A Contradictory Role of A1 Adenosine Receptor in Carbon Tetrachloride and Bile Duct Ligation Induced Liver Fibrosis in Mice

Ping Yang, Zheyi Han, Peng Chen, Lin Zhu, Shiming Wang, Zichun Hua and Jianfa Zhang

Center for Molecular Metabolism, Nanjing University of Science & Technology, Nanjing, 210094, China (P.Y., P.C., S.W., J.Z.)
Xijing Hospital, Fourth Military Medical University, Xi’an, 710032, China (Z.H.)
Department of Endocrinology, Nanjing Jinling Hospital, Nanjing, 210002, China (L.Z.)
The State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing, 210090, China (Z.H.)
Running Title Page

Running title: Role of $A_1$AR in hepatic fibrosis

Corresponding author: Jianfa Zhang

Address: Center for Molecular Metabolism, Nanjing University of Science and Technology, 200 Xiaolingwei Street, Nanjing, 210094, China

Tel (fax): +86-25-84318533

Email: jfzhang@mail.njust.edu.cn

Text pages: 13

Table: 1

Figures: 8; Supplemental figure: 1

References: 36

Words in Abstract: 219

Words in Introduction: 467

Words in Discussion: 533

Abbreviations: $A_1$AR, A$_1$ adenosine receptor; CCl$_4$, carbon tetrachloride; BDL, bile duct ligation; HSC, hepatic stellate cell; $\alpha$-SMA, $\alpha$-smooth muscle actin; ECM, extracellular matrix; AST, aspartate aminotransferase; ALT, alanine aminotransferase; RT-PCR, quantitative real-time polymerase chain reaction; mRNA, messenger RNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CYP2E1, cytochrome P450 2E1; UCP2, uncoupling protein 2; MMP, matrix metalloproteinase; TIMP, tissue inhibitor metalloproteinase; TGF-$\beta$, transforming growth factor beta; TNF-$\alpha$, tumor necrosis factor alpha; PDGF, platelet-derived growth factor; IL, interleukin; WT, wild-type.

Recommended section: Gastrointestinal, Hepatic, Pulmonary, and Renal
Abstract

Mice lacking A₁ adenosine receptors (A₁AR) were thought to protect from developing fatty liver, however the contribution of A₁AR to hepatic fibrosis has not been explored. Here we found the expression of A₁AR was decreased in fibrotic liver induced by chronic carbon tetrachloride (CCL₄), but increased in that induced by bile duct ligation (BDL). Therefore we examined whether A₁AR contributes to hepatic fibrosis in CCl₄ and BDL animal models utilizing A₁AR knockout mice, respectively. Compared with wild type (WT) mice, hepatic fibrosis resulting from chronic CCl₄ exposure was attenuated in A₁AR⁻/⁻ mice with markedly decreased collagen deposition and reduced hepatic stellate cell (HSC) activation, whereas BDL A₁AR⁻/⁻ mice displayed a significant increase in hepatic fibrosis. Hepatocyte damage was reduced in A₁AR⁻/⁻ mice following a single injection of CCl₄, with down-regulation of CYP2E1 and UCP2 gene expression in livers which resulted in impaired liver sensitivity to CCl₄. However, BDL caused severe bile infracts in livers of A₁AR⁻/⁻ mice, with significantly elevated levels of bile acid as compared to WT mice. CCl₄ and BDL resulted in different expression pattern of genes involved in fibrogenesis in A₁AR⁻/⁻ mice. These results indicate that A₁AR participates in the pathogenesis of hepatic fibrosis with complex mechanism and the effect of targeting adenosine and its receptors should be cautiously evaluated in the prevention of hepatic fibrosis.
Introduction

Adenosine is a natural metabolite that plays an important role in many physiological and pathological processes. Extracellular adenosine levels are elevated in areas where cell damage has occurred which arises from by the release of adenosine or adenine nucleotides. Extracellular adenosine acts mainly through activation of G protein-coupled adenosine receptors, A₁, A₂A, A₂B, and A₃, each of which have been cloned from various tissues and species. There is growing evidence that adenosine receptors could be promising therapeutic targets in a wide range of conditions, including cerebral and cardiac ischemic diseases, sleep disorders, immune and inflammatory disorders and cancer (Guo et al., 2001; Ohta and Sitkovsky, 2001; Fishman et al., 2002; Porkka-Heiskanen et al., 2002; Stenberg et al., 2003; Reichelt et al., 2005).

