Loss of A<sub>1</sub> Adenosine Receptor Attenuates Alpha-naphthylisothiocyanate-Induced Cholestatic Liver Injury in Mice

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Cholestasis has limited therapeutic options and is associated with high morbidity and mortality. The A<sub>1</sub> adenosine receptor (A<sub>1</sub>AR) was postulated to participate in the pathogenesis of hepatic fibrosis induced by experimental extrahepatic cholestasis; however, the contribution of A<sub>1</sub>AR to intrahepatic cholestatic liver injury remains unknown. Here, we found that mice lacking A<sub>1</sub>AR were resistant to alpha-naphthyl isothiocyanate (ANIT)-induced liver injury, as evidenced by lower serum liver enzyme levels and reduced extent of histological necrosis. Bile acid accumulation in liver and serum was markedly diminished in A<sub>1</sub>AR<sup>−/−</sup> mice compared with wild-type (WT) mice. However, biliary and urinary outputs of bile acids were significantly enhanced in A<sub>1</sub>AR<sup>−/−</sup> mice. In the liver, mRNA expression of genes related to bile acid transport (Bsep and Mrp2) and hydroxylation (Cyp3a11) was increased in A<sub>1</sub>AR<sup>−/−</sup> mice. In the kidney, A<sub>1</sub>AR deficiency prevented the decrease of glomerular filtration rate caused by ANIT. Treatment of WT mice with A<sub>1</sub>AR antagonist DPCPX also protected against ANIT hepatotoxicity. Our results indicated that lack of A<sub>1</sub>AR gene protects mice from ANIT-induced cholestasis by enhancing toxic biliary constituents efflux through biliary excretory route and renal elimination system and suggested a potential role of A<sub>1</sub>AR as therapeutic target for the treatment of intrahepatic cholestasis.

Key Words: A<sub>1</sub>AR; ANIT; cholestasis; bile acid; DPCPX.
ATTENUATED CHOLESTASIS IN A1AR−/− MICE

Steven Sun, Ruixia Mu, Daisheng Lu, Dapeng Li, Liang Yan, Qun Ruan, Chen Sun, Shi-jia Li, Xiao-long Liang, Zhihong Chen, Zhi-hua Tang, and Yue-xin Wang

Evidence indicating that adenosine receptors could be promising therapeutic targets in a wide range of conditions, including cerebral and cardiac ischemic diseases, sleep disorders, immunity and inflammatory disorders, and cancer (Fishman et al., 2002; Guo et al., 2001; Ohta and Sitkovsky, 2001; Porkka-Heiskanen et al., 2002; Reichelt et al., 2005; Stenberg et al., 2003).

A1 adenosine receptor (A1AR) is widely expressed in peripheral tissues and is well characterized for its important role in many organs, including brain, heart, and kidney (Brown et al., 2001; Johansson et al., 2001; Lee et al., 2004; Matherne et al., 1997; Shen and Kurachi, 1995). In the liver, A1AR involves in the development of mouse liver diseases, such as alcoholic fatty liver disease, hepatic ischemia reperfusion injury, and carbon tetrachloride (CCl4) or bile duct ligation (BDL) induced liver fibrosis (Kim et al., 2008; Peng et al., 2009; Yang et al., 2010). However, whether A1AR contributes to liver injury during intrahepatic cholestasis is not known. In this study, we examined the possible role of A1AR in a mouse model of intrahepatic cholestasis, using the hepatotoxic compound ANIT. We demonstrated the effects of genetic loss and pharmacological blockade of A1AR on ANIT-induced hepatobiliary toxicity. Finally, we tried to explore the underlying mechanisms whereby A1AR could participate in this model of liver injury.

METHODS

Animals. Male C57BL/6 wild-type (WT) mice and A1AR−/− (C57BL/6 background) mice were used in this study. The A1AR−/− mice were generated and genotyped as described previously (Sun et al., 2001). All mice were maintained in 12-h light/dark cycles with lights on at 0700 h and given free access to water and standard mouse chow. 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. The ANIT (Sigma, St Louis, MO) dissolved in olive oil was injected intraperitoneally (i.p.) at the dose of 75 mg/kg. The WT and A1AR−/− mice were randomly divided into four groups (n = 5) and sacrificed at 0, 12, 24, and 48 h after ANIT administration, respectively. The blood, tissues (liver and kidney), bile (from the gallbladder), and urine were collected.

