions that are histologically characterized by macrovesicular hepatic steatosis [1, 2]. NAFLD affects individuals who have not consumed alcohol in amounts considered harmful to the liver. The spectrum of NAFLD is broad, extending from simple steatosis through nonalcoholic steatohepatitis (NASH) to cirrhosis and liver failure [3, 4]. Among them, the term nonalcoholic steatohepatitis has recently been proposed to identify a fatty liver disease that is accompanied by diffuse fatty infiltration and inflammation [5, 6]. Although the pathologic findings in NASH and alcoholic liver disease are similar, the exact mechanisms that cause NASH are still unclear. Recently, a “2-hit hypothesis” was proposed to explain the development of NASH. Namely, steatosis as the first hit followed by a second hit that includes inflammation, oxidative damage, and fibrosis [5]. Many factors, such as cytokines, endotoxin, oxidative stress, and insulin resistance, have been proposed as the causes for the second hits of NASH [5, 6]. Several therapies, including diet [7] and antioxidants [8], have been tried to treat patients with NASH. However, no therapeutic strategy has been established for NASH as yet.

Two drug therapies have been recently proposed. One is peroxisome proliferator-activated receptor gamma (PPAR-γ) ligand therapy to improve insulin resistance [9]. Because NASH is a metabolic disease, it is reasonable to assume that an improvement of insulin resistance by PPAR-γ ligand will result in the amelioration of NASH. Another therapy is the
use of angiotensin II type I receptor antagonists. Recently, angiotensin II, the principal effector of the renin–angiotensin system, has been reported to have a crucial role in the pathogenesis of hepatic steatosis [10] and hepatic fibrosis [11] in a rat model of NASH. In addition, administration of the angiotensin II type 1 receptor antagonist losartan, was reported to improve liver biochemical indices of hepatic necroinflammation in NASH patients [12]. However, the mechanism of action of angiotensin II type 1 receptor antagonists has not been fully investigated yet. To clarify various questions about the recently proposed drug therapies, we investigated the inhibitory effect of angiotensin II type 1 receptor antagonists on the development of NASH in a choline-deficient, l-amino acid-defined diet (CDAA) animal model. In the present study, we have clarified 2 important points: (1) Whether the inhibitory effect on the development of NASH can be obtained with any angiotensin II type 1 receptor antagonist; and (2) whether amelioration of NASH is due to a blockade of the angiotensin II type 1 receptor, or other mechanisms such as the activation of PPAR-γ. Our findings may contribute to the establishment of a therapy for patients with NASH in the near future.

Materials and methods

Reagents and diets

PPAR-γ ligand, pioglitazone, and angiotensin II type I receptor antagonists, telmisartan and valsartan, were kind gifts from Takeda Pharmaceutical Co. (Tokyo, Japan), Nippon Boehringer Ingelheim Co. (Tokyo), and Novartis Pharma Co.

Fig. 1 Choline-deficient, l-amino acid-defined diet (CDAA), NASH animal model. (a) Typical photographs of tissue sections from rats fed a CDAA diet or a CSAA control diet, showing steatosis, inflammation, oxidative stress, and fibrosis. Steatosis, inflammation, oxidative stress, and fibrosis were evaluated by Oil red O (red), hematoxylin and eosin, nitrotyrosine (brown), and Masson trichrome (blue) staining, respectively (original magnification, ×100). (b) Quantitative parameters of steatosis, inflammation, and fibrosis. The tissue content of triglycerides (TG), inflammatory cell number, and percentage of collagen area were measured using quantitative parameters of steatosis, inflammation, or fibrosis, respectively. Data are expressed as the mean values ± SEM from 10 samples. (c) Comparison of other parameters in CDAA and CSAA control rats. Expression of collagen I and α-SMA protein in the liver from CDAA rats was evaluated by Western blot analysis and expressed as fold increase compared to that in CSAA control rats. Serum levels of TGF-β, TNF-α, ALT, leptin, and adiponectin were measured and expressed as the mean values ± SEM (n = 10)
(Tokyo), respectively. The CDAA and choline-sufficient, I-
amino acid-defined diet (CSAA) were obtained in powdered
form from Dyets (Bethlehem, PA; product Nos. 518753 and
518754) [13].