A₁ adenosine receptor (A₁AR) is widely expressed in peripheral tissues and well characterized of its important role in many organs, including: brain, heart, and kidney (Shen and Kurachi, 1995; Matherne et al., 1997; Brown et al., 2001; Johansson et al., 2001; Lee et al., 2004). Also, prior studies have indicated a critical role of adenosine-depend metabolism in maintaining normal liver function (Migchielsen et al., 1995; Boison et al., 2002). And there are some studies demonstrated that adenosine and its receptors may play a critical role in the development of liver fibrosis. This was supported by the fact that CD73 (ecto-5'-nucelotides which convert 5'-AMP into adenosine) knockout mice which have a lower extracellular adenosine concentration in the livers are resistant to CCl₄ induced liver fibrosis (Peng et al., 2008). And mice lacking adenosine A₂ receptors diminished liver fibrosis induced by hepatotoxin (Chan et al., 2006). Recently, it is reported that activation of endogenous A₁AR protected against hepatic ischemia reperfusion injury in mice (Kim et al., 2008), while A₁AR deficient mice were found to be protected from developing
alcohol-induced fatty liver (Peng et al., 2009). It reveals critical and diverse role of A1AR in liver physiological and pathological processes.

Here we examined the role of A1AR in mice hepatic fibrosis by employing two different experimental fibrosis models: Carbon tetrachloride (CCL4) and bile duct ligation (BDL). We characterized the distinct roles of A1AR between chronic CCL4 and BDL induced liver fibrosis. The totally different expression patterns involved in fibrogenesis were found in CCL4 and BDL model of A1AR−/− mice. We found that impaired liver sensitivity to CCL4 in A1AR−/− mice was associated with down-regulation of CYP2E1 and UCP2 gene expression in livers after CCL4; and BDL caused severe bile infracts in livers of A1AR−/− mice, with significantly elevated levels of bile acid as compared to WT mice. Thus, hepatic fibrosis resulting from chronic CCL4 exposure was attenuated in A1AR−/− mice, whereas BDL A1AR−/− mice displayed a significant increase in hepatic fibrosis. These results indicate that A1AR plays a contradictory role in CCL4 and BDL induced liver fibrosis in mice.
Materials and Methods

Animal Models

Male C57BL/6 (WT) mice and A1AR\textsuperscript{-/-} (C57BL/6 background) mice were maintained under standard laboratory conditions, with 12-hour light/12-hour dark cycles and free access to food and water at all stages of the experiments. All animal care and use procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee at the Nanjing University of Science and Technology.

For acute carbon tetrachloride (CCl\textsubscript{4}, PubChem Substance ID: 24859204)-induced liver injury, age-matched (6- to 8-week old) male WT and A1AR\textsuperscript{-/-} mice were injected intraperitoneally (i.p.) with a single CCl\textsubscript{4} dose of 600 μL/kg of body weight as a 12% (vol/vol) solution in olive oil. Animals were randomly divided into 5 groups (n=5) and sacrificed at 0, 12, 24, 48, 96 hours after CCl\textsubscript{4} treatment, respectively. Whole blood was obtained for aminotransferases measurements and the liver was collected for histological and mRNA analyses.

For chronic CCl\textsubscript{4}-induced liver fibrosis, age-matched (6- to 8-week old) male WT and A1AR\textsuperscript{-/-} mice were biweekly i.p. injected with CCl\textsubscript{4} (600 μL/kg in olive oil, 12% v/v) for 4 weeks (n=5). Control mice were received an equal volume of oil. Mice were sacrificed 24 h after the last injection. At the time of sacrifice, the liver was removed and divided into three portions: (a) fixed in 10% buffered formaldehyde formalin and embedded in paraffin; (b) snap frozen at -80 ºC for immunohistochemistry; (c) homogenized in Trizol (Invitrogen, Carlsbad, CA) for RNA isolation.

