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An angiotensin II type 1 receptor antagonist, olmesartan medoxomil, improves experimental liver fibrosis by suppression of proliferation and collagen synthesis in activated hepatic stellate cells

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1 We studied the effect of a new angiotensin II type 1 (AT_1) receptor antagonist, olmesartan medoxomil (olmesartan), on the fibrogenic responses in rat hepatic stellate cells (HSCs) and liver fibrogenesis.

2 Olmesartan (1 mg kg^{-1} per day) was orally administered to fibrotic rats, induced by bile duct ligation. Liver hydroxyproline content, the mRNA expression of collagen α1(I) and α-smooth muscle actin (α-SMA), and plasma levels of transforming growth factor-β1 (TGF-β1) were significantly reduced by olmesartan treatment, suggesting that olmesartan improved liver fibrosis. Interestingly, AT_1 receptors were found to be expressed in α-SMA-positive cells in the fibrotic area of livers in bile duct-ligated rats by immunohistochemical analysis. Olmesartan treatment reduced the number of these cells.

3 *In vitro* experiments showed that angiotensin II (Ang II) treatment induced proliferation and collagen synthesis, and upregulated the profibrogenic cytokines, TGF-β1 and connective tissue growth factor (CTGF), in rat primary HSCs. Olmesartan blocked all these effects of Ang II.

4 Based on these results, since activated HSCs were found to express AT_1 receptors and Ang II is thought to play an important role in the pathogenesis of liver fibrosis by binding to these receptors, olmesartan may act as a potent antifibrotic drug to suppress the proliferation, collagen synthesis and the expression of profibrogenic cytokines in activated HSCs by blocking these receptors.
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Keywords: Olmesartan medoxomil; hepatic stellate cells; liver fibrosis; angiotensin II; angiotensin II type 1 receptor; transforming growth factor-β1; α-smooth muscle actin; connective tissue growth factor; collagen; bile duct ligation

Abbreviations: ACE, angiotensin-converting enzyme; ALT, alanine aminotransferase; Ang II, angiotensin II; AST, aspartate aminotransferase; BDL, bile duct ligation; CTGF, connective tissue growth factor; DAB, 3, 3′-diaminobenzidine tetrahydrochloride; ECM, extracellular matrix; FAM, 6-carboxyfluorescein; GBSS, Gey's balanced salts solution; HBSS, Hanks' balanced salts solution; HSC, hepatic stellate cell; olmesartan, olmesartan medoxomil; PDGF, platelet-derived growth factor; RAS, rennin–angiotensin system; α-SMA, α-smooth muscle actin; TAMRA, 6-carboxytetramethylrhodamine; TCA, trichloroacetic acid; TGF-β, transforming growth factor-β

Introduction

Chronic liver injury is caused by a variety of insults, including viral hepatitis, alcohol and drug abuse, and autoimmune hepatitis. Injury often continues for months to years and causes liver fibrosis, which eventually results in liver cirrhosis, the end-stage of liver disease. Currently, there is no established therapy to delay or reverse the progression of liver fibrosis. Liver fibrosis is characterized by the excess production and deposition of extracellular matrix (ECM), and hepatic stellate cells (HSCs) are a major source of ECM (Li & Friedman, 1999). HSCs, which are normally quiescent, possess vitamin A droplets and produce small amounts of ECM components. Upon liver injury, HSCs undergo morphological transdifferentiation to myofibroblast-like cells, which are characterized as having a proliferative, fibrogenic and contractile phenotype. This process is known as 'activation'. Activated HSCs express α-smooth muscle actin (α-SMA) and produce an excess of collagen and other ECM components. As a result, liver fibrosis is developed. Several compounds have been shown to prevent experimental liver fibrosis by the suppression of HSC activation, inhibition of collagen production, promotion of ECM degradation or induction of HSC apoptosis (Ueki *et al*., 1999; Bruck *et al*., 2001; Tada *et al*., 2001; Wright *et al*., 2001; Kon *et al*., 2002). However, there is no evidence of their efficacy against human chronic liver disease.

Angiotensin II (Ang II) plays a central role in the regulation of systemic blood pressure and fluid homeostasis. The action of Ang II is mediated by mainly two subtypes of receptors, angiotensin II type 1 (AT_1) receptors and type 2 (AT_2) receptors, which are distributed in many kinds of organs and tissues. Recently, several lines of evidence have suggested that the rennin–angiotensin system (RAS) plays an important role

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in the pathogenesis of organ fibrosis (Brilla, 2000; Sun *et al*., 2000). In mesangial cells and other cell types, Ang II has been shown to promote the proliferation and collagen synthesis (Ray *et al*., 1991; Wolf *et al*., 1992; Kagami *et al*., 1994; Weber *et al*., 1994; Tharaux *et al*., 2000). Moreover, the expression of transforming growth factor-β (TGF-β), the key cytokine in the development of cardiac and renal fibrosis, is increased by Ang II (Weber, 1997). Blockade of the RAS by angiotensin-converting enzyme (ACE) inhibitors or by AT_1 antagonists has been shown to improve the progression of organ fibrosis (Ishidoya *et al*., 1995; Kim *et al*., 1995; Molteni *et al*., 2000). In the liver, Ang II is considered to play a role in the regulation of intrahepatic circulation (Schneider *et al*., 1999). Recently, it has been reported that Ang II induces proliferation and contraction of human HSCs, and TGF-β expression in rat HSCs, which are mainly mediated by AT_1 receptors (Bataller *et al*., 2000; Yoshiji *et al*., 2001), and that ACE inhibitors or AT_1 antagonists attenuate the progression of liver fibrosis *in vivo* (Ramos *et al*., 1994; Jonsson *et al*., 2001; Ohishi *et al*., 2001; Paizis *et al*., 2001; Yoshiji *et al*., 2002). These reports suggest that Ang II and RAS might play an important role in the pathogenesis of liver fibrosis.

Olmesartan medoxomil (olmesartan), (5-methyl-2-oxo-1,3-dioxolen-4-yl) methoxy-4-(1-hydroxy-1-methylethyl)-2-propyl-1-{4-[2-(tetrazol-5-yl)-phenyl] phenyl}methylimidazol-5-carboxylate, CS-866, is a new AT_1 antagonist. It is a prodrug containing an ester moiety, which is rapidly cleaved to release the active form of olmesartan (RNH-6270) after oral administration (Mizuno *et al*., 1995). This drug has a potent and long-lasting action, which is effective when given in a once-daily dose regimen, and is now sold on the U.S. and European market for treating hypertension. Olmesartan also might be effective for cardiac and kidney disorders (Koike *et al*., 2001; Mizuno *et al*., 2002). In this study, we estimated the effect of olmesartan on liver fibrogenesis, and to investigate in detail the mechanism of fibrogenic actions of Ang II and its main receptor, AT_1, we studied the effects of Ang II and olmesartan on the proliferation, collagen synthesis, and the expression of profibrogenic cytokines, TGF-β and connective tissue growth factor (CTGF), in activated HSCs *in vitro*.

