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Deletion of angiotensin II type I receptor reduces hepatic steatosis☆

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***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.**

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***Keywords*: Lipid metabolism; Renin-angiotensin system; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Peroxisome proliferator-activated receptor α**

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) encompasses a wide spectrum of liver pathology – from simple steatosis alone, through necroinflammatory disorder referred to as non-alcoholic 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

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Abbreviations: AT1R, angiotensin II type 1 receptor; ARB, angiotensin II type 1 receptor blocker; PPAR, peroxisome proliferator-activated receptor; Ang, angiotensin; WT, wild-type; MCD, methionine–choline deficient; TBARS, thiobarbituric acid-reactive substances; TG, triglyceride; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; HSC, hepatic stellate cell; siRNA, small interfering RNA; DMSO, dimethyl sulfoxide; FFA, free fatty acid; PBS, phosphate buffered saline; AST, aspartate aminotransferase; ALT, alanine aminotrasferase; ApoB, apolipoprotein B; ACSL, acyl-CoA synthetase; VLDL, very low density lipoprotein.

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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]. A clinical report has also demonstrated that losartan, an ARB, 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]. An animal study using obese Zucker rats 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 PPARγ-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 two 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. Materials and methods

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 performed according to the guidelines 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. β-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 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 dimethyl sulfoxide (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].

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