For extrahepatic cholestasis induced liver fibrosis, age-matched (6- to 8-week old) male WT and A1AR\textsuperscript{-/-} mice were underwent BDL. BDL was performed as described elsewhere (Miyoshi et al., 1999). Briefly, under pentobarbital anesthesia, the common bile duct was ligated with 4-0
surgical silk after a midline abdominal incision. Sham-operated mice had their common bile duct exposed and manipulated but not ligated. On day 2 post-surgery, mice were sacrificed to obtain serum samples for determination of bile acid (n = 4-6). Other animals were sacrificed at 10 days after BDL to study hepatic fibrosis (n=6-7). At the time of sacrifice, liver tissues were collected for histological and immunohistochemical studies, as well as mRNA analyses.

Assessment of Biomarkers of Hepatic Injury and Cholestasis

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, as well as serum bile acid concentration were determined by an Olympus (Tokyo,Japan) AU2700 automatic biochemical analyzer.

Histological Analysis and Morphometric Collagen Determination

The liver sections imbedded in paraffin were cut (5 μm) and stained with hematoxylin-eosin for routine examination or Masson’s trichrome for visualization of hepatic collagen deposition. Fibrosis was determined histologically by measuring the density of fibrosis in four to six (×100) digital image captured from slides of each mouse stained with Masson’s trichrome using NIH image J 1.30 (NIH, Bethesda, MD).

α-SMA Immunohistochemistry

To detect α–smooth muscle actin (α-SMA), slides of paraffin embedded liver sections were deparaffinized and rehydrated. Immunohistochemical staining for α-SMA (Roche, CH) with a mouse monoclonal antibody against alpha-smooth muscle actin was performed according to the
manufacturer’s protocol. The quantification of alpha-SMA-positive area was performed in four to six (×100) digital images captured from slides of each mouse.

**Quantitative Real-time Polymerase Chain Reaction (PCR)**

Total RNA from liver samples was extracted using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. Reverse transcript reaction using KeyGene reverse transcript enzyme according to the manufacturer’s protocol. Primer sequences for the genes studied are listed in Table 1, and PCR was performed with the SYBR Green PCR Kti (Applied Biosystems) following the manufacturer’s instruction and carried out on the ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA) in 20μl volume. Relative expression in comparison to *Gapdh* was calculated by the comparative CT method.

**Immunohistochemistry for Cytokeratin 19**

Livers collected from 10-day BDL mice were used for cytokeratin (CK19) immunostaining. Immunohistochemical staining for CK19 with a mouse monoclonal antibody (Roche, CH) was performed according to the manufacturer’s protocol.

**Statistical Analysis**

All data are expressed as the mean ± standard error of the mean. Statistical analysis was performed using Student’s t test when only two value sets were compared, and one-way analysis of variance followed by Tukey’s multiple comparison test when the data involved three or more groups. *P* less than 0.05 was considered as a statistically significant difference.
Results

A Reciprocal expression of A1AR Occurred Between CCl4 and BDL Induced Hepatic Fibrosis in WT Mice.

To investigate whether A1AR participate in the development of mice liver fibrosis, we analyzed the mRNA expression of A1AR in WT mice after chronic CCl4 administration or BDL. Intriguingly, the expression of A1AR was decreased by nearly 3-fold after CCl4, whereas was increased by nearly 3-fold after BDL (Fig. 1A, B). To explore whether the reciprocal expression pattern of A1AR implicated its contrary role in two different fibrosis models, we utilized A1AR−/− mice to exam the role of A1AR in CCl4 and BDL induced liver fibrosis, respectively.

Hepatic Fibrosis Was Reduced in Livers of A1AR−/− Mice After CCl4 Treatment.