Methods

Animals

All experiments were carried out in accordance with the Animal Experimentation Guidelines of Sankyo Co. , Ltd Sprague–Dawley (SD) male rats were obtained from Japan SLC Inc. (Sizuoka, Japan), and maintained in a room under a temperature controlled at $23\pm2^{\circ}C$ and a 12-h light–dark lighting cycle. The animals were allowed a standard pellet chow and water *ad libitum*. Anesthesia was performed by intraperitoneally injecting nembutal (Dainippon Pharmaceutical, Osaka, Japan) at a dose of $50\,mg\,kg^{-1}$.

Isolation of rat hepatic stellate cells (HSCs)

Rat HSCs were prepared from SD male rats (>500 g body weight) by the method of Kawada *et al*. (1998) with some modifications. The liver was perfused through the portal vein with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salts solution (HBSS; Sigma Chemical Co., St. Louis, MO, U.S.A.) containing 0.06% EGTA at a flow rate of $10\,ml\,min^{-1}$ at 37°C for 10 min. The liver was then perfused with HBSS containing 0.1% pronase E (Merck, Damstadt, Germany), followed by 0.02% pronase E and 0.125% collagenase (type IV; Sigma). After perfusion, the digested liver was excised, minced, and incubated with gentle stirring in HBSS containing 0.05% pronase E, 0.05% collagenase, $20\,\mu g\,ml^{-1}$ DNase I (Roche Diagnostics, Mannheim, Germany) for 30 min at pH 7.3. After passage through a mesh with a pore size of 150 μm in diameter, the cells were centrifuged twice at $400\times g$ in Gey's balanced salts solution (GBSS) at 4°C for 7 min. The HSC-enriched fraction was obtained by centrifugation in GBSS containing 8.2% Nycodenz (Daiichi Pure Chemicals, Tokyo, Japan) at $1400\times g$ for 20 min. The HSCs in the upper white layer were washed twice by centrifugation. The cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY, U.S.A.) containing 10% fetal calf serum (FCS), $100\,\mu g\,ml^{-1}$ gentamicin sulfate (Gibco BRL), $100\,\mu g\,ml^{-1}$ streptomycin sulfate, and $100\,U\,ml^{-1}$ penicillin G sodium (Gibco BRL) on 96-well or six-well tissue culture plates (FALCON, Becton Dickinson, Franklin Lakes, NJ, U.S.A.) at 37°C under a 5% CO_2 atomosphere. The culture medium was replaced 2 days after plating and then every 2–3 days. Before reaching confluency, the cells were used in each experiment.

Measurement of cell proliferation

The proliferation rate of HSCs was determined by measuring the amount of [^{3}H]thymidine incorporated into the cellular DNA. The media were replaced with serum-free DMEM containing Ang II (human, Sigma) with or without RNH-6270 and then pulsed for 48 hr with $0.5\,\mu Ci\,ml^{-1}$ [methyl-^{3}H]thymidine (Daiichi Pure Chemicals). At the end of the pulsing period, the plates were frozen and stored at −80°C. After thawing, the cells were incubated with 0.05% trypsin/$0.53\,mmol\,l^{-1}$ EDTA solution (Gibco BRL) at 37°C. Cellular DNA was then fixed on a glassfilter (Filtermat A, Wallac) using a cell harvester (Harvester 96, Tomtec). The radioactivity was counted by a liquid scintillation counter (Betaplate 1205, Wallac).

Measurement of collagen synthesis

The rate of collagen synthesis in rat primary HSCs was determined by measuring the amount of [^{3}H]proline incorporated into collagenase-digestible macromolecules. The media were replaced with Ang II in serum-free DMEM containing $0.5\,mmol\,l^{-1}$ 3-aminopropionitrile (Tokyo Kasei Kogyo,Tokyo, Japan) and $0.1\,mmol\,l^{-1}$ L-ascorbic acid (Sigma) in the presence or absence of RNH-6270, and then pulsed for 48 h with $0.5\,\mu Ci\,ml^{-1}$ L-[2,3,4,5-^{3}H]proline (Daiichi Pure Chemicals). At the end of the pulsing period, the plates were frozen and stored at -80°C. After thawing, the cells were precipitated twice with 10% trichloroacetic acid (TCA; Nacalai Tesque, Kyoto, Japan) by centrifugation and washed twice with ethanol–ether (3 : 1) mixture. After drying overnight, the pellet was digested with $5\,mg\,ml^{-1}$ collagenase type VII (Sigma), dissolved in $50\,mmol\,l^{-1}$ Tris-HCl, and $5\,mmol\,l^{-1}$ $CaCl_2$, at pH 7.4, and at 37°C for 90 min, and precipitated twice with 10% TCA and

5% tannic acid (Sigma) by centrifugation. The supernatants from the two centrifugations were pooled, and 50 μl of the mixture was placed on a Deepwell LumaPlate (Packard). After drying overnight, the radioactivity was counted by a microplate scintillation counter (TopCount HTS; Packard).

Establishment of an in vivo *liver fibrosis model and administration of olmesartan*

SD male rats (200–250 g body weight) were used. Liver fibrosis was induced by common bile duct ligation (BDL), as previously described (Kountouras *et al*., 1984). The common bile duct was double-ligated and cut between the ligatures. About 10% of the rats died from surgical complications within a week after the operation, and were not included in the study. On Day 7, the surviving rats were randomly divided into two groups. In one group, olmesartan, dissolved in distilled water containing 0.5% carboxymethyl cellulose (Nacalai Tesque), was orally administered at a dose of 1 mg kg^{-1} six times a week from Day 7 to Day 20 after the operation (BDL/Olmesartan; $n=10$). In another group, the bile duct-ligated rats received the vehicle for the same time period (BDL; $n=10$). The control animals for BDL received a sham-operation, in which they were subjected to a midline incision and manipulation of the common bile duct without ligation. They received a vehicle injection for the same time period (Sham; $n=5$). On Day 21, the animals were killed under ether anesthesia. Blood was collected from the aorta, and plasma was prepared by centrifugation. Plasma samples were frozen and stored at -80°C. The liver and spleen were washed with saline and weighed. The specimens of liver were immediately snap-frozen and stored at -80°C for TaqMan polymerase chain reaction (PCR) analysis. Portions of the liver lobes were also fixed in 10% buffered formalin (Wako Pure Chemical Industries, Osaka, Japan) and embedded in paraffin for histological analysis.