To examine the effects of pharmacological block of A1AR on ANIT-induced liver injury, some mice were treated with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 1 mg/kg, i.p.), a potent and selective antagonist of A1AR, twice daily for 2 days (n = 5). DPCPX (Sigma) was dissolved in 1% (vol/vol) dimethyl sulfoxide. 0.75% (vol/vol) 1 M NaOH in saline. ANIT was administered 15 min after the first dose of DPCPX. The animals were sacrificed 48 h after ANIT administration and the blood, tissues, bile, and urine were collected.

Adenosine extraction and quantification. Adenosine was extracted from liver samples using 0.4 N perchloric acid and analyzed by high-performance liquid chromatography (HPLC), as described previously (Sun et al., 2005). HPLC (Waters 1525 system; Millipore Corp., Bedford, MA) analysis was performed on a reverse phase (C18) column at a flow rate of 1.5 ml/min. Buffer A was 20 mM NH4H2PO4, pH 5.1; buffer B contained 20 mM NH4H2PO4 and 20% methanol, pH 5.1. The amount of buffer A was changed linearly between the following time points: 0–4 min, 100%; 4–6 min, 100–60%; 6–8 min, 60–0%; 8–12 min, 0%; and 12–18 min, 0–100%. Hepatic adenosine concentrations were normalized for total protein content, and values were given as μg/g protein.

Bile flow rate measurement. WT and A1AR−/− mice were administered with a single dose of ANIT (75 mg/kg) for 24 h (n = 5). For bile collection study, mice were anesthetized with pentobarbital (45 mg/kg) after 6 h fasting. The abdomen was opened and, after distal ligation of the common bile duct, the gallbladder was cannulated with a polyethylene catheter (PE-10) (Smit et al., 1993). Hepatic bile was collected for 1 h while mice were kept under anesthesia at 37°C under a heating lamp. Bile volume was measured gravimetrically, assuming the density to be 1 g/ml.

Serum biochemistry analysis. Serum was collected at 0, 12, 24, and 48 h after ANIT treatment. The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were determined using an AU2700 automatic biochemical analyzer (Olympus, Tokyo, Japan).

Bile acid and bilirubin analysis. Bile acids were extracted from liver samples using 75% ethanol, as previously described (Chen et al., 2009). Total bile acids in the liver, serum, bile, and urine were measured using a commercial assay kit (Bio-Quant, San Diego, CA). Total bilirubin in the serum and urine were measured with a Sigma Kit in accordance with the manufacturer’s protocols.

H&E and Oil Red O staining of liver sections. Liver samples were collected and fixed in 10% formalin. The samples were then embedded in paraffin, sliced into 5 μm in thickness, and stained with hematoxylin and eosin (H&E) for histological analysis. Quantification of the biliary infarct area was performed in five digital images captured from slides of each mouse using National Institutes of Health (Bethesda, MD) Image J. The numbers of neutrophils in the portal tracts and necrotic zones were counted in 8 high-power fields (magnification ×400) under a light microscope. For Oil Red O staining, liver sections were flash-frozen and embedded in frozen medium. The 5-μm sections were cut and stained with Oil Red O for 20 min. After being washed with 60% isopropanol, the sections were counterstained with hematoxylin.

Renal function analysis. Blood samples were collected at 48 h after ANIT treatment. Blood urea nitrogen (BuN), serum uric acid (UA), and serum creatinine levels were measured with an automatic analyzer using an enzymatic method (Hitachi 7180, Tokyo, Japan). Serum Cystatin C (CysC) was determined with a particle-enhanced immunoturbidimetric assay (Dako, Glostrup, Denmark) according to the manufacturer’s protocols.