Initially, we sought to examine the role of A1AR in mice liver following chronic CCl4 administration. WT and A1AR−/− mice were biweekly i.p. injection with CCl4 for 4 weeks to induce liver fibrosis. Hepatic collagen deposition was evaluated by morphometric analysis of Masson’s trichrome staining. After 4 weeks CCl4 treatment, WT mice developed severe hepatic fibrosis, with obvious fibrotic septa visible, but A1AR−/− mice suffered only mild fibrosis (Fig. 2A-E). Because HSCs were the principal hepatic cell type responsible for collagen deposition in the liver, we next investigated the presence of activated HSCs in WT and A1AR−/− mice after toxin treatment. HSCs were distributed primarily in fibrotic septa, and there were significantly more α-SMA positive cells in the livers of CCl4-treated WT mice than A1AR−/− mice as detected by immunohistochemistry (Fig. 2F-J). Consistent with the histological appearance, hepatic fibrogenesis was suppressed in A1AR−/− mice as indicated by the reduction in RNA expression of
markers of fibrogenesis including Acta-2 and Coll1α procollagen (Fig. 2K, L). These data demonstrate that lack of A1AR attenuates CCl4-induced hepatic fibrosis.

**Hepatic Fibrosis Was Enhanced in Livers of A1AR−/− Mice After BDL.**

To further elucidate the critical role of A1AR in hepatic fibrosis, we also examined a distinct model of fibrosis induced by BDL. Contrary to attenuated hepatic fibrosis in A1AR−/− mice exposed to CCl4, after BDL, A1AR−/− mice developed more severe fibrosis than WT mice as demonstrated by enhanced collagen deposition (Fig. 3A-D) and morphometric quantification (Fig. 3E). During BDL-induced hepatic fibrosis, the expression of α-SMA was also detected at a high level in the fibrotic septa of A1AR−/− mice (Fig. 3F-J). In addition, mRNA expression for Acta-2 and Coll1α, but not Col3α1, was increased in A1AR−/− mice compared with WT mice (Fig. 3K-M), indicating that lack of A1AR enhances BDL induced hepatic fibrosis.

**A1AR Deficiency Reduced The Acute Reactivity to Liver Injury Induced by a Single CCl4 Injection.**

In order to determine whether decreased hepatic fibrosis induced by CCl4 in A1AR−/− mice was associated with reduced acute liver injury after CCl4 exposure, the extent of hepatic necrosis was analyzed and serum transaminase levels were quantified after a single dose of CCl4. In both WT and A1AR−/− mice, centrilobular necrotic lesions were present on day 1 (punctuate necrosis), peaked on day 2, and had almost completely recovered on day 4 after the CCl4 treatment (Supplemental fig. 1A-J). Histological examination showed that the extent of CCl4-induced hepatic necrosis was reduced in A1AR−/− mice compared with WT mice at 48h after CCl4 injection. Consistent with
histological results, serum AST and ALT activities, hallmarks of liver injury, tend toward a decrease in $A_1AR^{-/-}$ mice compared with WT mice after CCl$_4$ injection (Fig. 4A, B). Next we found at the early stage of CCl$_4$ treatment, both $CYP2E1$ and $UCP2$ gene expression was significantly decreased in $A_1AR^{-/-}$ mice compared with WT mice (Fig. 4C, D). Downregulation of $CYP2E1$ following CCl$_4$ injection was considered as an adaptive mechanism for decreasing toxicity (Wong et al., 1998), and CCl$_4$ induces the expression of $UCP2$, which reduced the efficiency of cellular ATP synthesis, had been proposed as causative factor in CCl$_4$-induced cell death (Demori et al., 2008). Thus, the decreased sensitivity of $A_1AR^{-/-}$ mice to CCl$_4$ induced liver injury may involve in downregulating $CYP2E1$ and $UCP2$ expression in the early stage.

