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Adenosine A_{2A} receptors play a role in the pathogenesis of hepatic cirrhosis

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1 Adenosine is a potent endogenous regulator of inflammation and tissue repair. Adenosine, which is released from injured and hypoxic tissue or in response to toxins and medications, may induce pulmonary fibrosis in mice, presumably *via* interaction with a specific adenosine receptor. We therefore determined whether adenosine and its receptors contribute to the pathogenesis of hepatic fibrosis.

2 As in other tissues and cell types, adenosine is released *in vitro* in response to the fibrogenic stimuli ethanol (40 mg dl^{-1}) and methotrexate (100 nM).

3 Adenosine A_{2A} receptors are expressed on rat and human hepatic stellate cell lines and adenosine A_{2A} receptor occupancy promotes collagen production by these cells. Liver sections from mice treated with the hepatotoxins carbon tetrachloride (CCl_4) (0.05 ml in oil, 50 : 50 v : v, subcutaneously) and thioacetamide (100 mg kg^{-1} in PBS, intraperitoneally) released more adenosine than those from untreated mice when cultured *ex vivo*.

4 Adenosine A_{2A} receptor-deficient, but not wild-type or A_3 receptor-deficient, mice are protected from development of hepatic fibrosis following CCl_4 or thioacetamide exposure.

5 Similarly, caffeine (50 mg kg^{-1} day^{-1}, po), a nonselective adenosine receptor antagonist, and ZM241385 (25 mg kg^{-1} bid), a more selective antagonist of the adenosine A_{2A} receptor, diminished hepatic fibrosis in wild-type mice exposed to either CCl_4 or thioacetamide.

6 These results demonstrate that hepatic adenosine A_{2A} receptors play an active role in the pathogenesis of hepatic fibrosis, and suggest a novel therapeutic target in the treatment and prevention of hepatic cirrhosis.
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Abbreviations: CCl_4, carbon tetrachloride; CSC, 8-(3-chlorostyryl)-caffeine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine

Introduction

Adenosine, a nucleoside produced by cells and tissues in response to a variety of physical and metabolic stresses, mediates physiological activities that include sedation, inhibition of platelet aggregation and vasodilatation (Ralevic & Burnstock, 1998). The effects of adenosine are mediated by a family of four G protein-coupled receptors, A_1, A_{2A}, A_{2B} and A_3. Engagement of the adenosine A_{2A} receptor promotes the resolution of inflammation (Chan & Cronstein, 2002), which accounts for at least some of the anti-inflammatory actions of many commonly used drugs, including salicylates, methotrexate and sulfasalazine (Cronstein *et al*., 1993; 1999; Chan & Cronstein, 2002), and in the liver, prevents inflammation and acute inflammatory injury (Ohta & Sitkovsky, 2001). Adenosine A_{2A} receptors also promote tissue repair, wound healing and matrix production (Montesinos *et al*., 1997; 2002; Victor-Vega *et al*., 2002).

In the liver, chronic injury due to alcohol ingestion, viruses, drugs (e.g., methotrexate) or metabolic derangements stimulates fibrosis leading to cirrhosis, a major cause of morbidity and mortality throughout the world. Ethanol, one of the most important causes of hepatic fibrosis/cirrhosis in the Western world, stimulates increased extracellular adenosine levels *in vitro* through its action on the nucleoside transporter (Nagy *et al*., 1990) and ethanol ingestion increases purine release into the bloodstream and urine in normal volunteers (Puig & Fox, 1984). Surprisingly, ethanol-induced increases in extracellular adenosine in the central nervous system and the periphery

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mediate many of the manifestations of ethanol inebriation *via* interaction with adenosine receptors (El Yacoubi *et al*., 2003). Methotrexate increases extracellular adenosine concentrations which mediate the anti-inflammatory effects of methotrexate both *in vitro* and *in vivo* (Cronstein *et al*., 1991; 1993). Both exogenous adenosine A_{2A} receptor agonists and endogenously generated adenosine play a role in wound healing increasing both angiogenesis and matrix production (Montesinos *et al*., 1997; 2002; Victor-Vega *et al*., 2002). Moreover, recent work by Blackburn *et al*. (2003) showed that mice partially deficient in adenosine deaminase die prematurely from pulmonary injury and fibrosis and that enzyme therapy, by decreasing tissue adenosine levels, inhibited IL-13 levels and fibrosis and alveolar damage in the lung (Sun *et al*., 2005). We therefore hypothesized that adenosine, released in response to toxins or drugs, binds to its receptors in the liver to promote fibrosis, a form of sustained wound healing in response to injury.