Quantitative real-time PCR. Total RNA from liver and kidney samples was extracted using TRIzol according to the manufacturer’s instructions. The reverse transcription reaction using KeyGene reverse transcription enzyme was performed 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 Kit (Applied Biosystems, Foster City, CA) following the manufacturer’s instructions on an ABI 7300 Real-Time PCR System (Applied Biosystems) in a 20-μl volume. Relative expression compared with that of Gapdh was calculated by the comparative cycle threshold method.

Western blot analysis. Fresh tissues were homogenized, and proteins were extracted with Extraction Reagents (KeyGEN) according to the manufacturer’s instructions. The extracted proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (0.45 μm). A1AR and β-actin antibodies purchased from Bioworld were used for 1:1000 dilutions. Western blot analysis was performed as general protocol.

Statistics. All data were expressed as mean ± SEM. Comparison of two groups was performed using Student’s t-test. Comparison of multiple groups was performed using one-way ANOVA followed by Tukey’s post hoc test. P values less than 0.05 were considered significant.

RESULTS

Hepatic Adenosine and A1AR Levels Were Increased Following ANIT Challenge

To investigate whether adenosine-A1AR signal involves in the development of ANIT-induced liver injury, hepatic adenosine and A1AR levels were measured following ANIT.
WT mice challenged with ANIT exhibited about a twofold increase in hepatic adenosine concentrations (Fig. 1A) and a fourfold increase in hepatic A₁AR mRNA levels (Fig. 1B). Similarly, renal mRNA expression of A₁AR was increased fivefold by ANIT (Fig. 1C). Western blot analysis revealed an induction of A₁AR protein in the liver and kidney after ANIT fivefold by ANIT (Fig. 1C). Western blot analysis revealed an induction of A₁AR protein in the liver and kidney after ANIT fivefold by ANIT (Fig. 1C). Western blot analysis revealed an induction of A₁AR protein in the liver and kidney after ANIT fivefold by ANIT (Fig. 1C). Unexpectedly, hepatic neutrophil infiltration and cytokine induction were not attenuated in A₁AR−/− mice compared with WT mice after ANIT treatment (Supplemental fig. 1). In addition, ANIT-associated hepatic lipid accumulation was indistinguishable between A₁AR−/− mice and WT mice (Supplemental fig. 2A). Overall, our results suggested that lack of A₁AR attenuates ANIT-induced hepatocyte and biliary epithelial cell damage.

### A₁AR Deficiency Attenuated Liver Injury Induced by ANIT

To examine the role of A₁AR in ANIT-mediated hepatotoxicity, serum liver enzymes were quantified and liver histology was evaluated in WT and A₁AR−/− mice after intragastrically administered with ANIT (75 mg/kg). In both WT and A₁AR−/− mice, serum activities of AST, ALT, and ALP were unchanged at 12 h, elevated at 24 h, and peaked at 48 h after ANIT treatment, but there was an attenuated increase observed in A₁AR−/− mice (Fig. 2A(a–c)). Again, the area under the curve (AUC) values for AST, ALT, and ALP in A₁AR−/− mice were significantly lower than that of WT mice (Fig. 2C(a–c)). Histological examination of the liver from 48-h ANIT-treated mice showed extensive hepatocyte necrosis around portal area and bile duct epithelial cell injury, as well as neutrophils infiltration (Fig. 2B). Liver sections from A₁AR−/− mice contained less area of necrotic hepatocytes than WT mice, which was also confirmed by morphometric quantification (Fig. 2C(d)). At higher magnification, bile duct destruction and degeneration were visible (Fig. 2B(e–h)). The morphological changes of bile duct observed in A₁AR−/− mice were ameliorated compared with WT mice.

