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—Original—

Loss of angiotensin converting enzyme II (ACE2) accelerates the development of liver injury induced by thioacetamide

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Abstract: Angiotensin converting enzyme II (ACE2), an angiotensin converting enzyme (ACE) homologue that displays antagonist effects on ACE/angiotensin II (Ang II) axis in renin-angiotensin system (RAS), could play a protective role against liver damages. The purpose of this study is to investigate whether inflammation-mediated liver injury could be affected by ACE2 derived pathways in the RAS. Eight-weeks-old wild-type (WT; C57BL/6) and *Ace2* KO (hemizygous *Ace2*$^{-/y}$) male mice were used to induce liver fibrosis by thioacetamide (TAA) administration (0, 100, and 200 mg/kg BW). The mice administrated with TAA could be successfully induced liver fibrosis in a TAA-dose dependent manner. Compared to WT mice, the results show that *Ace2* KO mice have high sensitive, and developed more serious reaction of hepatic inflammation and fibrosis by TAA administration. The physiological and pathological examinations demonstrated higher serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels, infiltration of white blood cells and fibrotic lesions within liver in the *Ace2* KO mice. The severe liver damage of *Ace2* KO mice were also confirmed by the evidence of higher expression of hepatic inflammation-related genes (*IL-6* and *Tnf*) and fibrosis-related genes (*Col1a1*, *Timp1* and *Mmp9*). *Ace2* gene deficiency could lead to a severe inflammation and collagen remodeling in the liver administrated by TAA, and the responses lead the pathogenesis of liver fibrosis. Our studies provided the main messages and favorable study directions of relationship of *Ace2* and liver disease.

Key words: angiotensin converting enzyme II, inflammation, liver fibrosis, renin-angiotensin system, thioacetamide

Introduction

The renin-angiotensin system (RAS) is a key regulator in maintaining multiple essential functions, such as blood pressure homeostasis by regulating salt and fluid balance [4]. In the classic pathway of the RAS, renin cleaves the precursor peptide angiotensinogen into angiotensin I (Ang I). Ang I is further hydrolyzed into angiotensin II (Ang II) by angiotensin converting enzyme (ACE). Ang II is the major effector peptide of RAS and acts via the angiotensin II type-I receptor (AT1R). Therefore, the ACE/Ang II/AT1R pathway is the classical RAS axis. The discovery of an ACE homolog, angiotensin converting enzyme II (ACE2), adds new complexity to RAS [8, 35]. ACE2 degrades Ang II to form angiotensin 1–7 (Ang-(1–7)), which acts the effects opposite to those of Ang II through its receptors on cell membrane, Mas [30, 36]. The ACE2/Ang-(1–7)/MAS

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pathway is a non-classical RAS axis. The classical RAS pathway (ACE/Ang II/AT1R axis) which is pro-inflammatory and pro-fibrotic; and non-classical (alternative) RAS pathway (ACE2/Ang-(1–7)/MAS axis) which is anti-inflammatory and anti-fibrotic, like a counter-regulatory arm of RAS to make a balance *in vitro* [11]. It is currently believed that a local balance between ACE/Ang II/AT1R and the ACE2/Ang-(1–7)/MAS axis is important in preventing inflammatory and fibrotic diseases [32].

The deleterious effects of the classical ACE/Ang II/AT1R axis in liver diseases are well described in the literature [21, 27]. Abnormal RAS function, i.e., the action of excess Ang II, has been indicated that inflammatory response is associated with the pathogenesis of liver injury and fibrosis [11, 12, 22]. ACE2 is predominantly detected in the heart, kidneys, testes and the gastrointestinal tract, but is expressed at a low level in the liver and lung [34]. There were studies indicating that liver ACE2 could regulate the balance of Ang-(1–7) and Ang II, and is a target for the therapy of liver diseases [3, 24].