Measurement of liver hydroxyproline content

Liver tissue (approximately 500 mg of wet weight) was hydrolyzed in 5 ml of 6 mol l^{-1} hydrochloric acid at 105°C overnight. The hydrolysate was centrifuged at $800\times g$ for 15 min and 20 μl of the supernatant was evaporated under vacuum. Then, the sediment was redissolved in 0.6 ml of 50% isopropanol and incubated with 0.1 ml of chloramine-T solution, containing 42 mg of chloramine-T (Wako) dissolved in 6.9 ml of acetate-citrate buffer and 0.21 ml of distilled water, for 10 min at room temperature. To this were added, 0.5 ml of Ehrlich's solution, including 5 g of *p*-dimethylaminobenzaldehyde (Wako) dissolved in 5.5 ml of 60% perchloric acid, and 26.8 ml isopropanol, and the mixture was incubated at 50°C for 90 min. After cooling, the absorbance was read at 558 nm. Hydroxyproline concentration was calculated from a standard curve prepared with high-purity hydroxyproline (Wako). Hydroxyproline levels were expressed in micrograms of hydroxyproline per gram liver. The values were multiplied by the liver weight to obtain the total liver hydroxyproline content.

Plasma AST and ALT assays

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the plasma were measured using Autoanalyzer (Hitachi 7250).

Measurement of TGF-β1 production

For the *in vitro* experiments, HSCs were incubated with Ang II in the presence or absence of RNH-6270 or platelet-derived growth factor-BB (PDGF-BB, rat; R&D Systems, Minneapolis, MN, U.S.A.) for 48 h. Culture supernatants were collected, frozen and stored at -80°C. For quantitation of total TGF-β1, the supernatants were treated with 1 mol l^{-1} HCl for 10 min to convert the latent form of TGF-β1 to the active form and neutralized with 1.2 mol l^{-1} NaOH and 0.5 mol l^{-1} HEPES. For the *in vivo* experiments, plasma samples were treated with 2.5 mol l^{-1} acetic acid for 10 min and neutralized with 2.7 mol l^{-1} NaOH and 1 mol l^{-1} HEPES. The activated samples were measured using TGF-β1 Human, Biotrak ELISA System (Amersham Biosciences, Piscataway, NJ, U.S.A.) according to the manufacturer's instructions.

RNA extraction and TaqMan PCR analysis

For the *in vitro* experiments, HSCs were incubated with Ang II with or without RNH-6270 for 24 h and total cellular RNA was isolated after lysis of the cells. For the *in vivo* experiments, total RNA was isolated from homogenates of whole livers on Day 21 after operation. RNA extraction was performed by the acid guanidinium thiocyanate–phenol–chloroform extraction method using TRIZOL reagent (Gibco BRL) according to the manufacturer's instructions. RNA purity and concentration were determined using a spectrophotometer (DU 7500, Beckman Coulter). Total RNA was converted to complementary DNA (cDNA) with TaqMan Reverse Transcription Reagents (Applied Biosystems, Branchburg, NJ, U.S.A.) using GeneAmp PCR System 9600 (Perkin-Elmer). For cDNA synthesis, 5 μg mRNA, 10 μl 10 × RT buffer, 22 μl $MgCl_2$ (25 mM), 20 μl dNTPs mixture (10 μM each), 5 μl random hexamers (50 μM), 2 μl RNase inhibitor (20 U/μl^{-1}), and 2.5 μl (50 U/μl^{-1}) Moloney Murine Leukemia Virus Reverse Transcriptase were added to make a total volume of 100 μl. Samples were incubated at 25°C for 10 min and 48°C for 30 min. Reactions were stopped by heating to 95°C for 5 min. The fluorescent TaqMan probes, and forward and reverse primers were designed with the software, Primer Express™, Ver.1.0 (Applied Biosystems, Foster City, CA, U.S.A.), and synthesized by Sigma Genosys Japan (Hokkaido, Japan). Primer and probe sequences used for this study are shown in Table 1. All the probes contained a fluorescence reporter (6-carboxyfluorescein [FAM]) at the 5′ end and a fluorescence quencher (6-carboxytetramethylrhodamine (TAMRA)) at the 3′ end. We used a probe and primers from the Rodent GAPDH Control Reagents (Applied Biosystems, Foster City, CA, U.S.A.) for internal calibration. Two-step PCR was carried out using an ABI PRISM 7700 Sequence Detector System (Perkin-Elmer). PCR conditions were as follows: 5 μl cDNA solution, 25 μl TaqMan 2 × PCR Master Mix (Applied Biosystems, Branchburg, NJ, U.S.A.), 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM), and 0.5 μl probe (20 μM) were added to make a total volume of 50 μl. The thermal cycler conditions were 2 min

Table 1 Primer and probe sequences used for detection of collagen α1(I), α-SMA and CTGF mRNA

Collagen α1(I)	
Forward primer	5′-CTCCCAGCGGTGGTTATGAC-3′
Reverse primer	5′-TGCTGGCTCAGGCTCTTGA-3′
Probe	5′-*FAM*-AAGATGGTGGCCGTTA CTACCGGGC-*TAMRA*-3′
α-SMA	
Forward primer	5′-CAACTGGTATTGTGC TGGACTCTG-3′
Reverse primer	5′-CTCCTTGATGTCACGGACGATCT-3′
Probe	5′-*FAM*-AGATGGCGTGACTCACAA CGTGCCT-*TAMRA*-3′
CTGF	
Forward primer	5′-CAATACCTTCTGCAGGCTGGA-3′
Reverse primer	5′-TTAGCCCGGTAGGTCTTCACA-3′
Probe	5′-*FAM*-TGCATCCGGACGCCTAAAA TTGCCA-*TAMRA*-3′

at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C followed by 1 min at 60°C. Standard curves to determine the mRNA content were generated using the GAPDH control. Control RNA (Rodent, 50 ng μl^{-1}) was serially diluted five-fold with 1 × TE (Wako) down to 80 pg μl^{-1}. The amplification of standard and sample cDNA was carried out in a MicroAmp Optical 96-well reaction plate (Applied Biosystems, Foster City, CA, U.S.A.). All standards and samples were assayed in duplicate. Each plate always contained the same standard. The threshold cycle (*Ct*) values were used to plot a standard curve in which *Ct* decreased in proportion to the log of the template copy number. The correlation coefficients of the standard curves were always more than 99%.