Animal treatment and experimental procedure

All animals were treated humanely according to the Na-
tional Institutes of Health guidelines and the AERI-BBRI
Animal Care and Use Committee guidelines. All animal ex-
periments were approved by the institutional animal care
and use committee of Yokohama City University School of
Medicine.

Briefly, male Fischer-344 rats (6 weeks, body weight 150–
160 g) were purchased from Japan SLC Inc. (Hamamatsu,
Shizuoka, Japan). They were quarantined for 1 week and then
housed in stainless steel, mesh cages under controlled con-
ditions of temperature (25 ± 2°C), humidity (50 ± 10%),
and lighting (12-hour light/dark cycle). The animals were
allowed free access to food and tap water throughout the
acclimatization and experimental periods.

After acclimatization for 1 week, the rats were divided into
7 experimental groups. Animals in groups 1–6 were given
the CDAA diet throughout the experiment, whereas those in
group 7 were given the CSAA diet as the control. Group 2
was given pioglitazone by gavage once a day at a dose of

Fig. 1 Continued.
Fig. 2 Effects of angiotensin II type 1 receptor antagonists and a PPAR-γ agonist on liver steatosis. (a) Typical photographs of liver tissue sections stained with Oil red O staining, as a marker of steatosis (original magnification, ×100). (b) Content of triglycerides (TG) in the livers of rats treated with pioglitazone (Pio, 1.875 mg/kg per day), valsartan (Val, 5 mg/kg per day), telmisartan (Tel, 5 mg/kg per day), or vehicle (Control). TG content is expressed as mg/g tissue, and data are expressed as mean values ± SEM of 10 animals.1.875 mg/kg per day [14, 15]. Group 3 was given valsartan at a dose of 5 mg/kg per day. Groups 4, 5, and 6 were given telmisartan at a dose of 1.7, 5, and 15 mg/kg per day, respectively [16]. After 12 weeks, the animals were humanely killed under ether anesthesia and samples were collected.

Measurement of serum biochemical markers

Serum alanine aminotransferase (ALT) was measured using Spotchem SP-4410 (Arklay Co, Kyoto, Japan). Leptin, adiponectin, fasting blood sugar, and fasting insulin levels were determined by radioimmunoassay using commercially available kits (Linco Research Inc., St. Charles, MO). Hepatic tumor necrosis factor (TNF)-α was measured by ELISA using the Quantikine Rat ELISA kit (R&D Systems, Minneapolis, MN). Transforming growth factor (TGF)-β1 expression level was measured using a TGF-β1 ELISA system (Amersham Biosciences Co, Piscataway, NJ).

Measurement of liver triglycerides content

Liver samples were homogenized in 50 mmol Tris/HCl buffer, pH 7.4, containing 150 mmol NaCl, 1 mmol EDTA, and 1 μmol PMSF. Triglycerides were analyzed enzymatically using a diagnostic kit [17] (Infinity, Thermo DMA, Arlington, TX) and measured by spectrophotometry (Beckman Coulter Inc, Fullerton, CA).

Marker protein expressions in liver

Expression of actin α smooth muscle (α-SMA) and collagen I was determined by Western blot analysis as described pre-
Effects of angiotensin II type 1 receptor antagonists and a PPAR-γ agonist on liver inflammation. (a) Typical photographs of liver tissue sections stained with hematoxylin and eosin staining (original magnification, ×100).

(b) Quantitative parameters of inflammation in control (Cont), pioglitazone-treated (Pio, 1.875 mg/kg per day), valsartan-treated (Val, 5 mg/kg per day), or telmisartan-treated (Tel, 5 mg/kg per day) rats. Serum alanine aminotransferase (ALT, a marker of liver inflammation) and TNF-α were measured and expressed as the mean values ± SEM of 10 animals. The number of infiltrating inflammatory cells in the liver was measured and expressed as the mean value for 10 animals.