In light of these recent findings, it is supposed that ACE2 may play a crucial role in injury development of liver damaged by toxic and drug molecules [21, 27]. Therefore, a mouse model with liver injury induced by thioacetamide (TAA) administration was performed using *Ace2* knockout (KO) mice. Although carbon tetrachloride (CCl_4) was used in the induction of hepatotoxic, but there were many experiments revealed that treatment of mice and rats with TAA induced liver cell damage, fibrosis and/or cirrhosis, associated with increase of oxidative stress and activation of hepatic stellate cells [7, 18]. We attempted to study the hepatic fibrosis-related mechanism via ACE2 regulation. Therefore, *Ace2* KO mice were administered TAA, and the physiological changes, expression of inflammation-related as well as fibrosis-related genes were investigated.

Materials and Methods

Liver injury induced by TAA administration

Eight weeks of age, hemizygous *Ace2* KO mice (B6; 129S5-*Ace2*^{tm1Lex}/Mmcd, *Ace2*$^{-/y}$; male) and WT mice (C57BL/6J; male) were used in the present study. The WT mice were come from the National Laboratory Animal Center (NLAC; Taipei, Taiwan). The *Ace2* KO mice were obtained from the Mutant Mouse Regional Resource Centers (MMRRC, USA) supported by the National Institutes of Health in USA and bred in the NLAC. All of the experimental protocols of animal were conformed to the Guide for the Care and Use of Laboratory Animals [23] and was approved by the Institutional Animal Care and Use Committee of National Chia Yi University. In each experiment, the mice were randomly divided into four groups and treated with various doses of TAA (0, 100 and 200 mg/kg body weight) (Alfa Aesar). TAA was given by intraperitoneal (i.p.) injection three times a week, for 8 consecutive weeks. The control group were given by i.p. injection of Dulbecco's Phosphate-Buffered Saline (DPBS, ThermoFisher Scientific) with the same treatment procedure of TAA groups. During the experimental period, the mice were housed in a controlled environment (12 h light/dark, temperature 22°C to 24°C) and fed standard mouse chow *ad libitum* (Laboratory Autoclavable Rodent Diet 5010, LabDiet, St. Louis, MO, USA) with free access to water.

The mice were sacrificed 12 h after the last TAA injection and blood and liver samples were isolated. Blood samples were centrifuged to obtain serum at 3,000 × g for 15 min at 4°C. Liver samples were dissected out and washed immediately with ice cold phosphate buffered saline (pH 7.4) to remove as much blood as possible. A piece of the liver sample was fixed in 10% formalin for histopathological examination. The remnants of the livers were immediately stored at −80°C.

Biochemical assays

Biochemical parameters, including serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels, were determined using an automated analyzer (ADVIA1800; SIEMENS, Munich, Germany).

Protein extraction of liver tissue

Frozen liver tissue, around 200 mg, was homogenized in 1 ml of lysis buffer (200 mM sucrose, 10 mM sodium fluoride, 20 mM Tris–HCl, 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, 0.1 mM sodium orthovanadate and 1% (v/v) Triton X-100). The tissue homogenate was centrifuged at 10,000 × g at 4°C for 15 min, and then the supernatant was harvested for protein quantitation and further analysis.

Table 1. Primers and annealing temperatures for reverse transcription PCR

Gene	Primer sequence (5'→3')	Product size	Annealing temperature
Col1a1	F: GAAACCCGAGGTATGCTTGA R: GACCAGGAGGACCAGGAAGT	275 bp	50°C
Timp1	F: TCTTGGTTCCCTGGCGTACT R: GTGGCAGGCAAGCAAAGTG	201 bp	62°C
Mmp9	F: CTGCATTTCTTCAAGGACGG R: AAGTCGAATCTCCAGACACG	320 bp	57°C
IL-6	F: ACGGCCTTCCCTACTTCACA R: CATTTCCACGATTTCCCAGA	129 bp	62°C
Tnf	F: GCCTCTTCTCATTCCTGCTTG R: CTGATGAGAGGGAGGCCATT	115 bp	62°C
Gapdh	F: GAGGGGCCATCCACAGTCTT R: TTCATTGACCTCAACTACAT	506 bp	62°C

IL-6: interleukin-6; *Tnf*: tumour necrosis factor alpha; *Col1a1*: collagen type I alpha 1; *Timp1*: tissue inhibitor of metalloproteinases 1; *Mmp9*: metalloproteinases 9; *Gapdh*: glyceraldehyde-3-phophate dehydrogenase.