Increased urinary excretion of bile acids and bilirubin was found with a prominent increase in WT mice (Figs. 3A–C; p < 0.05). Increased urinary excretion of bile acids and bilirubin was found after ANIT treatment. Urinary bile acid concentrations in A₁AR−/− mice were significantly higher than those in WT mice after ANIT treatment (Fig. 3D; p < 0.05). Notably, urinary bilirubin concentrations in ANIT-A₁AR−/− mice were increased by sevenfold compared with ANIT-WT mice (Fig. 3E; p < 0.05). In contrast, bile acid concentrations in bile were dramatically reduced in both genotypes by ANIT, with a higher level observed in A₁AR−/− mice compared with ANIT-WT mice (Supplemental fig. 2A). Overall, our results suggested that lack of A₁AR attenuates ANIT-induced hepatocyte and biliary epithelial cell damage.

### Effects of A₁AR Deficiency on Bile Flow

To further investigate the differences in biliary secretion of bile acid and bilirubin between WT and A₁AR−/− mice, additional bile collection studies were made (Table 2). Bile flow rate was increased in A₁AR−/− mice compared with WT mice at baseline (p > 0.05). Biliary output rates of bile acids and bilirubin tended to be increased in A₁AR−/− mice (p = 0.07). A single dose of ANIT resulted in a reduction of bile flow to almost zero by 24 h in WT mice. A₁AR deficiency prevented the impairment of bile flow caused by ANIT. However, the bile flow rate

### TABLE 1

<table>
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<tr>
<th>Primer Sequences Used for Real-Time RT-PCR</th>
<th>Genes</th>
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<td>MCP-1</td>
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was still lower compared with untreated mice. Moreover, differences in bile composition were observed between WT and A1AR−/− mice after ANIT administration. Biliary bile acid and bilirubin concentrations were significantly increased in A1AR−/− mice compared with WT mice (p < 0.05). As a consequence, bile excretion rates of bile acid and bilirubin were markedly increased in A1AR−/− mice (p < 0.05).

Liver Gene Expression Patterns in WT and A1AR−/− Mice After ANIT Treatment

Firstly, we examined hepatic mRNA expression of basolateral transporters after ANIT treatment. In agreement with previous observations (Cui et al., 2009; Tanaka et al., 2009), ANIT significantly downregulated the expression of bile acid basolateral uptake transporter Ntcp, Oatp1a1, and Oatp1b2 in both genotypes (Fig. 4A). In contrast, ANIT markedly upregulated the basolateral efflux transporter Ostβ expression. There was no change in mRNA levels for Mrp3 and Mrp4 after ANIT treatment (Fig. 4B). However, for all basolateral transporters described above, no statistical difference was observed between WT and A1AR−/− mice.

Secondly, gene expression of canalicular efflux transporters was determined (Fig. 4C). ANIT treatment significantly upregulated Bsep and Mdr2 expression in both genotypes. The mRNA levels for Bsep were higher in A1AR−/− mice than in WT mice both at baseline or following ANIT treatment. Similarly, Mdr2 mRNA expression was increased in ANIT-A1AR−/− mice compared with ANIT-WT mice. Otherwise, Mrp2 mRNA expression was induced by ANIT treatment in A1AR−/− mice, but not in WT mice. In addition, protein levels of Bsep and Mdr2 were also increased in A1AR−/− mice than in WT mice after ANIT treatment (Supplemental fig. 2B).

Thirdly, we examined the mRNA expression of genes related to bile acid synthesis (Cyp7a1 and Cyp27a1) and detoxification (Cyp3a11) (Fig. 4D). The mRNA levels of Cyp7a1 and Cyp27a1 in A1AR−/− mice were declined to the same level as that in WT mice by ANIT. Cyp3a11 mRNA expression, which was increased in A1AR−/− mice by ANIT treatment, was unchanged in WT mice.

Adaptive Response in Kidney After ANIT Treatment

Because there was an enhanced urinary excretion of bile acids and bilirubin in A1AR−/− mice, we next determined the expression of putative renal bile acid transporters. ANIT treatment markedly increased the mRNA levels for bile acid export transporter Mrp2, Mrp3, and Mrp4 in both WT and A1AR−/− mice (Fig. 5A). Renal Mrp2 and Mrp3 expression was similarly in ANIT-A1AR−/− mice compared with ANIT-WT mice, but Mrp4 expression was significantly lower. Otherwise, ANIT downregulated the main reuptake transporter Asbt in WT mice, but not in A1AR−/− mice (Fig. 5A). The changes in renal transporter expression in A1AR−/− mice should not be a cause for enhanced urinary excretion, but probable be a result of reduced cholestasis.