Histopathologic and immunohistochemical evaluations

Liver samples were excised and embedded in Tissue-Tek OCT compound (Sakura Finetek U.S.A Inc, Torrance, CA) and paraffin for histologic analysis. Five-micrometer-thick sections of formalin-fixed and paraffin-embedded sections were processed routinely for hematoxylin and eosin staining. The presence of collagen, as an index of fibrosis in the lesions, was examined in Masson’s trichrome-stained preparations. Serial cryostat sections were used for staining nitrotyrosine, as an index of oxidative stress, using a polyclonal rabbit anti-nitrotyrosine antibody (Upstate Biotechnology Inc, Iowa city, IA) diluted 1:2000 as previously described [22, 23]. As a negative control, normal mouse or rabbit IgG was used instead of the primary antibody. The OCT-embedded samples were serially sectioned at 4 µm. For the evaluation of fatty deposition, the liver tissues were stained with oil red O. For quantification, 50 fields were microscopically examined at a 40× magnification using a grid of 0.0625 mm² with 100 points. Values were expressed as the number of stained points per 100 points. The rest of liver tissues was frozen and stored in liquid nitrogen or at −70°C until use for molecular and biochemical determinations.

Measurement of PPAR-γ activation

The PPAR-γ response element (PPRE) was transfected into 70% confluent Huh7 cells using Lipofectamin 2000 (Invitrogen, Carlsbad, CA). The cells were treated with 50 nmol PPRE for 24 hours before measurement by reporter assay. We used control medium as the control. To perform the reporter assay, Huh7 cells were seeded at a density of 1 × 10⁴...
Fig. 4 Effects of angiotensin II type 1 receptor antagonists and PPAR-γ agonist on hepatic fibrosis. (a) Typical photographs of liver tissue sections stained with Masson trichrome staining, as a marker of fibrosis (original magnification, ×100). (b) Quantitative parameters of fibrosis in control (Cont), pioglitazone-treated (Pio, 1.875 mg/kg per day), valsartan-treated (Val, 5 mg/kg per day), or telmisartan-treated (Tel, 5 mg/kg per day) rats. Collagen and α-SMA protein levels were detected by Western blot analysis and were expressed as fold increase in comparison with those in CSAA rats (nonfibrosis liver). The serum level of TGF-β was also measured. Data are expressed as mean values for 10 animals.

Figure 4:

A) Typical photographs of liver tissue sections stained with Masson trichrome staining, as a marker of fibrosis. (a) CDAA-control, CDAA + Pioglitzone, CDAA + Valsartan (5mg/kg). (b) CDAA + Telmisartan (1.7mg/kg), CDAA + Telmisartan (5mg/kg), CDAA + Telmisartan (15mg/kg).

B) Quantitative parameters of fibrosis in control (Cont), pioglitazone-treated (Pio), valsartan-treated (Val), or telmisartan-treated (Tel) rats. Collagen and α-SMA protein levels were detected by Western blot analysis and were expressed as fold increase in comparison with those in CSAA rats (nonfibrosis liver). The serum level of TGF-β was also measured. Data are expressed as mean values for 10 animals.

Statistical analysis

All results are expressed as mean values ± SE. Statistical comparisons were made using Student’s t-test or Scheffé’s multiple comparison test after the analysis of variances. The results were considered significantly different at P < .05.