Gelatin zymography assay

Method of gelatin zymography was used for the detection of hepatic MMP-9 activity following the previous protocol mentioned by Hung *et al.* [6]. The tissue protein was mixed with 2X zymography sample buffer (50% glycerol, 8% SDS, 0.02% bromophenol blue, and 125 mM Tris-HCl, pH 6.8), and then analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. After electrophoresis, the gels were equilibrated twice for 30 min in renaturing buffer (2.5% Triton) to remove the SDS, and then incubated in reaction buffer (200 mM NaCl, 5 mM $CaCl_2$, 0. 02% Brij-35, and 50 mM Tris-HCl, pH 7.5) at 37°C for 12–18 h. After the development of enzyme activity, the gels were then stained with Coomassie blue for 30 min and then destained with destain buffer for 2–4 h. The presence of MMP-9 activity in the gels was indicated by unstained transparent zones. Enzyme activity of MMP-9 in the gel slab was scanned and quantified by Scion Image software (Scion, Frederick, MD, USA).

RNA isolation and quantification

Liver total RNA was extracted according to the standard protocol of TRIzol Plus RNA Purification System (Invitrogen). In brief, 100 mg of liver tissue was homogenized in 1 ml TRIzol reagent. An amount of 200 μl chloroform was following added and completely mixed, and the mixture was centrifuged at 10,000 × g for 20 min. The aqueous phase was transferred to a new tube, the RNA was precipitated from the aqueous phase by mixing with 500 μl isopropyl alcohol. The sample was centrifuged at 10,000 × g at 4°C for 20 min. Supernatant was removed and the RNA pellet was then washed twice with 1 ml of 75% cold ethanol. The pallet dried and RNA dissolved in 20–30 μl of diethylpyrocarbonate-treated water.

The integrity and relative amounts of total RNA were evaluated by electrophoresis on denaturing 1.2% agarose gel followed by ultraviolet visualization of SYBR Green II -stained RNA. The total RNA was quantified by measuring absorbance at 260 nm.

RT-PCR

The MMLV Reverse Transcription kit (Protech Technology) was used to synthesize cDNA. Briefly, 2.5 μg of total RNA was reverse transcribed in a reaction that contained 1 × reverse transcription buffer, 1 mmol/L dNTPs, 0.2 μg/μl Random Hexamers, 0.5 U/μl RNase inhibitor and 1 μl of MMLV reverse transcriptase (Protech Technology) in a total volume of 20 μl. After a 60 min incubation at 45°C and deactivation of reverse transcriptase at 70°C for 10 min, RT-PCR was performed using a LabCycler 48 (Sensoquest GmBH, Göttingen, Germany) and a GenTaq DNA polymerase kit (GMbiolab). Reactions contained 20 pmol/l of each primer and 5 μl cDNA in a total volume of 25 μl. PCR specificity was confirmed using agarose gel electrophoresis. Expression of the glyceraldehyde-3-phophate dehydrogenase (*Gapdh*) gene was used as an internal standard. Primer pairs for the gene expression are listed in Table 1.