We report here that endogenously released adenosine plays an important role in the pathogenesis of hepatic fibrosis. In initial studies, we observed that two known hepatotoxins, ethanol and methotrexate, stimulate increased adenosine release from cultured hepatoma (HepG2) cells. Occupancy of the adenosine A_{2A} receptor stimulates collagen production by hepatic stellate cell lines. Treatment of mice with the hepatotoxins carbon tetrachloride (CCl_4) or thioacetamide at doses that stimulate hepatic fibrosis leads to increased adenosine release from liver slices cultured *ex vivo*, and adenosine A_{2A} receptor expression is upregulated in thioacetamide-treated murine liver homogenates. Unlike otherwise genetically identical wild-type mice, adenosine A_{2A} receptor knockout mice were protected from developing hepatic fibrosis in response to CCl_4 and thioacetamide. Moreover, an adenosine A_{2A} receptor antagonist (ZM241385) and the nonselective adenosine receptor antagonist caffeine, but not selective adenosine A_1 or A_{2B} receptor antagonists, prevented hepatic fibrosis in these animal models.

Methods

Cell cultures and reagents

HepG2 cells were passaged from cells originally obtained from ATCC. Immortalized rat hepatic stellate cells (Vogel *et al*., 2000) and LX-2 human hepatic stellate cells were used in some studies. LX-2 cells are derived from normal primary human stellate cells that have been immortalized by selection in low serum (Xu *et al*., 2005); they express all key markers of activated stellate cells *in vivo*. CGS-21680, 8-cyclopenthyldipropylxanthine (DPCPX), caffeine, 3-propylxanthine (enprofylline), 8-(3-chlorostyryl)-caffeine (CSC), collagenase type VII (C2799), CCl_4, thioacetamide, ascorbic acid, β-aminoproprionitrile and Cremophor EL were purchased from Sigma (St Louis, MO, U.S.A.); ZM-241385 from Tocris (Ballwin, MO, U.S.A.) and ^{14}C-labelled proline from Moravek Biochemicals (Brea, CA, U.S.A.).

Adenosine extraction and quantification by HPLC

Extraction and quantification of adenosine were conducted as previously described (Cronstein *et al*., 1993).

Quantification of collagen production by ^{14}C-proline incorporation

Collagen was measured by modification of the method previously described by Mauviel *et al*. (1991). Rat hepatic stellate cells and LX-2 cells were grown to near confluence before treatment. Cells were treated with ascorbic acid ($50\,\mu g\,ml^{-1}$, 8 h) and then pulsed with ^{14}C-labelled proline (Moravek Biochem., Brea, CA, U.S.A.) to which β-aminoproprionitrile was added ($50\,\mu g\,ml^{-1}$) for 16 h. Adenosine receptor agonist was incubated with cells (37°C, 5%CO_2, 16–24 h) with or without the addition of adenosine receptor antagonists (CSC, DPCPX, enprofylline, 10 μM each, three wells per condition) (Desai *et al*., 2005). Supernates were collected following treatment and collagen extracted by the addition of ethanol (3 : 1 v : v) and the precipitate redissolved in 1% SDS following centrifugation at $6000 \times g$ for 5 min and electrophoresed on 7% polyacrylamide gel. Collagen was identified as a high molecular weight ($\sim$220 kDa), collagenase-sensitive protein visible on SDS–PAGE (Figure 2c). Radioactivity was quantified following phosphorimager exposure (10 and 21 days for rat hepatic stellate cells and LX-2 cells, respectively) using ImageQuant software v.5.0 (Figure 2a) and band intensity determined using Kodak 1D software v. 2.0.1, adjusted to relative protein density on Coomassie blue-stained gels.