Histological analysis

Liver sections were incubated with xylene and hydrated through several washes in ethanol and distilled water to remove the paraffin. The sections were either stained with Masson-trichrome or subjected to immunohistostaining using antibodies against α-SMA or AT_1 receptor. For AT_1 immunohistostaining, sections were heated with 10 mmol l^{-1} sodium citrate buffer (pH 6.0) at 120°C for 10 min. Endogenous peroxidase activity was quenched by the addition of 3% (v v^{-1}) hydrogen peroxide for 5 min. Unspecific binding sites were blocked by Block Ace (Dainippon Pharmaceutical) for 30 min. Polyclonal anti-rabbit AT_1 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) diluted 1 : 200 in Block Ace was used. The sections were incubated for 30 min with the primary antibody at room temperature, the secondary antibody (goat anti-rabbit IgG, biotinylated, Santa Cruz; diluted 1 : 100 in Block Ace) for 30 min, and lastly, with peroxidase-conjugated streptavidin (Nichirei, Tokyo, Japan). For α-SMA immunohistostaining, after endogenous peroxidase inactivation, sections were incubated for 60 min with mouse monoclonal anti-human smooth muscle actin/HRP (DAKO Japan, Kyoto, Japan) at room temperature. The immunoreactivity was detected by the addition of 3,3′-diaminobenzidine tetrahydrochloride (DAB, DAKO Japan) at room temperature. Between each step, the sections were washed three times with tris-buffered saline (TBS, DAKO Japan) for 5 min. The sections were counterstained with Carazzi's hematoxylin (Muto Pure Chemicals, Tokyo, Japan), dehydrated and mounted.

Statistical analysis

Results were expressed as the mean ± s.e. For comparisons between two groups, statistical analysis was performed by Student's *t*-test (when the F-test was significant, the Welch test was carried out). For comparisons among three or more groups, Dunnett's test was performed. When a data result was less than the detection limit, the minimum value was used. $P<0.05$ was considered statistically significant. The SAS® System Release 8.2 (TS2M0) for Windows® (SAS Institute Inc., Cary, NC, U.S.A.) was used for the statistical analysis.

Results

Effect of olmesartan on in vivo *liver fibrosis*

Liver fibrosis was induced by bile duct ligation in rats, and thereafter, olmesartan was orally administered at a dose of 1 mg kg^{-1} six times a week from Day 7 to Day 20. Two animals of the BDL group and three animals of the BDL/olmesartan group died during Days 8–11 after bile duct ligation presumably due to surgical complications. The survival rate was not statistically different between these two groups (Fisher's exact test, Table 2). Final body weight was significantly lower than those in the BDL group compared with the Sham group ($P<0.05$), but olmesartan treatment had no effect. Liver and spleen weights were also increased in BDL group by 1.8- and 1.9-fold, respectively ($P<0.001$ for both), and were significantly lower in BDL/olmesartan group compared with BDL group ($P<0.01$ for liver, $P<0.05$ for spleen). Plasma AST and ALT levels were increased in BDL group by 5.3- and 2.6-fold, respectively ($P<0.001$ for both, Table 2). Olmesartan had no effect on these parameters.

Liver collagen accumulation was measured by determining the hydroxyproline content in the livers. Compared with the

Table 2 Biological parameters of olmesartan-treated fibrotic rats

	Sham	*BDL*	*BDL/ olmesartan*
Survival rate	5/5	8/10	7/10
Final body weight (g)	304 ± 7*	275 ± 8	250 ± 15
Liver weight/body weight (%)	3.41 ± 0.07‡	6.06 ± 0.15	5.46 ± 0.24†
Spleen weight/body weight (%)	0.23 ± 0.01‡	0.43 ± 0.02	0.37 ± 0.02*
Plasma AST (IU l^{-1})	67.2 ± 1.4‡	353 ± 51	499 ± 135
Plasma ALT (IU l^{-1})	38.8 ± 1.0‡	101 ± 9	122 ± 24

Note: Rats were treated as described in Methods. Sham, Sham-operated rats receiving the vehicle ($n=5$); BDL, bile duct-ligated rats receiving the vehicle ($n=8$); BDL/olmesartan, bile duct ligation followed by olmesartan treatment from Day 7 to Day 20 ($n=7$). Blood, livers, and spleens were collected on Day 21. AST, aspartate aminotransferase; ALT, alanine aminotransferase. Values are means ± s.e. of these separate experiments. *$P<0.05$, †$P<0.01$, ‡$P<0.001$ vs BDL (by *t*-test).

Sham group, liver hydroxyproline per gram of liver tissue was increased 1.8-fold ($P<0.01$) and three-fold for total liver content ($P<0.01$) in BDL group, and in the BDL/olmesartan group, it was reduced by 45% ($P<0.05$) and 54% ($P<0.01$), respectively (Figure 1). We also determined the plasma levels of the fibrogenic cytokine, TGF-β1, at the time of killing. Plasma TGF-β1 levels were increased 1.7-fold in BDL group ($P<0.05$). Olmesartan treatment reduced these levels by 79% ($P<0.05$, Figure 2).

Histological analysis showed a marked bile duct proliferation in the livers of bile duct-ligated rats (Figure 3b). In contrast, the proliferation in olmesartan-treated rats was minimal (Figure 3c). Masson-trichrome staining (Figure 3a–c) showed that collagen deposition was enlarged from the area of bile duct proliferation in bile duct-ligated rats (Figure 3b), and olmesartan treatment decreased the fibrotic area (Figure 3c). To identify activated HSCs, immunostaining for α-SMA was performed (Figure 3d–f). The increase in α-SMA-positive cells, which were stained brown, was observed in the