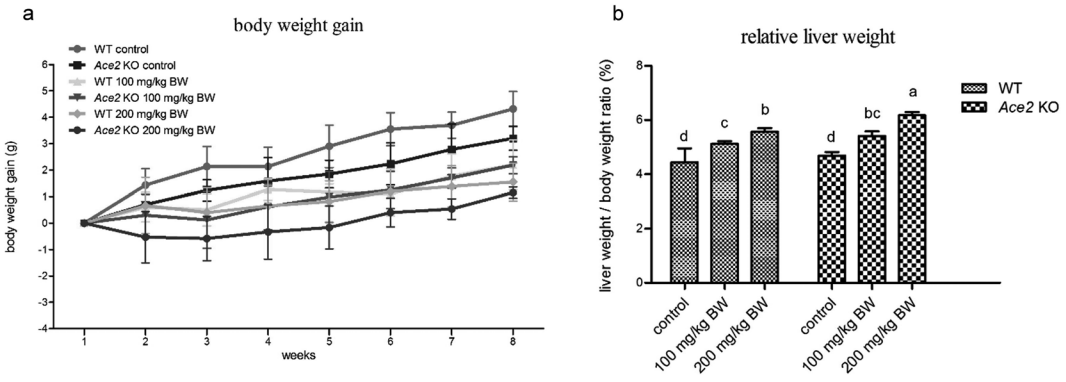

Fig. 1. The changes in body weight and relative liver/body weight ratio of the mice after 8 weeks of TAA administration. The WT and *Ace2* KO mice were i.p. injected with TAA or DPBS for 8 weeks, and body weights were measured at the time of each injection. The relative liver/body weight ratio is the final liver weight as a percentage of total body weight. Results are shown as the means ± SD (n=8) for each group. Different letters show a significant difference between the treatment groups (P<0.05).

Histological determination

The liver samples were excised from the experimental animals of each group, washed with normal saline, fixed in 10% formalin and processed for paraffin embedding following the microtome technique. The sections were cut at 6 μm thickness, processed in an alcohol-xylene series and were stained with Masson's trichrome. The tissue sections were microscopically examined for the evaluation of histopathological changes.

Statistical analysis

For all of the values of each group, mean ± SD is a measure that is used to quantify the amount of variation. To compare the quantitative results between two groups, Student's *t*-test was applied for presumably distributed variables. One-way analysis of variance (ANOVA) method was applied to test the statistical differences when the group numbers were more than two. A possibility value (p) less than 0.05 was considered statistically significant.

Results

Physiological changes

Ace2 KO and WT mice were administered TAA for 8 weeks and their body weight was examined each week (Fig. 1a). The beginning body weight of *Ace2* KO mice and WT mice was 24.7 ± 1.9 g and 24.6 ± 1.6 g, respectively. After 8 weeks without TAA, both WT and *Ace2* KO mice markedly increased in body weight. There was an insignificant increase in body weight found in the mice given TAA. The *Ace2* KO mice given a high dose of TAA had significant decreases in body weight (Fig. 1a).

The liver weight of the mice treated with TAA was increased, especial in the mice treated with a high dose of TAA, relative to the untreated mice. The relative liver/body weight ratio was calculated and the ratios significantly increased in a TAA dose-dependent manner (Fig. 1b).

As shown in Fig. 2, the animals administered TAA for 8 weeks showed significantly increased serum AST, ALT and ALP levels in both WT and *Ace2* KO mice. These hepatotoxic factors increased in a manner that indicated TAA-dose dependence. Moreover, the *Ace2* KO mice treated with a high dose of TAA (i.e., 200 mg/kg body weight) showed significantly increased serum AST, ALT and ALP levels compared with the WT mice.

Pathological changes

Histopathological examinations showed that TAA administration induced hepatocyte necrosis with inflammatory cell infiltration and fibrotic lesions (Fig. 3a). Severe hepatic lesions (proliferated of ECM, blue area) induced by TAA were remarkably worse both in the WT and *Ace2* KO mice (Fig. 3b), but increasing numbers of Masson's trichrome stained liver sections were found in the *Ace2* KO mice compared with the WT mice at 100 mg/kg BW.