Increased passive glomerular filtration also facilitates renal bile acid elimination under cholestasis (Trauner et al., 2005); so studies were undertaken to determine whether there is a difference in the glomerular function between WT and
A1AR−/− mice. WT mice that received ANIT yielded higher levels of serum urea nitrogen, creatinine, and UA than untreated mice. However, these changes were not observed in A1AR−/− mice when treated with ANIT (Figs. 5B–D). To examine any difference in the glomerular filtration rate (GFR), CysC, which is a novel serum marker of the GFR (Song et al., 2009), was measured in WT and A1AR−/− mice. ANIT treatment caused an increase in serum CysC concentrations in WT mice, but not in A1AR−/− mice (Fig. 5E). Urinary KIM-1 and NGAL concentrations were reduced in A1AR−/− mice compared with WT mice after ANIT treatment (Supplemental fig. 2C).

These results suggested that a higher GFR in ANIT-A1AR−/− mice may function to enhance bile acid and bilirubin elimination via the urine.
DPCPX Treatment Protects Mice Liver From ANIT-Induced Liver Injury

The selective A₁AR antagonist DPCPX was used to further characterize the effects of pharmacological block of A₁AR on ANIT-induce hepatotoxicity. Treatment of WT mice with DPCPX apparently attenuated serum levels of AST, ALT, and ALP raised by ANIT (Fig. 6A). This effect of DPCPX was absent in A₁AR⁻/⁻ mice, suggesting that DPCPX exerts its protective role through antagonism of A₁ARs. Consistently, histological examination observed that treatment with DPCPX significantly suppressed hepatocyte necrosis in periportal area in ANIT-WT mice, but not in ANIT-A₁AR⁻/⁻ mice (Fig. 6B(a–f)). Morphometric analysis also showed a reduced degree of necrotic foci when treated WT mice with DPCPX (Fig. 6B(i)). Bile duct epithelial cell damage was also improved by DPCPX treatment observed at higher magnification (Fig. 6B(g and h)). Thus, pharmacological block of A₁AR attenuated ANIT-induced liver injury.

Quantitative Changes in Bile Acid and Bilirubin Levels After DPCPX Treatment

Serum and hepatic bile acid, and serum bilirubin levels were all markedly increased by ANIT. Treatment of WT mice with DPCPX resulted in substantial reduction in these levels (Figs. 7A–C; p < 0.05). Urinary excretion of bile acid and bilirubin increased after ANIT treatment. DPCPX treatment resulted in a further increase in urinary bile acid and bilirubin concentrations (Figs. 7D and E; p < 0.05). In contrast, biliary bile acid levels were dramatically decreased by ANIT. DPCPX treatment caused a threefold augmentation of bile acid concentrations in
bile (Fig. 7F; $p < 0.05$). These results suggested that pharmacological block of A1AR stimulates bile acid and bilirubin elimination and attenuates cholestasis.

**DISCUSSION**

Extracellular adenosine released from cells during increased stress mediates a diverse set of physiopathological functions (Stiles, 1992). Adenosine molecular signal is important in maintaining normal liver function (Boison et al., 2002; Migchielsen et al., 1995). Previous studies have revealed an essential role of A1AR in the pathogenesis of mouse liver disease. It has been reported that activation of endogenous A1AR protects mice from hepatic ischemia reperfusion injury by reducing apoptotic and necrotic cell death (Kim et al., 2008). However, mice lacking A1AR were protected from developing alcohol-induced fatty liver through decreasing fatty acid synthesis (Peng et al., 2009).