**$A_1AR$ Contributed to Liver Injury in the BDL Mouse.**

Then we investigated the hepatocyte injury in the BDL model of WT and $A_1AR^{-/-}$ mice. Histopathological examination of liver specimens from 10-day BDL WT mice displayed severe cholestatic hepatitis with widespread bile infarctions, along with bile ductular proliferation, portal edema, and hepatocellular damage. Livers form 10-day BDL $A_1AR^{-/-}$ mice exhibited significantly enhanced injury (Fig. 5A-D). In addition, bile infarcts were also significant evident in $A_1AR^{-/-}$ mice (Fig. 5E). Those results implicated lack of $A_1AR$ enhanced cholestatic liver injury. Interestingly, $A_1AR^{-/-}$ mice showed about 3 times higher in levels of serum bile acids than that in WT mice after 2 days of BDL (Fig. 5F). Finally, we observed lack of $A_1AR$ enhanced biliary proliferation in the BDL mice by CK19 immunohistochemistry staining (Fig. 5G, H). These results indicate that $A_1AR$ plays a protective role in liver injury induced by BDL involving in downregulation of bile acid levels.
CCL4 and BDL Caused Different Expression Pattern of Fibrogenic Cytokines in A1AR-/- Mice

To further characterize the differential impact of A1AR between the CCl4 and BDL fibrosis model, a series of growth factors and cytokines mRNA expression was quantitated in hepatic lysates of WT and A1AR-/- mice after either CCl4 or BDL. Almost all of these growth factors and cytokines showed an elevated expression in fibrotic liver compared with normal liver (Fig. 6). However, the expression patterns of those genes were different between CCl4 group and BDL group in A1AR-/- mice. Following CCl4 treatment, hepatic mRNA expression of TGFβ2, TNFα, IL-1β and IL-6 decreased in A1AR-/- mice compared with WT mice (Fig. 6B, C, E, F). No differences were observed with respect to increases in mRNA for TGFβ1 and PDGF (Fig. 6A, D). Inversely, following BDL, hepatic mRNA expression of IL-1β and TGFβ1 in A1AR-/- mice increased significantly as compared to that in WT mice (Fig. 6G, K). No difference of mRNA levels for TNFα, PDGF and IL-6 were observed (Fig. 6I, J, L).

Matrix Degrading Metalloproteinases (MMP) and Tissue Inhibitors of Metalloproteinases (TIMP) Expression Pattern in WT and A1AR-/- Mice Following CCl4 and BDL.

Matrix degradation regulates the development of fibrosis (Bataller and Brenner, 2005) and collagen degradation occurs as a consequence of changes in the balance of the matrix degrading metalloproteinases (MMP) and their specific tissue inhibitors, the TIMP (Kasahara et al., 1997). Then we examined the expression of MMP-2, MMP-13 TIMP-1 and TIMP-2 by quantitative real time RT-PCR in WT and A1AR-/- mice. Almost all of those genes showed an elevated expression in
fibrosis liver compared with normal liver. As show in Fig 7, there was no significant difference in the mRNA levels of those genes between WT and $A_2AR^{+/+}$ mice after CCl$_4$ treatment (Fig. 7A-D), whereas, BDL caused a significant increase in $MMP-13$ expression and a decrease in $TIMP-2$ expression in $A_2AR^{+/+}$ mice compared with WT mice (Fig. 7F, H).
Discussion

Adenosine is a multifunctional nucleoside and plays an important role in maintaining normal physiological process. It has been reported that adenosine displays contradictory effects on regulation of cell growth (MacLaughlin et al., 1997; Fishman et al., 1998; Merighi et al., 2002). Adenosine triggers a survival signal via A3 receptor activation, and it also kills the cell through A2A receptor inducing a signaling pathway that involves protein kinase C and mitogen-activated protein kinases (Merighi et al., 2002). The protective effects of adenosine in myocardial and cerebrovascular ischemia are well known (Ely and Berne, 1992; Sweeney, 1997). Furthermore, adenosine affects the immune system by exerting immunosuppressive and anti-inflammatory activities (Ralevic and Burnstock, 1998).