Inflammation and fibrosis pathogenesis

By analyzing the expression of cytokines related to tissue inflammation, it could be confirmed that TAA

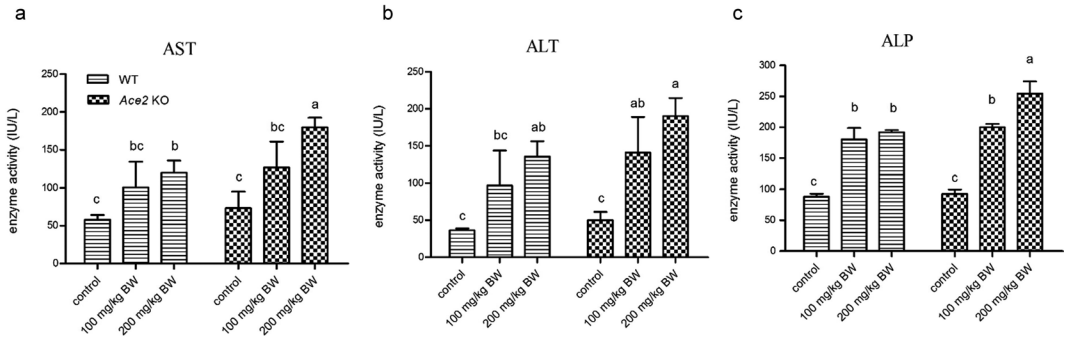

Fig. 2. Changes in serum AST, ALT and ALP levels of the mice after 8 weeks of TAA administration. The WT and *Ace2* KO mice were i.p. injected with 100 and 200 mg/BW of TAA or DPBS for 8 weeks and then serum samples were collected for the assays. Results are shown as the means ± SD (n=8) for each group. Different letters show a significant difference between the treatment groups (P<0.05). (AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase)