MMP-14 protein level determination by Western blotting

MMP-14 expression was determined semiquantitatively by Western blot. LX-2 cells were treated in DMEM media (Gibco, Carlsbad, CA, U.S.A.) with or without CGS-21680. After 24 h treatment, whole-cell lysates were collected using lysis buffer, then fractionated on 10% SDS–PAGE and transferred onto a nitrocellulose membrane (Bio Rad, Hercules, CA, U.S.A.). Protein samples ($20\,\mu g\,lane^{-1}$) were mixed with 5 $\times$ loading buffer (50 mM Tris-HCl, pH 6.8; 2% SDS; 0.1% bromophenol blue; 10% glycerol) and 2-mercaptoethanol (96 mM) and heated for 5 min. The nitrocellulose membrane was blocked for 2 h at 4°C in blocking solution (3% BSA in 1 $\times$ Tween 20 Tris-buffered saline (TTBS) (TTBS consists of: 20 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween 20), then immersed in blocking solution containing a 1 : 5000 dilution of primary antibody for anti-MMP-14 (Chemicon, Temecula, CA, U.S.A.) and incubated for 1 h at room temperature. After incubation with alkaline phosphatase-labeled anti-rabbit secondary antibody (Santa Cruz, Santa Cruz, CA, U.S.A.), proteins were visualized using the ECF kit (Amersham Biosciences, Piscataway, NJ, U.S.A.) and quantitated with ImageQuant intensity software program. MMP-14 bands were normalized to β-actin.

Determination of metalloproteinase activity by gelatin zymography

Supernates were fractionated on a 10% SDS–PAGE with 10% gelatin (Bio Rad, U.S.A.). Protein samples ($15\,\mu l\,lane^{-1}$) were mixed with double volume of 1 $\times$ zymogram sample buffer (Bio Rad, U.S.A.). The gel was incubated with renaturation buffer (Bio Rad, U.S.A.) for 30 min at room temperature, then incubated in developing buffer (Bio Rad, U.S.A.) overnight at 37°C with gentle agitation and stained with 0.5% Coomassie

Blue in 40% methanol and 10% acetic acid for 1 h at room temperature and destained. MMP controls were obtained from Sigma, U.S.A.

Reverse transcription–polymerase chain reaction (RT–PCR) and real-time quantitative PCR analyses for metalloproteinases

Total mRNA was isolated from LX-2 cells at approximately 80% confluence in T-25 flasks using Micro-FastTrack (Invitrogen, Carlsbad, CA, U.S.A.) following treatment with adenosine A_{2A} receptor agonist, MRE94 (4 h, 5% CO_2, 37°C, 1 μM). Reverse transcription (RT) was performed using the GeneAmp RNA Core Kit (Applied Biosystems, Branchburg, NJ, U.S.A.) in a volume of 50 μl using oligo dT primers and MuLV reverse transcriptase according to the protocol of the manufacturer. Real-time quantitative PCR analyses were performed with SYBR® green and a Cepheid Smart Cycler (Sunnyvale, CA, U.S.A.) to measure mRNA levels of MMP-14. Aliquots (4 μl) of RT products were subjected to PCR in 25 μl reactions with SYBR® green (PE) using primers 5′-ggctacagcaatatggctac-3′ (forward), 5′-ttgccatttgagaccctgga-3′ (reverse). The reaction was run in the Smart Cycler at an initial 95°C for 300 s and then at 95°C for 60 s and 58°C for 45 s and 72°C for 45 s for 35 cycles. The optic signal was recorded at the end of every 72°C extension step. Initial real-time PCR amplifications were examined by agarose gel electrophoresis to verify that the primer pairs amplified a single product of the predicted size, and the identities of the products were confirmed by sequencing. GAPDH RNA levels were measured as controls for each RT reaction. The real-time PCR data were analyzed with the Smart Cycler® software (version 1.2b) to calculate the threshold cycle values for the different samples and are presented as mRNA copy number normalized to GAPDH.