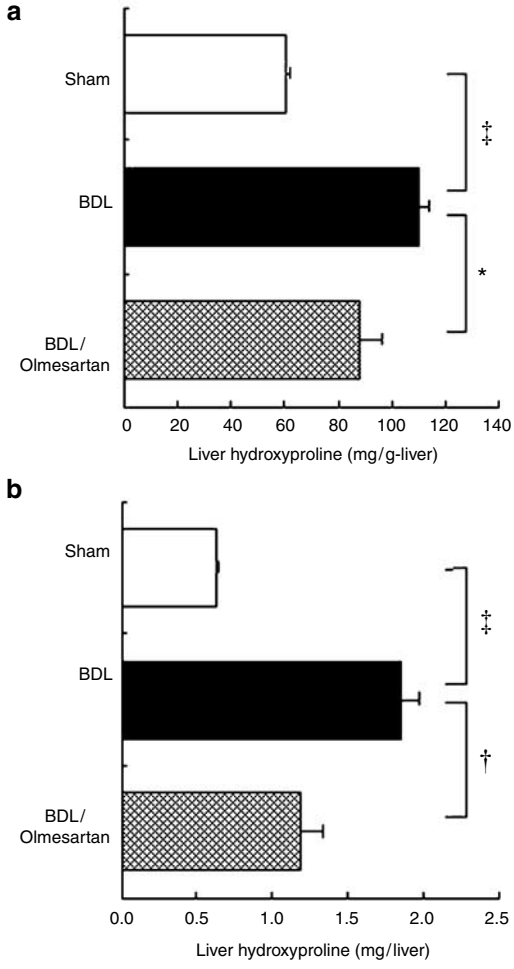

Figure 1 Effect of olmesartan on liver hydroxyproline content in bile duct-ligated rats. Liver hydroxyproline per gram of liver tissue (a) and total liver content of hydroxyproline (b) are shown. Rats were treated as described in Methods. Sham, Sham-operated rats receiving the vehicle ($n=5$); BDL, bile duct-ligated rats receiving the vehicle ($n=8$); BDL/olmesartan, bile duct ligation followed by olmesartan treatment from Day 7 to Day 20 ($n=7$). Liver sections were prepared on Day 21. Values are means$\pm$s.e. of these separate experiments. *$P<0.05$, †$P<0.01$, ‡$P<0.001$ vs BDL (by t-test).

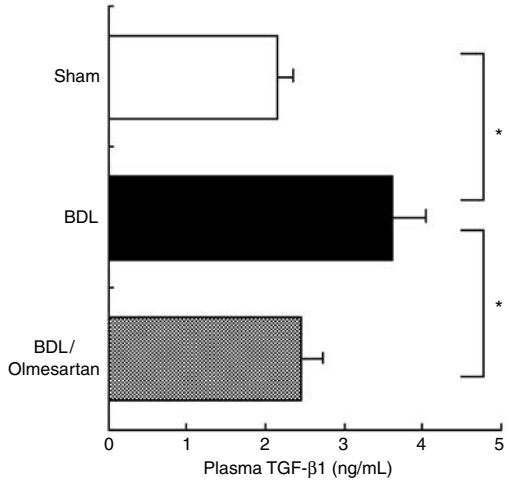

Figure 2 Effect of olmesartan on plasma TGF-β1 levels in bile duct-ligated rats. Rats were treated as described in Methods. Sham, Sham-operated rats receiving the vehicle ($n=5$); BDL, bile duct-ligated rats receiving the vehicle ($n=8$); BDL/olmesartan, bile duct ligation followed by olmesartan treatment from Day 7 to Day 20 ($n=7$). Blood was collected on Day 21. Values are means$\pm$s.e. of these separate experiments. *$P<0.05$ vs BDL (by t-test).

livers of bile duct-ligated rats (Figure 3e). These cells were localized in the area of collagen deposition. Olmesartan administration reduced the increase of these cells in the livers of bile duct-ligated rats, which correlated with the reduction of collagen accumulation (Figure 3c,f). Immunostaining for AT_1 receptor (Figure 3g–i) showed that these receptors were increased in the livers of bile duct-ligated rats (Figure 3h). Moreover, the receptors were localized in the area of collagen deposition where accumulation of α-SMA-positive cells was seen. Olmesartan administration seemed to reduce the increase in AT_1 expression (Figure 3i).

We determined the mRNA levels of collagen α1(I) and α-SMA in bile duct-ligated rats by TaqMan PCR analysis. These mRNA levels were increased in the BDL group by 3.7-fold compared with the Sham group ($P<0.001$ for both, Figure 4). Olmesartan treatment significantly reduced the increase in mRNA levels of collagen α1(I) and α-SMA by 44% ($P<0.05$) and 52% ($P<0.05$), respectively.

Effects of Ang II and olmesartan on the fibrogenic responses in activated HSCs in vitro

To investigate the mechanism of fibrogenic actions of Ang II and its main receptor, AT_1, we estimated the effects of Ang II and olmesartan on the fibrogenic responses in activated HSCs in primary cultures. Olmesartan medoxomil is a prodrug that is rapidly metabolized to the pharmacologically active form, RNH-6270. Therefore, we used RNH-6270 for the *in vitro* experiments. First, we studied the effects of Ang II and RNH-6270 on the proliferation in activated HSCs. Ang II induced a significant increase of HSC proliferation ($P<0.01$ at $10\,nmol\,l^{-1}$, $P<0.001$ at $10\,\mu mol\,l^{-1}$), as measured by the amount of [^{3}H]thymidine incorporated into the cells (Figure 5). At $10\,\mu mol\,l^{-1}$, RNH-6270 completely blocked the Ang II-induced proliferation of HSCs ($P<0.01$). We also studied the effects of Ang II and RNH-6270 on collagen synthesis in