Previous studies have already implicated a contradictory role of adenosine or adenosine receptors in liver fibrosis. Liver fibrosis is an exceedingly complex pathophysiologic syndrome (Bataller and Brenner, 2005). Some findings suggest adenosine and adenosine receptors function as profibrotic signal in liver fibrosis (Chan et al., 2006; Peng et al., 2008), while others implicate a protective role of adenosine in the process of fibrogenesis (Andrade et al., 2008; Hashmi et al., 2007; Hernández-Muñoz et al., 1997). Our studies extended those observations by elucidating a contradictory role of A1AR in the pathogenesis of liver fibrosis. We examined the susceptibility of A1AR<sup>-/-</sup> mice to liver fibrosis induced by either CCl4 or BDL and found a contradictory role of A1AR in two different models. Mice lacking A1AR were protected from developing liver fibrosis induced by chronic CCl4 administration, but susceptible to liver fibrosis induced by BDL.

A1AR played a different role between CCl4 and BDL induced liver fibrosis due to different liver injury response to CCl4 vs BDL. It is well known that CYP2E1 was required for the CCl4 induced
hepatotoxicity (Wong et al., 1998; Taïeb et al., 2005; Avasarala et al., 2006). CCl₄ metabolism by CYP2E₁ in the hepatocytes produces the highly oxidative stress (Weber et al., 2003). In early stage after CCl₄, loss of A₁AR function resulted in decreased levels of CYP2E₁, reducing hepatotoxicity by CCl₄. Meanwhile, we observed, an impaired UCP2 expression in A₁AR⁻/⁻ mice following CCl₄ compared with WT mice, which might be obligated for decreased death of hepatocytes after CCl₄ treatment (Demori et al., 2008).

Contrarily, bile acid level was markedly elevated in A₁AR⁻/⁻ mice after BDL, which was responsible for more liver injury (Zollner et al., 2006; Trauner et al., 2008), consequently upregulating profibrotic cytokines (such as TGFβ1 and IL-1β), increasing HSC activation and eventual collagen synthesis. Also, BDL caused an increased MMP-13 expression and decreased TIMP-2 expression, responding for the excessive accumulation of collagen in A₁AR⁻/⁻ mice.

In summary, the present studies demonstrate a contradictory role of A₁AR in CCl₄ and BDL induced mice liver fibrosis (Fig.8). In CCl₄-induced liver fibrosis, reduction of acute liver injury by decreasing CYP2E₁ and UCP2 expression and downregulation of profibrotic mediators is the key event leading to decreased fibrosis in A₁AR⁻/⁻ mice, whereas, in BDL-induced liver fibrosis, enhanced biliary infarcts and cholangiocyte proliferation due to elevation of bile acid levels should be the primary causation leading to increased fibrosis in A₁AR⁻/⁻ mice. Interestingly, A₁AR exhibits a reciprocal expression pattern in WT mice following two different insults, and it is likely to be an adaptive protection mechanism.
Acknowledgements

We are indebted to Dr. MR Blackburn for providing A1/AR-deficient mice.
References


Lee HT, Gallos G, Nasr SH and Emala CW (2004) A1 adenosine receptor activation inhibits...


Shen WK and Kurachi Y (1995) Mechanisms of adenosine-mediated actions on cellular and


Footnotes

* Ping Yang and Zheyi Han contributed equally to this work.

This work was supported by the National Science Foundation of China (30730030) and a Program for New Century Excellent Talents in University.
**Figure legends**

**Figure 1**

*Reciprocal expression of *A1AR* between CCl4 and BDL induced hepatic fibrosis.* WT mice were treated with the hepatotoxin CCl4 or subjected to BDL to induce hepatic fibrosis. Effects of CCl4 (A) and BDL (B) on the expression of *A1AR* were determined by quantitative real time RT-PCR. *, significantly different from the control group.