In addition, our previous study demonstrated a contradictory role of A1AR in two different models of mouse liver fibrosis (Yang et al., 2010). In this study, we aimed to extend previous findings considering the effects of A1AR on intrahepatic cholestasis, using xenobiotic ANIT, a well-known chemical that produces biliary injury and cholestasis. We presented here the first evidence that mice lacking A1AR are protected from the accumulation of bile acids and subsequent development of liver injury induced by ANIT, in association with an augmentation of bile acid excretion in the bile and urine.

Bile acid transport at canalicular membrane of the hepatocyte is mediated by ATP-binding cassette transporters, including Bsep, Mrp2, and Mdr2. Canalicular export of toxic bile acids is probably an alternative escape route during intrahepatic cholestasis. In this study, we found that hepatic mRNA and protein levels of canalicular transporter Bsep and Mdr2 are all upregulated in A1AR−/− mice (Fig. 4C; Supplemental fig. 2B). The functional outcomes of the increased canalicular transporter expression were supported by the data concerning bile secretion (Table 2). A1AR deficiency partly prevented the block of bile secretion caused by ANIT. Biliary output rates of bile acid and bilirubin were markedly enhanced ANIT-A1AR−/− mice compared with ANIT-WT mice. These data suggested that decreased bile acid retention in A1AR−/− mice is partially due to induction of an adaptive canalicular transporter response, which could facilitate the elimination of bile acids via canalicular export systems.

Adaptive responses to cholestasis also occur in the kidney in an effort to eliminate excess bile acids and toxic compounds from circulation. Liver disease is often accompanied by impaired renal function. A1ARs are expressed throughout the kidney and have been linked to physiological regulation in the afferent arterioles and proximal tubule. In the ANIT model, kidney failure occurs, as evidenced by elevated serum creatinine, urea nitrogen, and UA levels (Figs. 5B–D). Loss of A1AR suppressed the increase of these biomarkers and prevented the decrease of GFR which were caused by ANIT treatment.

**FIG. 4.** Gene expression in livers of vehicle- and ANIT-treated WT and A1AR−/− mice. Total RNA was isolated from both vehicle- and ANIT-treated mouse liver and gene expression was measured using real-time RT-PCR. Relative hepatic mRNA levels of (A) basolateral uptake transporters (Ntcp, Oatp1a1, and Oatp1b2), (B) basolateral efflux transporters (Ostβ1, Mrp3, and Mrp4), (C) canalicular efflux transporters (Bsep, Mrp2, and Mdr2), and (D) bile acid-metabolizing enzymes (Cyp7a1, Cyp27a1, and Cyp3a11) were determined in WT and A1AR−/− mice 48h after ANIT treatment. Values are expressed as the percentage of WT controls. Open bars, WT mice; black bars, A1AR−/− mice. *Significantly different from the control group treated with vehicle. #Significantly different from the WT group.
Moreover, urinary excretion of bile acids and bilirubin was significantly higher in A1AR−/− mice than in WT mice during ANIT-cholestasis (Figs. 3D and E). Our results indicated that lack of A1AR attenuates ANIT-cholestasis partially though preventing kidney failure, which could maintain bile acid elimination in the urine.

The protective effects achieved by genetic loss of A1AR were further proved by pharmacological blockade. Treatment with A1AR antagonist DPCPX protected WT mice against ANIT hepatotoxicity (Fig. 6). DPCPX treatment also attenuated ANIT-induced cholestasis, accompanied by increased bile acid excretion into bile and urine (Fig. 7). Cholestasis has limited therapeutic options and is associated with high morbidity and mortality. Ursodeoxycholic acid (UDCA) is a representative drug used in the clinical treatment of cholestatic liver disease. UDCA is a tertiary bile acid with known anticholestatic properties, probable by stimulation of hepatobiliary secretion (Paumgartner and Beuers, 2002). Unfortunately, UDCA therapy needs tedious course and has limitations regarding ANIT-induced intrahepatic cholestasis (Ando, 1992). The search for more specific and effective therapies for intrahepatic cholestasis is needed. A1AR antagonists are safety and become an attractive therapeutic option for treatment of patients with heart failure and renal impairment (Hoche, 2010). Our results suggested a potential role of A1AR antagonist as further treatment strategy for intrahepatic cholestasis.