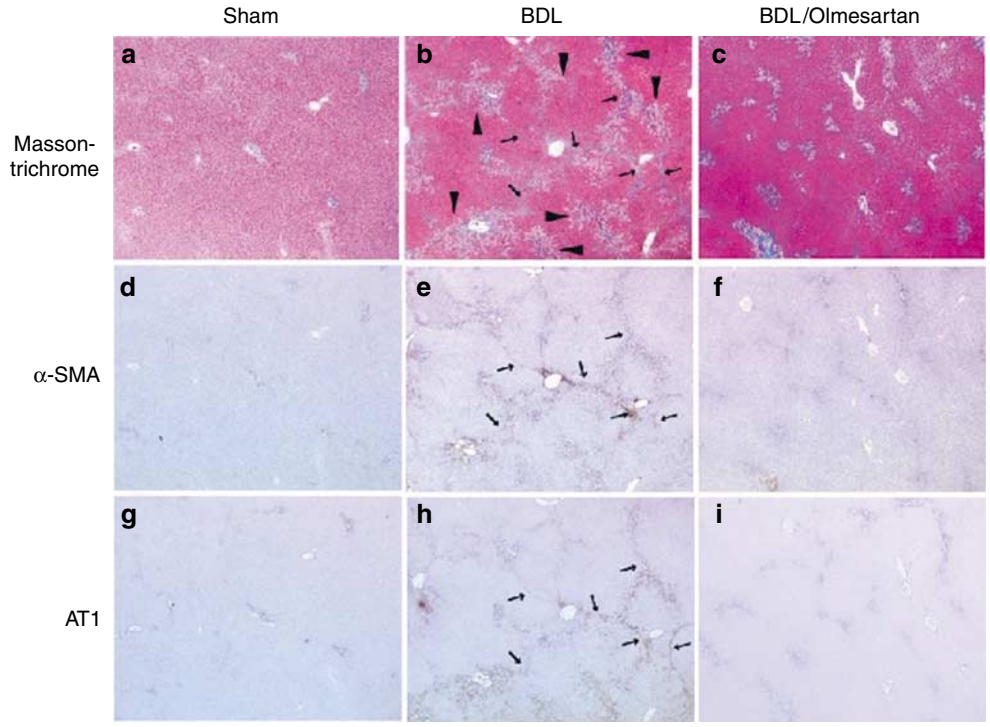

Figure 3 Histological analysis of livers from olmesartan-treated fibrotic rats. Liver sections were examined by Masson-trichrome staining (a–c) or immunohistochemical staining using antibodies against α-SMA (d–f) or AT_1 receptor (g–i). Rats were treated as described in Methods. Sham, Sham-operated rats receiving the vehicle (a, d, g); BDL, bile duct-ligated rats receiving the vehicle (b, e, h); BDL/olmesartan, bile duct ligation followed by olmesartan treatment from Day 7 to Day 20 (c, f, i). Liver sections were prepared on Day 21. The three images on the left, in the middle and on the right are serial. Representative sections of rats in each group are shown. Arrowheads in (b) indicate the bile duct proliferation. Arrows in (b), (e) and (h) indicate the site of fibrosis, the α-SMA-positive cells, and the AT_1-positive cells, respectively. Original magnification $\times 25$.

activated HSCs. As shown in Figure 6, Ang II treatment increased collagen synthesis 4.3-fold at $10\,nmol\,l^{-1}$ ($P<0.001$) and 9.3-fold at $10\,\mu mol\,l^{-1}$ ($P<0.001$) in HSCs, as measured by [^{3}H]proline incorporation into the cells. It was reduced by 85% with an RNH-6270 treatment of $10\,\mu mol\,l^{-1}$ ($P<0.001$).

We next studied the effects of Ang II and olmesartan on the expression of fibrogenic marker genes in rat primary HSCs. TGF-β is thought to play a central role in the activation of HSCs leading to the establishment of a myofibroblast-like phenotype in an autocrine manner. Therefore, we studied the production of TGF-β1 protein by assaying the supernatants of activated HSCs in primary cultures. Stimulation of HSCs with Ang II caused a significant increase in TGF-β1 levels in culture supernatants at $1\,nmol\,l^{-1}$-$10\,\mu mol\,l^{-1}$ in a dose-dependent manner ($P<0.001$ at $>1\,nmol\,l^{-1}$, Figure 7a). PDGF is the most potent mitogen for HSCs. In this study, PDGF also induced TGF-β1 production in HSCs ($P<0.001$). Interestingly, Ang II enhanced the PDGF-induced production of TGF-β1 ($P<0.05$ at $100\,nmol\,l^{-1}$, $P<0.01$ at $1\,\mu mol\,l^{-1}$, $P<0.001$ at $10\,\mu mol\,l^{-1}$). RNH-6270 blocked the TGF-β1 induction by Ang II treatment in HSCs, almost completely ($P<0.001$, Figure 7b).

CTGF may be a downstream mediator of the fibrogenic effect of TGF-β in HSCs. TaqMan PCR analysis showed that CTGF mRNA was increased 1.9-fold in $10\,\mu mol\,l^{-1}$ Ang II-treated HSCs ($P<0.001$, Figure 8). RNH-6270 treatment completely blocked the Ang II-induced CTGF mRNA expression ($P<0.01$).

Discussion

We showed that a new AT_1 receptor antagonist, olmesartan medoxomil, reduced collagen accumulation in the livers of fibrotic rats, and was thus linked to the antifibrogenic effect on activated HSCs. Recently, ACE inhibitors or AT_1 receptor antagonists have been shown to attenuate the progression of liver fibrosis *in vivo* without inducing any antihypertensive effects (Ramos *et al*., 1994; Jonsson *et al*., 2001; Ohishi *et al*., 2001; Paizis *et al*., 2001; Yoshiji *et al*., 2001; 2002). Paizis *et al*. similarly used the BDL model for the estimation of an AT_1 receptor antagonist, irbesartan. They showed that irbesartan reduced liver collagen α1(I) mRNA levels in this model, although hydroxyproline content was not significantly different. We demonstrated that olmesartan reduced both collagen deposition and the mRNA expression in the livers of bile duct-ligated rats. In this study, olmesartan did not reduce hepatocyte injury as indicated by the plasma AST and ALT levels, although it did improve liver fibrosis. This suggests that olmesartan may have direct effect on fibrosis rather than indirect effect mediated by hepatoprotection.

To investigate the mechanism of the fibrogenic actions of Ang II and its main receptor, AT_1, we examined the effects of Ang II and olmesartan on activated HSCs *in vitro*. We found that the Ang II treatment increased proliferation and collagen synthesis in rat HSCs. These inducible effects of Ang II were blocked by the active metabolite of olmesartan, RNH-6270. These results indicate that Ang II increases proliferation and

