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Up-regulation of components of the renin–angiotensin system in liver fibrosis in the rat induced by CCL_4

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ABSTRACT

The purpose of this study was to investigate the components of renin–angiotensin system (RAS), liver function and histology in liver fibrogenesis in the rats induced by low-dose chronic carbon tetrachloride (CCL_4) administration and evaluate the relationship between biochemical variables and components of RAS. Male Sprague–Dawley (SD) rats were randomly divided into the CCL_4 group which received intraperitoneal injection of 40% CCL_4 dissolved in olive oil every three days for four consecutive weeks (Initial dose was 5 mL/kg, other dose: 3 mL/kg) and the control group which received the same dose of olive oil. The micro-structure of the liver was examined by H&E. Hepatic Ang II and Ang(1–7) was detected. Real-time PCR and Western-blot were performed to determine the gene and protein expression of the RAS. The components of RAS were all up-regulated in CCL_4 group, and the increased extent of ACE-Ang II-AT1 axis was higher than the ACE2-Ang(1–7)-Mas axis. There was a significant correlation between ACE and ACE2 gene expression, AT1 and MAS gene expression, Ang II and Ang(1–7) in the liver of rats. ACE (or ACE2) gene expression strongly correlated with the index of liver injury significantly. These results suggest hepatic fibrogenesis induced by chronic CCL_4 administration may be associated with the relationship of ACE-Ang II-AT1 axis and ACE2-Ang(1–7)-MAS axis.

1. Introduction

Recent evidence indicates that the tissue renin–angiotensin system (RAS) takes part in the pathogenesis of liver fibrosis (Paizis et al., 2002; Bataller et al., 2005; Yang et al., 2005; Nabeshima et al., 2006), angiotensin converting enzyme (ACE), a dipeptidyl carboxypeptidase, is a key enzyme in RAS as it converts angiotensin I (Ang I) to angiotensin II (Ang II). Ang II mediates pivotal biological actions (by the AT1 receptor) involved in tissue repair, myofibroblast proliferation, infiltration of inflammatory cells and collagen synthesis (Bataller et al., 2000; Bataller et al., 2003b; Yoshiji et al., 2003; Yang et al., 2005). The importance of the ACE-Ang II-AT1 receptor axis in hepatic fibrosis is supported further by studies which have shown that inflammation and fibrosis in response to both CCL_4 treatment and BDL (bile duct ligation) are attenuated in AT1-knockout mice (Kanno et al., 2003; Yang et al., 2005). Furthermore, systemic infusion of angiotensin II can stimulate proliferation of bile duct epithelial cells, exacerbate liver fibrosis and increase serum transaminases and endotoxin levels in BDL rat livers (Bataller et al., 2003a; Bataller et al., 2005). In almost all of the published studies, both Angiotensin converting enzyme inhibitor (ACEI) and Ang II receptor blocker (ARB) have been established to have beneficial effects for the attenuation of fibrosis and down-regulation of key inflammatory and profibrotic cytokines involved in the pathogenesis of hepatic fibrosis (Wei et al., 2000; Jonsson et al., 2001; Ohishi et al., 2001; Yoshiji et al., 2001).

More recently, the discovery of ACE2 (Donoghue et al., 2000; Tipnis et al., 2000), a homologue of ACE, has revolutionized our understanding of the RAS. It can degrade Ang II to Ang(1–7) and cleave Ang I to generate Ang1–9. Ang1–9 has no known effects, but is converted to Ang(1–7) by ACE (Rice et al., 2004).The actions of Ang(1–7) are mainly mediated by the G-protein-coupled receptor MAS. ACE2, together with Ang(1–7) and MAS, represents another axis [ACE2-Ang(1–7)-MAS axis] of the RAS (Santos et al., 2008). This axis can be viewed as the principal counter-regulatory mechanism for the ACE-Ang II-AT1 receptor axis. Apparently, ACE2 acts as a key regulator controlling the balance between these two axes by simultaneously diminishing Ang II and forming Ang(1–7). The discovery of ACE2 has stimulated investigations into the potential role of this enzyme in the prevention and treatment of diseases. In experimental models of hypertension and diabetes, the expression of tissue ACE2 is reduced, contributing to Ang II-mediated tissue injury (Crackower et al., 2002; Tikellis et al., 2003). ACE2 is also a functional receptor for the severe acute respiratory syndrome (SARS)

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coronavirus, which facilitates the infection of target cells (Li et al., 2003; Imai et al., 2005; Guo et al., 2008).

Several studies recently reported that hepatic ACE2 expression was up-regulated in cirrhotic human livers or in a liver injury model in the rats (Paizis et al., 2005; Herath et al., 2007; Huang et al., 2009). However, changes in the two axes of the RAS and the ratio of ACE/ACE2 Ang II/Ang(1–7) and AT1/Mas have not been determined after CCL_4 induced liver injury. In the current study, we aimed to examine the contents of the ACE-Ang II-AT1 axis and the ACE2-Ang(1–7)-MAS axis in a model of liver fibrosis induced by CCL_4. Specifically, we evaluated the correlation between the ACE-Ang II-AT1 axis and the ACE2-Ang(1–7)-MAS axis, and the relationship between biochemical variables and the two axes of the RAS in liver tissue.