RT and real-time PCR for collagen

Total RNA from treated LX-2 cells was isolated using Trizol (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's protocol. RT was performed using the GeneAmp RNA Core Kit (Applied Biosystems, Branchburg, NJ, U.S.A.) in a volume of 50 μl using oligo dT primers and MuLV reverse transcriptase according to the manufacturer's protocol. Real-time PCRs were performed using the SYBR Green PCR Kit (Applied Biosystems, Foster City, CA, U.S.A.) following the manufacturer's instructions and carried out on the Mx3005P™ Q-PCR system (Stratagene, La Jolla, CA, U.S.A.). Aliquots of RT reactions were subjected to PCR in 25 μl reactions with SYBR® green (PE) using primers for Col1a1 (5′-tgttcagctttgtg gacctccg-3′ (forward), 5′-ccgttctgtacgcaggtgattg-3′ (reverse)), Col3a1 (5′-gaagatgtccttgatgtgc-3′ (forward), 5′-agccttgcgtgttcg atat-3′ (reverse)), MMP-9 (5′- gaagatgctgctgttcagc-3′ (forward), 5′-gaagacgtcgtgcgtgtc-3′ (reverse)) and GAPDH [5′-acc atcatccctgcctctac-3′ (forward) and 5′-cctgttgctgtagccaaat-3′ (reverse)). The thermal cycling conditions included an initial 95°C for 300 s; then 95°C for 60 s, 58°C for 45 s and 72°C for 45 s for 40 cycles. For each assay, standards, a no-template and no-RT controls were included to verify the quality and cDNA specificity of the primers. The initial number of copies for each template was calculated by Mx3005P software and was normalized to GAPDH.

Semiquantitative RT–PCR for adenosine A_{2A} receptors

Total RNA was isolated from frozen fibrotic and non-fibrotic livers using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). Mouse brain RNA was extracted as positive control for the A_{2A} receptor. Single-stranded complementary DNA (cDNA) was synthesized and subjected to the PCR. The synthesized cDNA was amplified using specific primers for A_{2A} receptor (forward: 5′-agcaacctgcagaacgtcac-3′ and reverse: 5′-aatgacagcacccagcaaat-3′) and GAPDH as the housekeeping gene (forward: 5′-ctacagtgaggaccaggttgtct-3′ and reverse: 5′-ggtctgggatggaaattgtg-3′). The PCR was performed in a GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer, Branchburg, NJ, U.S.A.) under the following conditions: 94°C for 5 min followed by 33 cycles (94°C for 45 s, 60°C for 1 min and 72°C for 1 min) and a final extension of 72°C for 7 min. PCR product was separated by electrophoresis on 2% agarose gel containing ethidium bromide (0.15 μg ml^{-1}), visualized with a UV transilluminator, and digitally photographed. The amplicon was quantitated densitometrically using Kodak Digital Science software.

In vivo *induction of hepatic fibrosis in adenosine A_{2A} receptor- or A_3 receptor-deficient mice*

Adenosine A_{2A} receptor-deficient mice (Chen *et al*., 1999) or A_3 receptor-deficient mice (Lee *et al*., 2002) and their respective wild-type littermate controls were treated with either of the known hepatic fibrosis-inducing agents, thioacetamide (100 mg kg^{-1} in PBS, intraperitoneally, three times weekly for 9 weeks) or CCl_4 (0.05 ml in corn oil, 50 : 50 v : v, subcutaneously, twice weekly for 6 weeks) ($n = 15$ per group). Animals were killed by CO_2 narcosis at the end of the treatment periods. Hepatic sections (five cross-sections per liver) were harvested and stained with picrosirius red as previously described (James *et al*., 1990) or hematoxylin and eosin (H&E). Digitized photomicrographs (entire cross-sections at ×10 magnification, five sections per liver) were quantitated for total area of red staining using SigmaScan Pro software v.5.0.0 (SPSS) and fibrosis was calculated as a percentage of total hepatic area and expressed as the average of five randomly selected tissue sections from each liver. Assessment of hepatic inflammation was performed histologically on H&E-stained sections by a qualified histopathologist in a blinded fashion using a modified Knodell scoring system (Knodell *et al*., 1981; Ishak *et al*., 1995). Sections were scored for zonal necrosis, confluent necrosis, lobular inflammation and portal inflammation (0–3 for each) and a composite score was calculated for each section (maximum score = 12). These studies were approved by Institutional Animal Care and Use Committee of NYU School of Medicine.

In vivo *administration of adenosine receptor antagonists*

C57BL/6 mice were treated with either of the known hepatic fibrosis-inducing agents CCl_4 (0.05 ml in oil, 50 : 50 v : v, subcutaneously, twice weekly for 6 weeks) or thioacetamide (100 mg kg^{-1} in PBS, intraperitoneally, three times weekly for 7 weeks). Treatment with the orally bioavailable adenosine receptor antagonists DPCPX (A_1 receptor, 50 mg kg^{-1} day^{-1} orally) (Andersson *et al*., 2000), enprofylline (A_{2B} receptor, 50 mg kg^{-1} day^{-1} orally) (Itoh *et al*., 1998) and caffeine