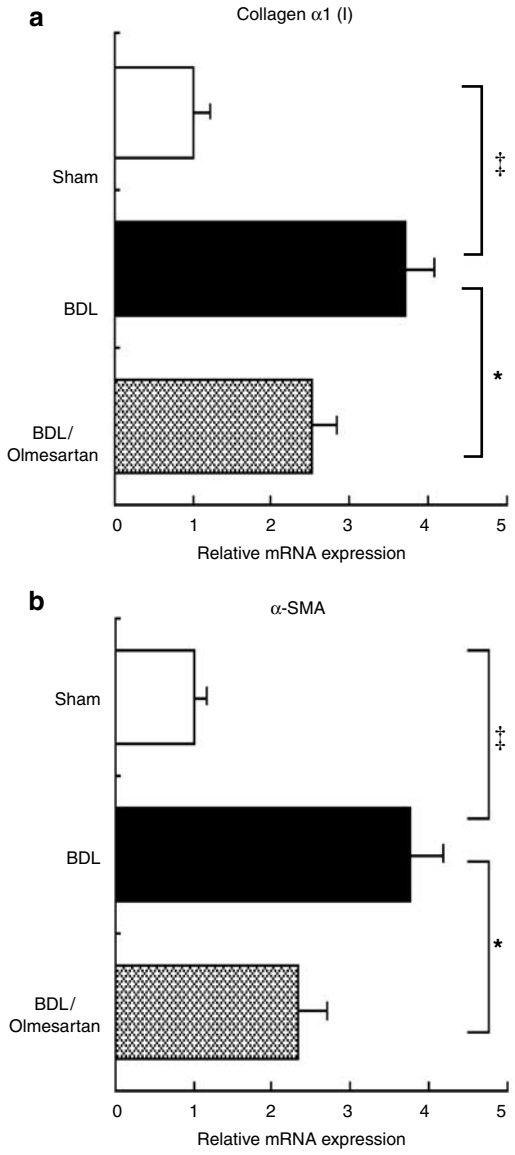

Figure 4 RNA analysis of livers from olmesartan-treated fibrotic rats. Rats were treated as described in Methods. Sham, Sham-operated rats receiving the vehicle ($n=5$); BDL, bile duct-ligated rats receiving the vehicle ($n=8$); BDL/olmesartan, bile duct ligation followed by olmesartan treatment from Day 7 to Day 20 ($n=7$). Total RNA was prepared from the frozen livers of each group on Day 21, and mRNA levels of collagen α1(I) (a) and α-SMA (b) were determined by TaqMan PCR analysis as described in Methods. RNA was normalized to that of GAPDH RNA. Values are means±s.e. of these separate experiments. $^{*}P<0.05$, $^{\ddagger}P<0.001$ vs BDL (by *t*-test).

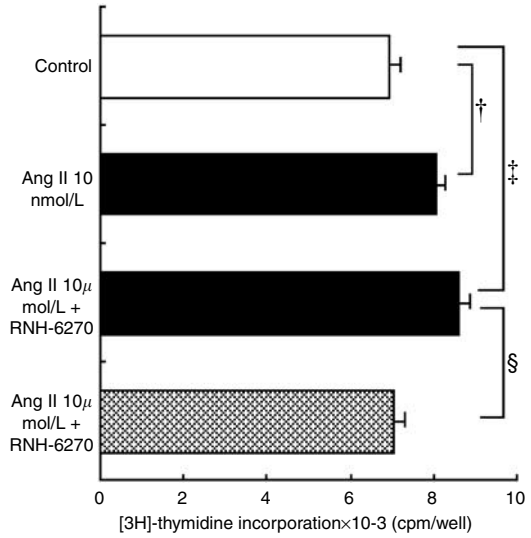

Figure 5 Effects of Ang II and RNH-6270 on DNA synthesis in rat HSCs. Cells were incubated with Ang II in the presence or absence of 10 μmol l^{-1} RNH-6270 in serum-free medium for 48 h, and DNA synthesis was measured by calculating the amount of incorporated [^{3}H]thymidine into cells, as described in Methods. Values are means±s.e. of eight separate experiments. $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$ vs Control (by Dunnett's *t*est); $^{\S}P<0.01$ vs Ang II 10 μmol l^{-1} (by *t*-test).

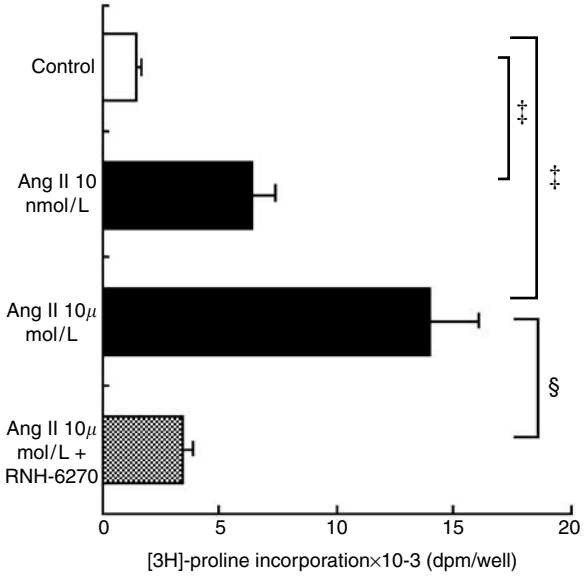

Figure 6 Effects of Ang II and RNH-6270 on collagen synthesis in rat HSCs. Cells were incubated with Ang II in the presence or absence of 10 μmol l^{-1} RNH-2670 in serum-free medium for 48 h, and collagen synthesis was measured by calculating the amount of incorporated [^{3}H]proline into cells, as described in Methods. Values are means±s.e. of eight separate experiments. Statistical tests were performed with logarithmically transformed values. $^{\ddagger}P<0.001$ vs Control (by Dunnett's test); $^{\S}P<0.001$ vs Ang II 10 μmol l^{-1} (by *t*-test).

collagen synthesis in HSCs, mainly mediated by AT_1 receptors, and that Ang II and AT_1 receptors may play an important role in the development of liver fibrosis.

Next, we studied the effects of Ang II and AT_1 receptors on the expression of profibrogenic cytokines. TGF-β mainly stimulates the activation and collagen synthesis of HSCs in an autocrine or paracrine manner (Matsuoka & Tsukamoto, 1990; Gressner, 1995). It has been reported that a blockade of TGF-β by the injection of a soluble type or dominant-negative type of TGF-β type II receptors into animals, prevented experimental liver fibrosis (George *et al*., 1999; Qi *et al*., 1999; Yata *et al*., 2002). Thus, this cytokine is highly involved in the pathogenesis of liver fibrosis. In the BDL model, TGF-β expression was upregulated in the liver and HSCs were shown to be their main source of production (Bissell *et al*., 1995). In this study, similar results such as the dose-dependent induction