**Figure 2**

*Reduced hepatic fibrosis in A1AR-/- mice caused by CCl4 treatment.* WT and *A1AR*--/- mice were biweekly i.p. injection with CCl4 (600μL/kg in olive oil, 12% v/v) for 4 weeks. A-D: Collagen deposition was evaluated by Masson’s trichrome staining after 4 weeks of CCl4 injection (×100). A, WT–control; B, *A1AR*--/-–control; C, WT + CCl4; D, *A1AR*--/- + CCl4. E: Digital image analysis quantification of Masson’s trichrome staining. F-I: Expression of alpha-SMA was determined by immunohistochemistry after 4 weeks of CCl4 injection (×100). F, WT-control; G, *A1AR*--/--control; H, WT + CCl4; I, *A1AR*--/- + CCl4. J: Digital image analysis quantification of alpha-SMA positive area. K-M: Relative hepatic mRNA levels for *Acta-2*, *Collα1* and *Col3α1* after CCl4 treatment were determined in WT and *A1AR*--/- mice by quantitative real time RT-PCR. *, significantly different from the control group treated with oil; #, significantly different from the WT group.

**Figure 3**

*Enhanced hepatic fibrosis in A1AR-/- mice caused by BDL.* WT and *A1AR*--/- mice were subjected to BDL for 10 days. A-D: Collagen deposition was evaluated by Masson’s trichrome staining after...
10 days of BDL (×100). A, WT–sham; B, A₁AR<sup>−/−</sup>–sham, C, WT + BDL; D, A₁AR<sup>−/−</sup> + BDL. E:

Digital image analysis quantification of Masson’s trichrome staining. F-I: Expression of alpha-SMA was determined by immunohistochemistry after 10 days of BDL (×100). F, WT-sham; G, A₁AR<sup>−/−</sup>-sham, H, WT + BDL ; I, A₁AR<sup>−/−</sup> + BDL. J: Digital image analysis quantification of alpha-SMA positive area. K-M: Relative hepatic mRNA levels for Acta-2, Col1α1 and Col3α1 after BDL were determined in WT and A₁AR<sup>−/−</sup> mice by quantitative real time RT-PCR. *, significantly different from the sham group; #, significantly different from the WT group.

Figure 4

The extent of CCl₄-induced hepatic injury was reduced in A₁AR<sup>−/−</sup> mice. WT and A₁AR<sup>−/−</sup> mice were i.p. injection with 0.6 ml/Kg CCl₄ for 12 h, 24 h, 48 h and 96 h. Serum aspartate aminotranferase (A) and alanine aminotransferase (B) activities were monitored at indicated time points. C: Relative hepatic mRNA expression of CYP2E1 was measured in WT and A₁AR<sup>−/−</sup> mice after treatment with CCl₄ for 12h. D: Relative hepatic mRNA expression of UCP2 was measured in WT and A₁AR<sup>−/−</sup> mice after treatment with CCl₄ for 48h. Relative hepatic mRNA expression was determined by quantitative real time RT-PCR. *, significantly different from the corresponding control group; #, significantly different from the WT group.

Figure 5

Hepatic injury was aggravated in A₁AR<sup>−/−</sup> mice after BDL. WT and A₁AR<sup>−/−</sup> mice were subjected to BDL. A-D: Representative photomicrographs of conventional H&E-stained liver sections from 10-day BDL WT and A₁AR<sup>−/−</sup> mice are shown (×100). E: Quantitative image analysis of bile
infarcts of 10-day ligated WT and A\textsubscript{1}AR\textsuperscript{−/−} mice. F: Serum bile acid levels were measured on day 2 post-surgery. G, H: Biliary proliferation was detected by immunohistochemistry for CK19 in liver section from 10-day ligated mice. * significantly different from the WT group.

**Figure 6**

**Expression of fibrogenic mediators in WT and A\textsubscript{1}AR\textsuperscript{−/−} mice.** Relative hepatic mRNA levels of \textit{TGF}β\textit{1}, \textit{TGF}β\textit{2}, \textit{TNF}α, \textit{PDGF}, \textit{IL}-1\textit{β} and \textit{IL}-6 were measured in WT and A\textsubscript{1}AR\textsuperscript{−/−} mice after chronic CCl\textsubscript{4} treatment (A-F) or BDL (G-L) by quantitative real time RT-PCR. * significantly different from the control group; # significantly different from the WT group.