(nonselective, 50 mg kg^{-1} day^{-1} orally) were administered in the drinking water. ZM-241385 is an A_{2A} receptor antagonist that is not orally bioavailable and was therefore administered intraperitoneally (25 mg kg^{-1} twice daily in a vehicle consisting of 15% Cremophor EL, 15% DMSO, 70% water). To achieve steady-state levels, all of the agents were given throughout the period of fibrosis induction and commenced 3 days before the first injection of either CCl_4 or thioacetamide. All mice also received intraperitoneal injections of vehicle. Animals were killed by CO_2 narcosis at the end of the treatment periods. Serum AST, ALT and alkaline phosphatase were assessed in all of the different treatment groups tested after CCl_4 or thioacetamide ingestion. No significant differences were observed among experimental groups with the exception of CCl_4-treated, A_{2A} receptor-deficient mice and mice treated with the A_{2A} receptor antagonist ZM-241385, both of which showed significantly increased AST and ALT values (Tables 1 and 2), consistent with previous observations by Ohta & Sitkovsky (2001). Hepatic sections were harvested and stained with picrosirius red. Digitized photomicrographs ($\times 10$ magnification) were quantitated blindly and modified Knodell scoring was performed as described above. These studies were approved by the Institutional Animal Care and Use Committee of NYU School of Medicine.

Table 1 Hepatic transaminases following thioacetamide administration

	n	*AST*	*ALT*	*AP*
$A_{2A}+/+$	6	216±52	93±10	69±19
$A_{2A}-/-$	9	184±69	211±50	20±6
$A_{2A}+/+$ + TAA	9	180±18	97±4	117±11
$A_{2A}-/-$ + TAA	11	252±80	206±59	64±14
Control + TAA	5	159±73	82±23	32±9
Caffeine + TAA	4	183±91	161±103	49±15
Enprofylline + TAA	5	458±211	191±107	68±43
ZM-241385 + TAA	5	259±137	189±6	37±12

Serum AST, ALT and alkaline phosphatase (AP) values of experimental animals following treatment with vehicle or thioacetamide (TAA) were determined in serum obtained at killing after treatment with thioacetamide, as described in Methods.

Table 2 Hepatic transaminases following carbon tetrachloride (CCl_4) administration

	n	*AST*	*ALT*	*AP*
$A_{2A}+/+$	5	708±191	48±23	36±15
$A_{2A}-/-$	14	129±22	37±9	163±45
$A_{2A}+/+$ + CCl_4	6	380±81	130±25	90±24
$A_{2A}-/-$ + CCl_4	21	1178±449*	308±78*	200±36
Control + CCl_4	5	344±60	349±72	156±19
Caffeine + CCl_4	5	309±49	431±122	40±22
Enprofylline + CCl_4	4	181±20	142±12	133±48
ZM-241385 + CCl_4	5	569±76**	870±177**	45±19

Serum AST, ALT and alkaline phosphatase (AP) values of experimental animals following treatment with vehicle or CCl_4 were determined in serum obtained at killing after treatment with CCl_4, as described in Methods.
*$P<0.05$ vs $A_{2A}-/-$ treated with vehicle or $A_{2A}+/+$ mice treated with CCl_4, two-way analysis of variance with Dunn's *post hoc* analysis.
**$P<0.001$, vs control mice, two-way analysis of variance with Dunn's *post hoc* analysis.

Quantification of hepatic hydroxyproline content

Tissue specimens were dried and hydrolyzed in 6 N HCl at 110°C for 24 h, and hydroxyproline content in liver specimens was measured colorimetrically as described previously (Stegemann & Stalder, 1967). Results were expressed as μg of hydroxyproline per mg of tissue. We verified the accuracy of our digitized picrosirius measurements by comparison with hepatic hydroxyproline content. Following administration of vehicle or thioacetamide to mice for 5 weeks, livers were harvested, bisected and five cross-sections through half of the liver were processed for histology and picrosirius red staining. Digital measurements of picrosirius red-stained collagen were made in cross-sections through five different sites, as above, and the mean picrosirius-stained collagen per liver was compared with the hydroxyproline content of the other half of the liver. These measures correlated extremely well ($r=0.987$, $n=13$, $P<0.0001$) confirming previous observations (James *et al*., 1990).