Results

Animal model of NASH

In the pathogenesis of human NASH, hepatic steatosis followed by inflammation, oxidative damage and fibrosis are observed [4, 24, 25]. As shown in Fig. 1a, steatosis, inflammation, oxidative stress, and fibrosis were observed in rats fed the CDAA. In contrast, the rats fed the CSAA diet developed no steatosis (Fig. 1a). In addition, other biochemical, inflammatory, and fibrosis markers showed a pattern similar to that observed in human NASH (Fig. 1b, c). Therefore, rats fed the CDAA diet were considered a good animal model of NASH [13, 15]. There were no significant differences between the CDAA and CSAA diet groups in the total amount of calories consumed nor in body weight (data not shown).
Effect of angiotensin II type 1 receptor antagonists and a PPAR-γ agonist on liver steatosis

We used 2 angiotensin II type 1 receptor antagonists, valsartan and telmisartan, and the PPAR-γ agonist pioglitazone, to treat rats fed the CDAA diet. Hepatic steatosis was markedly suppressed by telmisartan in comparison to the control (Fig. 2a). The effect of telmisartan on hepatic steatosis was dose dependent (1.7–15 mg/kg). The content of triglycerides (TG) in the liver was also suppressed by telmisartan (Fig. 2b). A similar effect was observed when CDAA rats were treated with pioglitazone. In contrast, valsartan exerted a weak suppressive effect on the development of hepatic steatosis. These results indicate that angiotensin II type 1 receptor antagonists suppress steatosis but their efficacy varies greatly.

Effect of angiotensin II type 1 receptor antagonists and a PPAR-γ agonist on liver inflammation

We next investigated the effect of the angiotensin II type 1 receptor antagonists and a PPAR-γ agonist on liver inflammation. As shown in Fig. 3a and b, the number of infiltrating inflammatory cells in the liver of rats treated with pioglitazone, valsartan, and telmisartan was significantly reduced compared to that in the control liver. However, no difference was found among the treatment groups. Other indexes of inflammation, such as ALT and TNF-α levels, were also significantly inhibited by pioglitazone, valsartan, and telmisartan. Treatment with pioglitazone or telmisartan inhibited ALT and TNF-α levels more effectively than valsartan; this difference was statically significant.

Effect of angiotensin II type 1 receptor antagonists and a PPAR-γ agonist on hepatic fibrosis

Hepatic fibrosis was markedly suppressed by valsartan and telmisartan as well as by pioglitazone (Fig. 4a). The indexes of fibrosis, namely, increases in collagen and α-SMA, were significantly suppressed by valsartan, telmisartan, and pioglitazone (Fig. 4b). Pioglitazone or telmisartan inhibited the increases in collagen and α-SMA levels more effectively than valsartan. A similar effect was observed on the TGF-β level.

Effect of angiotensin II type 1 receptor antagonists and a PPAR-γ agonist on lipid metabolism and fat accumulation in the whole body

Food intake was similar in all groups (Fig. 5a). The liver TG content was significantly suppressed by pioglitazone
and telmisartan, but not by valsartan (Fig. 5b). In addition, the serum level of TG rats was lower in telmisartan-treated rats than in valsartan-treated rats (420 ± 48 versus 562 ± 63 mg/dL). Subcutaneous fat accumulation was significantly suppressed by valsartan and telmisartan. However, pioglitazone did not inhibit subcutaneous fat accumulation (Fig. 5c). In contrast, visceral fat accumulation was significantly suppressed by pioglitazone and telmisartan, but not by valsartan (Fig. 5d). These results indicate that telmisartan suppresses hepatic steatosis more effectively than valsartan, and that the effect of telmisartan might be due to an improvement of lipid metabolism in the whole body. In fact, the serum level of leptin that is related to fat accumulation, was significantly reduced by pioglitazone and telmisartan, but not by valsartan (Fig. 6b). The serum level of adiponectin, which is an anti-inflammatory and anti–insulin-resistant adipokine, tended to increase in pioglitazone and telmisartan-treated animals (Fig. 6b).

**Effect of angiotensin II type 1 receptor antagonists and a PPAR-γ agonist on PPRE-dependent transcription**

We next examined the effect of angiotensin II type 1 antagonists on PPRE-dependent transcription in cultured hepatic cells Huh7 strain, because PPRE-dependent transcription is one of the most important factors involved in lipid metabolism and liver steatosis [26–28]. Valsartan-treated cells showed no significant alterations of PPRE-dependent transcription compared with the control (Fig. 6a). In contrast, PPRE-dependent transcription was significantly activated in telmisartan-treated cells, and an almost similar effect was observed in pioglitazone-treated cells. These results indicate that telmisartan is a potent PPAR-γ agonist and, therefore, it is different from other angiotensin II type 1 receptor antagonists such as valsartan.