**FIG. 5.** Adaptive regulation in kidney in ANIT model of cholestasis. (A) Relative mRNA levels of putative renal bile salt transporters (Mrp2, Mrp3, Mrp4, and Asbt) were determined 48h after ANIT treatment. Effects of ANIT on glomerular function were evaluated by measuring (B) blood urea nitrogen (BuN), (C) serum creatinine, (D) serum uric acid (UA), and (E) serum Cystatin C (CysC) concentrations. *Significantly different from the control group treated with vehicle. #Significantly different from the WT group.
FIG. 6. Protection against ANIT-induced hepatotoxicity by A<sub>1</sub>AR selective antagonist DPCPX. WT and A<sub>1</sub>AR<sup>−/−</sup> mice were treated with DPCPX (1 mg/kg, ip) or vehicle twice daily for 2 days, and ANIT was administered 15 min after the first dose. (A) Serum (a) AST, (b) ALT, and (c) ALP activities were measured in WT and A<sub>1</sub>AR<sup>−/−</sup> mice 48 h after ANIT treatment. (B) Representative H&E-stained liver sections from each treatment group at ×100 magnification (a–f) and ×400 magnification (g and h). (a) vehicle/vehicle-treated WT mice; (b) Vehicle/vehicle-treated A<sub>1</sub>AR<sup>−/−</sup> mice; (c) vehicle/ANIT-treated WT mice; (d) vehicle/ANIT-treated A<sub>1</sub>AR<sup>−/−</sup> mice; (e) DPCPX/ANIT-treated WT mice; (f) DPCPX/ANIT-treated A<sub>1</sub>AR<sup>−/−</sup> mice; (g) vehicle/ANIT-treated WT mice; (h) DPCPX/ANIT-treated WT mice; (i) Quantitative image analysis of bile infarcts of vehicle/ANIT and DPCPX/ANIT-treated WT and A<sub>1</sub>AR<sup>−/−</sup> mice. *Significantly different from the vehicle/vehicle group. #Significantly different from the vehicle/ANIT group. **Significantly different from the vehicle/ANIT-treated WT mice.

FIG. 7. Protection against ANIT-induced cholestasis by DPCPX. WT mice were treated with DPCPX (1 mg/kg, ip) or vehicle twice daily for 2 days, and ANIT was administered 15 min after the first dose. (A) Serum bile acids, (B) serum bilirubin, (C) hepatic bile acids, (D) urinary bile acids, (E) urinary bilirubin, and (F) biliary bile acids were measured 48 h after ANIT treatment. *Significantly different from the control group. #Significantly different from the ANIT group.
Although A1AR deficiency protected mice against ANIT toxicity, liver injury induced by BDL was increased as we previously reported. ANIT and BDL represent two different experimental cholestasis models, intrahepatic and extrahepatic, respectively. ANIT causes intrahepatic cholestasis by damaging the cholangiocytes lining the bile ducts, whereas BDL causes extrahepatic cholestasis by blocking the drainage of bile from the liver to the duodenum. It is well known that drugs that stimulate bile secretion are disadvantage of treating extrahepatic cholestasis. These drugs further augment the biliary pressure and thus have harmful effects in extrahepatic biliary obstruction. We hypothesis that increased bile secretion in A1AR−/− mice may provide the liver against toxic effects of ANIT, but increase liver injury caused by BDL.

In summary, A1AR deficiency reduces the susceptibility of mice to ANIT-induced hepatotoxicity. This anticholestatic effect is associated with adaptive pathways both in the liver and kidney. In the liver, genetic loss of A1AR promotes bile acid elimination by activation of canalicular efflux transporters (Bsep and Mdr2) and bile acid-detoxifying enzyme Cyp3a11. In the kidney, elevated GFR in A1AR−/− mice may facilitate bile acid and bilirubin elimination through kidney. Moreover, our findings suggest that A1AR antagonism may provide a novel therapy for intrahepatic cholestatic liver disease, probably by augmenting bile acid elimination.

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