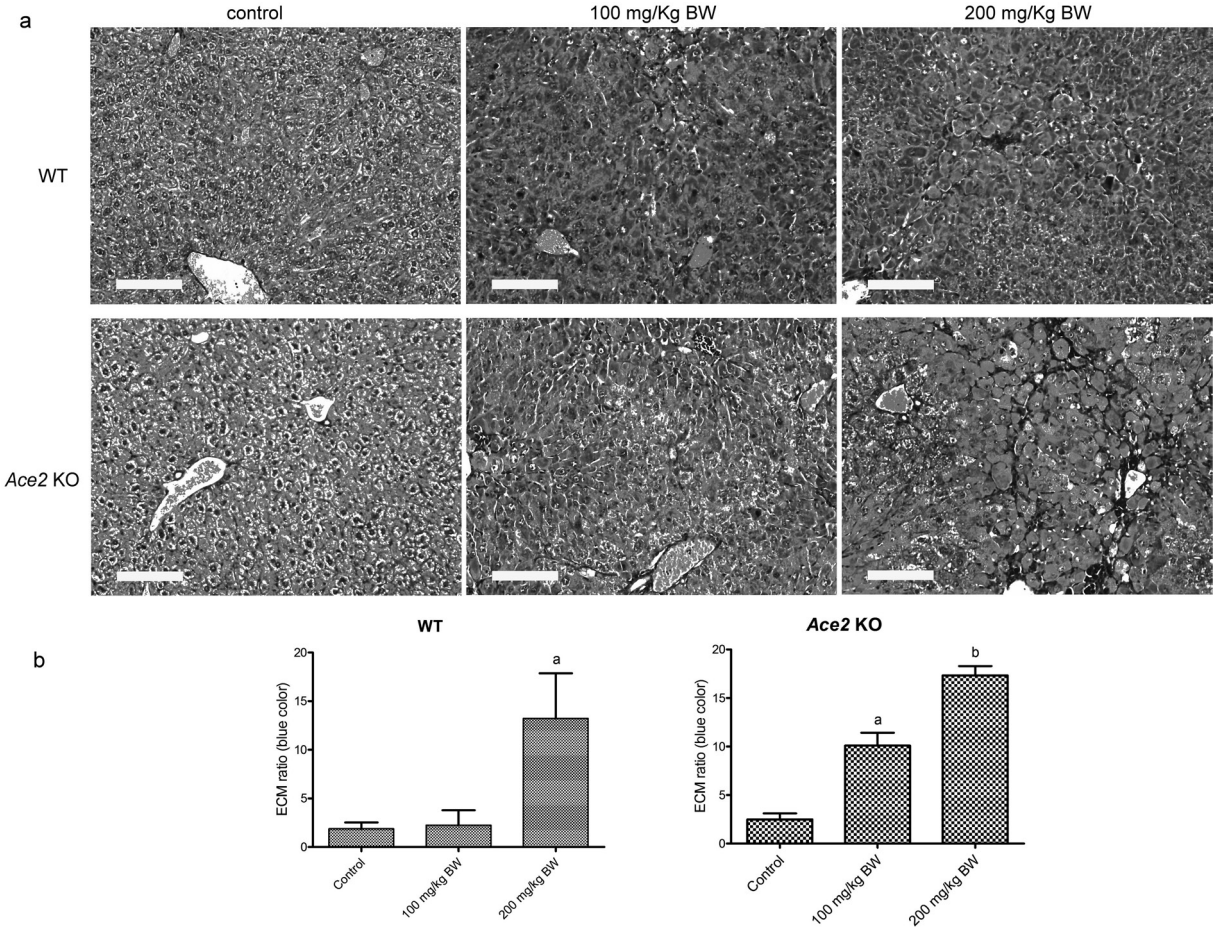

Fig. 3. Histopathological features of the mice after 8 weeks of TAA administration. All mice were sacrificed and the liver was removed, fixed and embedded in paraffin. Histopathological photomicrographs of the mouse livers were stained with Masson's trichrome and blue staining shows the fibrosis areas (a). The extracellular matrix (ECM) ratio (blue staining) increased represent by different TAA treatment (b). Scale bars indicate 125 μm.

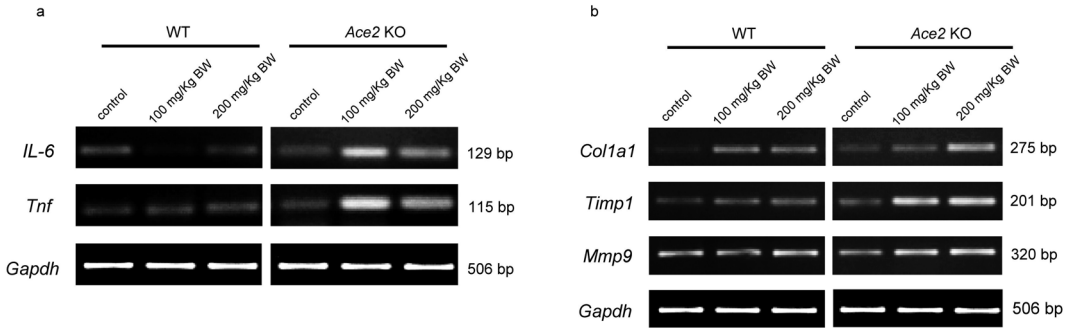

Fig. 4. Expression levels of inflammation and fibrosis related genes in the mice livers after treatment with TAA. WT and *Ace2* KO mice were i.p. injected with TAA or DPBS for 8 weeks, and RNA was then extracted from the liver tissue. Reverse-transcription PCR (RT-PCR) was used to analyze the RNA expression level of inflammatory *IL-6* and *Tnf* genes (a), and fibrosis related *Col1a1*, *Timp1* and *Mmp9* genes (b). *Gapdh* was used as an internal control (*IL-6*: interleukin-6; *Tnf*: tumor necrosis factor alpha; *Col1a1*: collagen type I alpha 1; *Timp1*: tissue inhibitor of metalloproteinases 1; *Mmp9*: metalloproteinases 9; *Gapdh*: glyceraldehyde-3-phosphate dehydrogenase).

administration induced hepatic inflammation in mice (Fig. 4). The expression levels of liver *IL-6* and *Tnf* in the WT mice treated with 100 and 200 mg/kg body weight TAA for 8 weeks were significantly higher (2- to 3-fold) than those in the control mice. The TAA-induced levels of liver *IL-6* and *Tnf* in the *Ace2* KO mice were significantly higher than those in the WT mice.