Toxin-induced hepatic release of adenosine in mice

C57BL/6 mice were treated with single doses of CCl_4 (0.05 ml in oil, 50 : 50 v : v, subcutaneously) or thioacetamide (100 mg kg^{-1} in PBS, intraperitoneally) and control mice were given vehicle alone. Animals were killed after 24 h and their livers harvested. Liver slices were incubated in growth medium (DMEM/10%FBS) overnight and adenosine concentrations in culture supernates were determined by HPLC as described above. Results were normalized to 2-chloro-adenosine standards and liver weights.

Measurements of ALT, AST and alkaline phosphatase

Whole blood was taken from mice at the time of killing (4 days after final dose following 6 weeks' treatment for CCl_4, 2 days after final dose following 7 weeks' treatment for thioacetamide) and the serum isolated and analyzed by the Clinical Laboratory of Bellevue Hospital.

Statistics

Data were analyzed by means of appropriate level of ANOVA and significance of differences between groups was determined by appropriate *post hoc* analysis. Comparison of digitized picrosirius red quantification of hepatic fibrosis and hepatic hydroxyproline content was made using Pearson's correlation coefficient. All statistical analyses were performed with SigmaStat software v. 2.03 (SSPS).

Results

Hepatocytes release adenosine following stimulation by methotrexate or ethanol

Methotrexate and ethanol are two hepatotoxins that may cause cirrhosis (Tobias & Auerbach, 1973; de la Monte *et al*., 1984). As they can both promote the release of adenosine (Nagy *et al*., 1990; Cronstein *et al*., 1991; Morabito *et al*., 1998), we investigated whether hepatocytes, the major target of most chronic liver injuries, generate adenosine in response

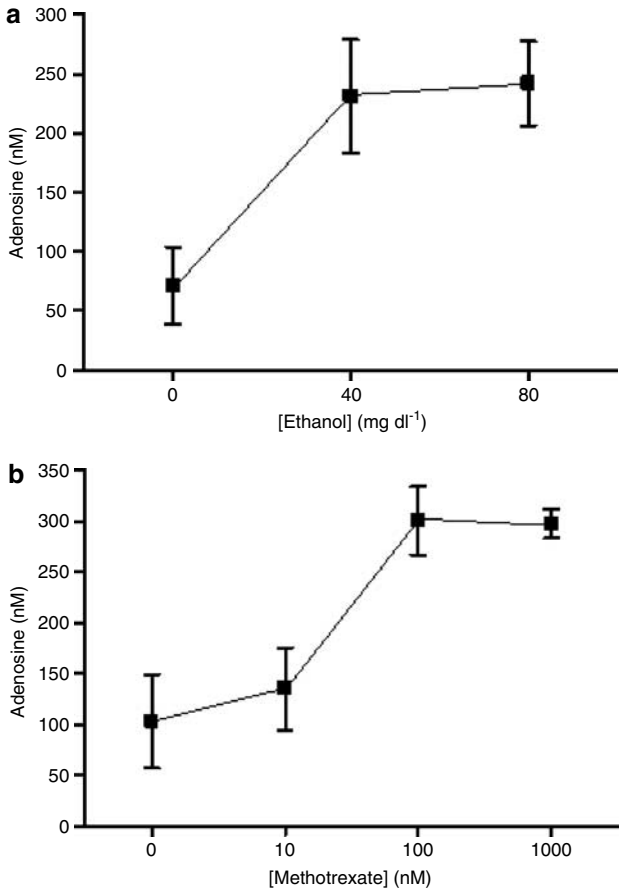

Figure 1 Hepatotoxins stimulate adenosine release from the hepatoma cell line HepG2. (a) Ethanol treatment of HepG2 cells for 3 h stimulated adenosine release into the supernate as measured by HPLC. At an ethanol concentration of 40 mg dl^{-1}, mean adenosine concentration rose from a control value of 71.5±32.6 to 231±47.6 nM, and this increase was sustained at an ethanol concentration of 80 mg dl^{-1} (242±35.7 nM) (one-way ANOVA, $n=4$, $P=0.003$). (b) Methotrexate treatment of HepG2 cells for 72 h stimulated extracellular adenosine release as measured by HPLC. Methotrexate, at a concentration of 10 nM, increased HepG2 cell adenosine release by three-fold (from 103±46.1 to 301±33.8 nM) and the increase in adenosine release was maintained at a methotrexate concentration of 100 nM (298±14.2 nM, one-way ANOVA, $n=4$, $P=0.023$).