**Discussion**

In the present study, we clearly demonstrated that telmisartan, but not valsartan, markedly attenuated hepatic steatosis and fibrosis in a rat model of NASH. The suppressive effect of telmisartan and valsartan on the development of steatosis may also be observed with other antagonists, but that of telmisartan seems to be the strongest.

From where does the difference between telmisartan and other angiotensin II type 1 receptor antagonists derive? One of the reasons is the difference in chemical structure. The structure of the angiotensin II type 1 receptor antagonists in clinical use today resembles that of valsartan; namely, they
are biphenyl tetrazole derivatives. In contrast, telmisartan is a non-tetrazole derivative, with a single carboxylic acid group instead of a large tetrazole ring [29]. Thus, telmisartan is structurally quite different from all other angiotensin II antagonists.

Recently, several reports have indicated that the structure of telmisartan resembles that of pioglitazone, a thiazolidinedione-type PPAR-γ ligand, and telmisartan is, therefore, considered to act as a partial agonist of PPAR-γ [29–31]. Namely, telmisartan influences the expression of PPAR-γ target genes that are involved in the metabolism of carbohydrates and lipids, as well as in the control of glucose, insulin, and TG levels. The present study clearly shows that telmisartan strongly activates PPAR-γ. This is a member of the nuclear hormone receptor superfamily and acts as a transcription factor that regulates the expression of multiple genes involved in the metabolism of carbohydrates and lipids as well as in inflammation [32–37]. The PPAR-γ agonist effect of telmisartan resulted in a stronger inhibition of NASH progression compared with other angiotensin II antagonists such as valsartan.

Activation of the PPAR-γ pathway would improve insulin resistance, dyslipidemia, adipokine secretion, inflammation, and cell proliferation, leading mainly to an improvement of hepatic steatosis, NASH first hit. Blockade of the renin–angiotensin pathway would improve oxidative stress, inflammation, and proliferation, leading to an improvement of hepatic fibrosis, NASH second hit. According to the present results and previous reports, both effects of telmisartan as angiotensin II antagonist and PPAR-γ agonist are great advantages for their therapeutic use in patients with NASH and other types of vascular hypertrophy [38, 39].

In recent years, NASH has been considered as a metabolic syndrome affecting mainly the liver, and visceral fat has been considered to play an important role on the progression of NASH. The majority of NASH patients present with complications like diabetes mellitus, hyperlipidemia, hypertension, and obesity. Therefore, many other agents targeting different components of the pathogenesis of NASH have been studied, including insulin-sensitizing agents, weight loss agents, and anti-hyperlipidemic agents [40–44]. Drug therapy for NASH is positively carried out when patients cannot control eating or drinking, or cannot modify their lifestyle with diet and exercise. Pioglitazone has been used to treat NASH and its effectiveness has been reported [11, 45]. Both telmisartan and pioglitazone ameliorated hepatic steatosis, inflammation, and fibrosis to a similar degree. However, from the viewpoint of systemic conditions such as lipid metabolism and body weight, pioglitazone-treated rats showed increases in body weight and subcutaneous inguinal fat; telmisartan-treated rats showed mild loss of body weight and marked decreases in both subcutaneous inguinal and epididymal visceral fat. These are the most different and important points of the therapeutic efficacy of pioglitazone and telmisartan on animal NASH. In facts, we gave medication (pioglitazone 15 mg/day, for 6 months) to our 32 outpatients who could not improve liver steatosis and serum ALT levels with exercise and diet. Almost half (n = 15) showed little or no effect on glucose, lipid metabolism, and became worse about insulin resistance and liver steatosis compared with before medication (data not shown).

In conclusion, telmisartan should be the drug of first choice for the treatment of patients with NASH in the near future.

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