Severe liver damage was also confirmed by higher expression of hepatic fibrosis-related genes (*Col1a1*, *Timp1* and *Mmp9*) detected in the *Ace2* KO mice compared with those gene expression in the WT mice (Fig. 4). Above results suggest that the inflammatory and fibrotic pathogenesis observed in the mice was related to TAA-induced liver injury.

Hepatic MMPs activity in TAA-induced liver injury

The activities of liver MMPs in the mice treated with TAA for 8 weeks were investigated to study the proposition that MMP-2 and MMP-9 may participate in the pathogenic development of liver injury. The liver MMP-2 activities in both *Ace2* KO and WT mice administered TAA were similar (data not shown). Whereas, the liver MMP-9 activity in the mice treated with TAA was significantly increased in a TAA dose-dependent manner **(**Fig. 5**)**. The overall trend of increased MMP-9 activity in the *Ace2* KO mice due to TAA administration with dosage dependently (100 mg/kg BW and 200 ng/kg BW) and similar to that in the WT mice, but no significant between WT and Ace2 KO mice (Fig. 5). The result illustrates that TAA administration led to an increasing hepatic MMP-9 activity in the mice.

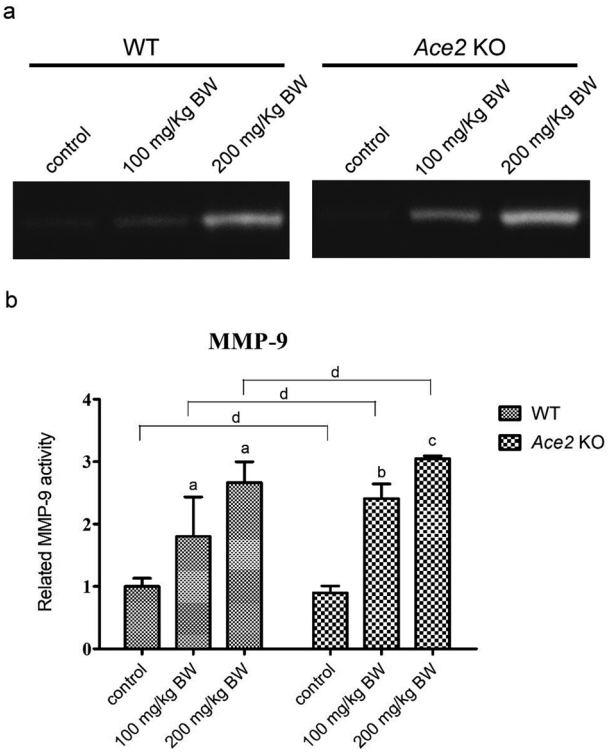

Fig. 5. Hepatic MMP-9 activity of the mice given TAA. The WT and *Ace2* KO mice were i.p. injected with TAA or DPBS for 8 weeks and total proteins were extracted from liver tissue. MMP-9 activity was analyzed using gelatin zymography (a). The means ± SD (n=4) are shown for each group (b). Different letters show a significant difference between the treatment groups ($P<0.05$).

Discussion

Our results show an induction of inflammation, liver fibrosis and an increase in hepatic MMP-9 activity in the mice administered TAA. In the *Ace2* KO mice, severe liver physiological and pathological changes were observed compared with those found in the WT mice. The results indicate that RAS, including the ACE2/Ang-(1–7) axis, plays a crucial role in TAA-induced liver fibrosis.