2. Materials and methods

2.1. Animal and experimental model

Male Sprague–Dawley (SD) rats weighing about 125 g ± 10 g were purchased from Shanghai Laboratory Animal Center, Chinese Academy Science (SIBC, CAS) and housed in a controlled environment in the animal facility with a 12 h light–dark cycle. After 1 week acclimatization, the rats were randomly divided into two groups, the control group and the CCL_4 group ($n = 8$ in each group).The CCL_4 group received intraperitoneal injection of 40% CCL_4 (Sigma–Aldrich, Co., USA) dissolved in olive oil every three days for four consecutive weeks (Initial dose was 5 mL/kg, other dose: 3 mL/kg) and the control group received the same dose of olive oil. All procedures were approved by the Investigation and Ethics Committee and the Institutional Animal Care and Use Committee of our institution.

2.2. Experimental protocol

2.2.1. Relative hepatic weight (RHW)

The rats weighed once a week and the weight of liver was measured at the end of the whole experiment.

$$\mathrm{RHW}(\%) = \text{hepatic weight}/\text{body weight}$$

2.2.2. Blood processing

Blood samples were obtained from the abdominal aorta at the end of the experiment. Serum was collected from the blood sample by immediate centrifugation at 3500*g* for 10 min at 4 °C. The serum was collected in tubes and stored at −70 °C for measuring liver function.

2.2.3. Biochemical and histological assessment of liver injury

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP) and albumin (ALB) concentrations were measured using kits that were purchased from Nanjing jiancheng Bioengineering Institute. For histological analysis, liver samples were fixed in 10% formaldehyde solution, dehydrated, embedded in paraffin, and cut into 3 μm-thick sections. Staining for hematoxylin and eosin (H&E) was carried out with standard techniques.

2.2.4. Radioimmunoassay for Ang II

The hepatic tissue (100 mg) was homogenized in 1 ml 0.9% NaCl (contain 10 μl 0.3 M Na_2EDTA, 5 μl 0.32 M dimercaptopropanol and 10 μl 0.34 M 8-hydroxyquinoline). After centrifugation, the supernatant was used to measure Ang II in a radioimmunoassay. The concentration of protein in liver served as the endogenous control. The kits were purchased from Beijing North Institute of Biological Technology and the intra- and inter-assay coefficients of variation were less than 10% and 15%, respectively.

2.2.5. ELISA for Ang(1–7)

The concentration of Ang(1–7) in liver homogenate (the homogenate was same as above) was measured by enzyme-linked immunosorbent assay (ELISA). The kits were purchased from R&D Systems.

2.2.6. Real-time polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from rat liver using TRNZOL reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Total RNA (2 μg) was subjected to first-strand cDNA synthesis using random primers, M-MLV reverse transcriptase and RNase inhibitor (SunShine Bio, Nanjing, China). The specific primers for the polymerase chain reaction (PCR) were as follows: ACE: 5′-ATGCCTCTGCGTGGGACTTC-3′ (forward) and 5′-TACTGCAGTGGCCCATCTC-3′ (reverse); ACE2: 5′-AATCGTAGGCTCTGGGC TTGG-3′ (forward) and 5′-TTCGATCAACTGGTTTCGGTTGTA-3′ (reverse); AT1:5′-C CCACTCAAGCCTGTCTACGAA-3′ (forward) and 5′-GTGTGCTTTGAACCTGTCACTCC-3′ (reverse); MAS:5′-TGACAGCC ATCAGTGTGGAGA-3′ (forward) and 5′-GCATGAAAGTGCCCACAGGA-3′ (reverse); β-actin:5′-CCCTGTGCTGCTCACCGA-3′ (forward) and 5′-ACAGTGTGGGTGACCCCGTC-3′ (reverse); The amplified product sizes were 112, 198, 120, 116 and 186 bp respectively, and RNA expression levels were quantified by SYBR Green real-time PCR using a real-time PCR detection system (Prism 7300; Applied Biosystems Inc., Foster City, USA). β-actin served as the endogenous control. In order to calculate differences in the expression level of each target gene, the ΔΔCT method for relative quantification was used according to the manufacturer's manual.

2.2.7. Western-blot analysis

Liver proteins for Western-blot were extracted with radio immunoprecipitation assay lysis buffer (RIPA, 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 10% glycerol, 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 mg/L aprotinin, 10 mg/L leuprptin) and the protein was quantified by bicinchoninic acid (BCA, Beyotime, shanghai China). Protein (100 μg) was run by SDS–PAGE on a 8% gel, and the separated proteins transferred to polyvinylidene difluoride membranes (PVDF; Millipore, Bedford, USA). After transfer, the membranes were blocked in 5% non-fat milk in tris-buffered saline with 0.1% Tween-20 (TBST) for an hour at room temperature and then incubated overnight at 4 °C with primary antibodies of ACE2 (1:300; Santa Cruz Biotechnology, CA, USA) and β-actin antibody (1:10,000; Cell Signaling, Beverly, USA), and then were washed 5 times for 10 min in TBST. Immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibodies (1:10,000, Abcam, America) and an enhanced chemiluminescence system (Pierce, Rockford, USA). The density of individual bands was quantified by densitometric scanning of the blots using Quantity One software (Bio-Rad, Hercules, CA, USA).

2.3. Statistical analysis

Data are expressed as the mean ± SE. Multiple comparisons were performed using one-way analysis of variance (ANOVA) and Bonferroni's test. Pearson correlation coefficient was used between the individual variables. $P < 0.05$ was considered significant. All statistical analyses were performed using SPSS16.0 software (SPSS, Chicago, USA).