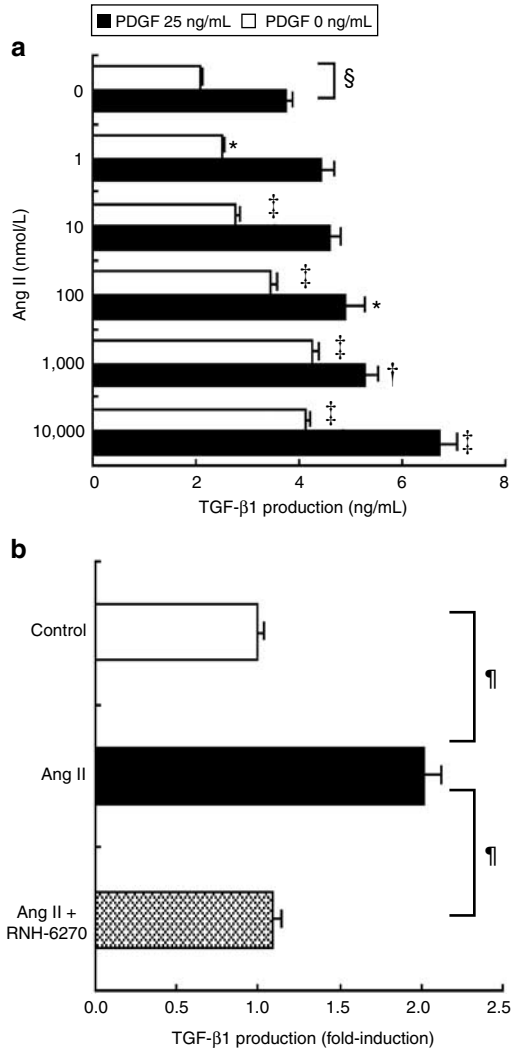

Figure 7 Effects of Ang II and RNH-6270 on TGF-β1 production in rat HSCs. Cells were incubated with 1 nmol l^{-1}–10 μmol l^{-1} Ang II in the presence or absence of 25 ng ml^{-1} PDGF-BB (a), and with 10 μmol l^{-1} Ang II in the presence or absence of 10 μmol l^{-1} RNH-6270 (b) in serum-free medium for 48 h. The collected culture supernatants were acidified, and then, total TGF-β1 amount in the supernatants was measured as described in Methods. Values are means ± s.e. of eight separate experiments. $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$ vs Ang II 0 nmol l^{-1} (by Dunnett's test); $^{\S}P<0.001$ vs PDGF 0 ng ml^{-1} (by *t*-test); $^{\P}P<0.001$ vs Ang II (by *t*-test).

of TGF-β1 production by Ang II and its blockade by RNH-6270 in rat HSCs were observed. Interestingly, Ang II enhanced the inducible effect of TGF-β1 production in rat HSCs by stimulation of PDGF, which is another key cytokine for HSC proliferation and activation (Pinzani *et al*., 1989). This suggests that PDGF and Ang II may jointly induce the activation of HSCs in the development of liver fibrosis. In the *in vivo* experiments, we also showed that plasma TGF-β1 levels, which were correlated with the progression of fibrosis in patients with chronic viral hepatitis (Murawaki *et al*, 1998; Flisiak *et al*., 2000), were increased in bile duct-ligated rats, and that olmesartan administration reduced these levels. These data indicate that AT_1 antagonism decreased TGF-β1 levels both in HSCs *in vitro* andfibrotic rats *in vivo*.

CTGF, a cysteine-rich protein belongs to the family of CNN, is thought to act as a downstream mediator of the

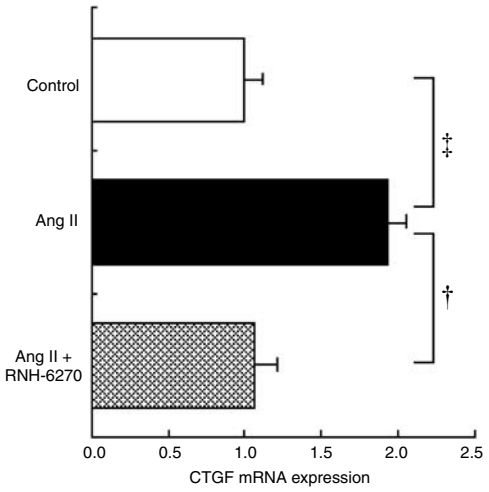

Figure 8 Effects of Ang II and RNH-6270 on CTGF mRNA expression in rat HSCs. HSCs were incubated with 10 μmol l^{-1} Ang II in the presence or absence of 10 μmol l^{-1} RNH-6270 in serum-free medium for 24 h. Total RNA was extracted from cells, and mRNA expression was determined by TaqMan PCR analysis as described in Methods. RNA was normalized to that of GAPDH RNA. Values are means ± s.e. of five separate experiments. $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$ vs Ang II (by *t*-test).

fibrogenic actions of TGF-β, and play an important role in the development of fibrosis in a variety of organs (Leask *et al*., 2002). It was reported that CTGF mRNA was upregulated in human liver cirrhosis or activated HSCs, and recombinant CTGF induced the activation of HSCs (Paradis *et al*., 1999; Williams *et al*., 2000; Paradis *et al*., 2002). Therefore, we next examined the effect of Ang II on CTGF mRNA levels in HSCs by TaqMan PCR analysis. As it was expected, Ang II treatment increased CTGF mRNA expression in activated HSCs, and RNH-6270 completely inhibited it. These results suggest that the increase in collagen synthesis by Ang II stimulation is to some extent mediated by the upregulation of two profibrogenic cytokines, TGF-β and CTGF, in an autocrine manner.

Olmesartan administration was initiated 7 days after BDL because administration of hypertensive agents may be harmful in postoperated animals. In addition, for clinical use, the drug must be effective against established liver fibrosis. On Day 7 in the BDL model, we demonstrated that many fibrogenic markers were already upregulated, and collagen deposition was initiated (data not shown). In this study, the histological analysis showed that collagen was excessively accumulated in the livers of bile duct-ligated rats, and that olmesartan administration improved this disorder. α-SMA-positive cells, which are associated with activated HSCs and possibly proliferating HSCs, were increased and localized in the area of collagen deposition. The increase of these positive cells was correlated with the mRNA levels of α-SMA by TaqMan PCR analysis. Olmesartan decreased the number of α-SMA-positive cells, suggesting that AT_1 receptor antagonism suppresses the activation and proliferation of HSCs in bile duct-ligated rats. As it was expected, these cells seemed to possess AT_1 receptors based on the AT_1 immunostaining. Recently, Paizis *et al*. (2002) showed that some key elements of RAS including AT_1 receptors were upregulated in bile-duct ligated rats. Bataller *et al*. (2000) reported that human HSCs expressed AT_1 receptors and that binding of Ang II to these receptors

induced the contraction and proliferation of human HSCs. Our data suggest that activated HSCs, which possess AT_1 receptors, may proliferate, express α-SMA and profibrogenic cytokines, TGF-β1 and CTGF, and produce an excess of collagen and other ECM components in response to Ang II, and that this may indicate the manner in which liver fibrosis progresses.

In conclusion, we demonstrated that a new AT_1 receptor antagonist, olmesartan medoxomil, improved liver fibrosis in bile duct-ligated rats, and that olmesartan suppressed the fibrogenic responses in activated HSCs including proliferation, collagen synthesis, and the expression of profibrogenic cytokines, TGF-β1 and CTGF, mediated by Ang II-induced AT_1 receptor stimulation. Recently, it has been reported that the AT_1 receptor antagonist was effective for patients in the early stages of chronic hepatitis C (Terui *et al*., 2002). Therefore, olmesartan medoxomil may be a potent antifibrotic drug for chronic hepatitis or liver cirrhosis.

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