Previous studies had noted that *IL-6* and *Tnf* were associated with various liver diseases [26, 29]. In our results, the hepatic *IL-6* and *Tnf* levels in *Ace2* KO mice were higher than those in WT mice after TAA administration. Additionally, we have detected that hepatic inflammatory and pathogenic lesions in the *Ace2* KO mice were more serious than those in the WT mice. The result, i.e., progression of liver injury in *Ace2* KO mice was more rapid than in WT mice, demonstrates that ACE2 may play a critical role in preventing liver damage from TAA administration. Consistent with this proposition, previous studies indicate that supplementation with recombinant ACE2 [24] or adenoviral constructs that express an *Ace2* gene [3] can reduce critical features of liver fibrosis in mouse models.

Previous studies have showed that RAS participates in the pathogenesis of several inflammatory diseases [2, 34]. Increased ACE2 could affect the concentration of inflammatory cytokines, such as *IL-6* and *Tnf* [31, 33]. These inflammatory cytokines are associated with MAPK derived signaling pathways [9]. Besides to inflammation, the role of ACE2 in liver diseases concerns with more special interests because a lot of research evidence suggest that abnormal RAS also participates in tissue remodeling and fibrosis after liver injury [9]. The most studies supporting a dominate role for RAS in liver fibrosis is the finding that blocking Ang II generation could attenuate liver fibrotic process in animal models of liver injury [24].

ACE2 is a key negative regulator of the RAS and functions to limit fibrosis through the degradation of Ang II and the formation of Ang-(1–7) [24]. ACE2 can degrade Ang II and create Ang-(1–7), which acts as counter-regulator of the classical ACE-Ang II-AT1R axis, named as non-classical ACE2-Ang-(1–7)- axis. Pereira *et al.* concluded that blocking endogenous Ang-(1–7) pharmacologically accelerates liver fibrosis [28]. Eliminating *Ace2* in mice exacerbates liver fibrosis following chronic carbon tetrachloride (CCL_4) administration or bile duct ligation [24]. It has been concluded that recombinant ACE2 plays a protective role in the liver by decreasing experimental liver fibrosis in a mouse model using *Ace2* KO mice [24]. Our results support the idea that ACE2 is necessary for reducing liver damage following an insult.

However, more research on liver fibrosis must be performed to ensure the particular role of local ACE2 [38]. Various studies have documented that hepatic ACE2 immunoreactivity is upregulated in human cirrhotic livers or in mouse injury models [15–17, 25]. There are contradictory results concerning ACE2 function in liver pathogenesis. While some studies show ACE2 increased the effects of liver fibrosis, others report the opposite [5, 16, 24]. This issue needs to be clarified because of the importance of local ACE2 in the liver.

There are currently at least 28 MMPs identified. Among known MMPs, the gelatinases (MMP-2, 72 kDa; MMP-9, 92 kDa) are the most studied because both enzymes have been shown to play important roles in the pathogenesis of progressive fibrosis [10, 14]. A lot of studies have examined MMP-2 and MMP-9 activity in experimental and clinical subjects with liver fibrosis [13]. MMP-9 is a major MMP in basement membrane-like extracellular matrix remodeling and has been shown to be expressed by inflammatory macrophages [37]. MMP-2 has been documented to be expressed by activated stellate cells [1]. Therefore, the increased hepatic MMP-9 detected in the present study is most likely an inflammatory response in the liver of mice administered TAA. During the fibrotic pathogenic process, inflammatory monocytes are recruited to the injured liver because TAA induces them to form pro-fibrotic macrophage populations. It has been reported that MMP-9 in the scar areas of active fibrogenesis in liver indicates hepatic stellate cells may be an important source of MMP-9 [19]. In a rodent liver fibrosis induced by bile duct ligation, hepatic MMP-9 activity increased 2 days after the treatment, reached maximal level at day 10, and remained high throughout the study period, suggesting that sustained tissue damage due to chronic cholestasis induces MMP-9 [20].

In conclusion, this is the first time to report that ACE2 deficiency promotes TAA-induced liver inflammation and MMP-9 activity, and this contributes to the pathogenesis of liver fibrosis. We propose that ACE2 may have a capability to protect the liver from TAA-induced in-

jury, but further studies are necessary to confirm these encouraging results.

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