**Figure 7**

**MMP and TIMP mRNA expression in WT and A\textsubscript{1}AR\textsuperscript{−/−} mice.** Relative hepatic mRNA levels of \textit{MMP}-2, \textit{MMP}-13, \textit{TIMP}1 and \textit{TIMP}2 were measured in WT and A\textsubscript{1}AR\textsuperscript{−/−} mice after chronic CCl\textsubscript{4} treatment (A-D) or BDL (E-H) by quantitative real time RT-PCR. * significantly different from the control group; # significantly different from the WT group.

**Figure 8**

**A proposed model for different contributions of A\textsubscript{1}AR between CCl\textsubscript{4} and BDL induced liver fibrosis.**
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward/Reverse</th>
<th>Primer (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>CATCCACTGGTGTGCTGCC AAGGCTGT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACAACCTGGTCTCAGTGTAGCCCA</td>
</tr>
<tr>
<td>A1AR</td>
<td>Forward</td>
<td>TGTGACCACCAACCCAGGTA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCAGAGACTGAGCAGAAGGAC</td>
</tr>
<tr>
<td>Acta-2</td>
<td>Forward</td>
<td>ATGAAGCCCAAGGAAGAAGGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATGTCGCCAGTGGCTGTGGTAG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward</td>
<td>ACGGCATGGATCTCAAGAAGAC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CGGACTCCGAAAGTCGACC</td>
</tr>
<tr>
<td>Tgf-β1</td>
<td>Forward</td>
<td>GTGAGACATGGATCGGAAGCAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCTGATCGTGGGCGGCTTGGCA</td>
</tr>
<tr>
<td>Tgf-β2</td>
<td>Forward</td>
<td>CCCCCACATCTCTGCTAATGT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCTAAAGCAGTCAGAGCATCA</td>
</tr>
<tr>
<td>PDGF-B</td>
<td>Forward</td>
<td>CAATTCCGGACTAGAGGAAGCAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTCCCGTTGAGAATCTCCAC</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward</td>
<td>CATCCAGCTTCAAATCTCGCAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CACACACCAGAGTGATCGTCA</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward</td>
<td>CATGTTCTCTGGAGAATCGTGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTACTCCAGTGGTATGCTGTAC</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Forward</td>
<td>CAAATCTTTAAACAAATTGGGCAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCACGATGCCTGCTGA</td>
</tr>
<tr>
<td>UCP2</td>
<td>Forward</td>
<td>GCTG GTGTTGCTGGAGAAGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACAGTGCAATGGCATTA CGG</td>
</tr>
<tr>
<td>Col1α1</td>
<td>Forward</td>
<td>ACGTCCTGGTGAAGTGGTGT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAGGGAAAGCTCTCTCTCTCT</td>
</tr>
<tr>
<td>Col3α1</td>
<td>Forward</td>
<td>TGTCCTCGAGGCTGGAAGAGGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTCCAGCATCCTTTGTGTGT</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Forward</td>
<td>GTGCAGCCTAAACACAGACAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTCTCGATGTTGCTGTGT</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Forward</td>
<td>AGGCCTTCAGAAAGGCCTTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCCTTGAGTGGATCCAGACC</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Forward</td>
<td>ATCAAGGCTGTTGGGAATG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTCAAGGCTCAAGGACAGA</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>Forward</td>
<td>TCCTCTACAGGGCGAGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CATTGCGTGAAGTCTGTTGGA</td>
</tr>
</tbody>
</table>
Figure 1

**Figure 1**

(A) Comparison of $A_1$ receptor mRNA levels between control (C) and CCI4 fibrosis groups.

(B) Comparison of $A_1$ receptor mRNA levels between Sham and BDL fibrosis groups.

Bars with an asterisk (*) indicate statistically significant differences.
Figure 2

WT Control  A₁AR⁻/⁻ Control  WT CCl₄  A₁AR⁻/⁻ CCl₄

A

B

C

D

E

F

G

H

I

J

K

L

M

Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8