to these agents. In HepG2 cells, a human hepatoma cell line, adenosine release into cell culture supernatants was quantitated following incubation with pharmacologically relevant concentrations of either ethanol or methotrexate (Figure 1). Adenosine concentrations in the supernatants of HepG2 cultures increased more than three-fold following 3-h incubation with ethanol (e.g. from 71.5±32.6 to 231±47.6 nM at 40 mg dl^{-1} of ethanol). Methotrexate, in pharmacologically relevant concentrations, also stimulated the release of adenosine into supernates of HepG2 cell cultures following incubation for 72 h (Figure 1b). Methotrexate, at a concentration of 100 nM, increased HepG2 cell adenosine release by three-fold (from 103±46.1 to 301±33.8 nM) and the increase in adenosine release was maintained at a methotrexate concentration of 1 μM (298±14.2 nM, one-way ANOVA, $n=4$, $P=0.023$).

Adenosine A_{2A} receptor stimulates collagen production by hepatic stellate cells

Hepatic stellate cells are the principal fibrogenic cell type in the liver (Li & Friedman, 1999; Friedman, 2000). To determine whether adenosine release could contribute to hepatic fibrosis, we studied the effects of adenosine receptor agonists and antagonists on collagen production by hepatic stellate cell lines. Prior studies have demonstrated that the hepatic stellate cell lines studied here are already activated and express α-smooth muscle actin and other activation markers (Reeves & Friedman, 2002; Taimr *et al*., 2003). We confirmed that LX-2 cells express α-smooth muscle actin and further found that treatment with adenosine receptor agonists did not alter the level of α-smooth muscle actin expression (data not shown). The adenosine A_{2A} receptor agonist, CGS-21680, increased collagen production by a rat hepatic stellate cell line (rHSC) (Vogel *et al*., 2000) in a dose-dependent fashion (Figure 2b), with identical results obtained on the human LX-2 stellate cell line (Figure 2a). Maximal increase in collagen release was seen with the highest concentration of CGS-21680 applied (10 μM), where collagen production increased by 20-fold above baseline (2045±470% control value). The increase in rHSC collagen production was almost completely abrogated by the addition of the adenosine A_{2A} receptor antagonist, CSC (10 μM) (Figure 2b). At a CGS-21680 concentration of 10 μM, preincubation of rHSC with CSC (10 μM) diminished CGS-21680-induced collagen production by 86% (from 2045±470 to 271±83% of control, $P<0.001$, $n=9$, two-way ANOVA). Blockade of the adenosine A_1 receptor by DPCPX (10 μM) or the A_{2B} receptor by enprofylline (10 μM) only minimally suppressed collagen release ($P=$NS) (Figure 2b). Message for Col1a1 but not Col3a1 was also increased by CGS-21680 treatment (24 h, 10 μM, 37°C), in part explaining the increase in total collagen seen (data not shown). Thus, the adenosine A_{2A} receptor stimulates collagen production by hepatic stellate cells, consistent with its known effects on dermal matrix formation during wound healing.

Adenosine A_{2A} receptor ligation suppresses metalloproteinase expression by hepatic stellate cells

To further determine the mechanism(s) by which adenosine A_{2A} receptors increase collagen production, we examined the expression and activity of metalloproteinases by LX-2 cells following incubation with CGS-21680. By gelatin zymography, CGS-21680 suppressed MMP-9 activity in a dose-dependent manner and at peak concentration (10 μM), MMP-9 activity was reduced by 66±13% ($n=3$, $P<0.001$) (Figure 2d), with a similar decrease in MMP-9 message (data not shown). In contrast, MMP-2 activity was unaltered by incubation with CGS-21680 at concentrations of up to 1 μM, but at a concentration of 10 μM, CGS-21680 inhibited MMP-2 activity by 53±8% ($n=3$, $P<0.001$) (Figure 2d). Finally, incubation of LX-2 cells with CGS-21680 suppressed MMP-14 (membrane-type matrix metalloprotease 1, MT-MMP-1) expression, as determined by Western blotting, in a dose-dependent manner. At a concentration of 10 μM, CGS-21680 suppressed MMP-14 expression by 48±12% ($n=4$, $P=0.004$) (Figure 2e). A 24 h treatment of LX-2 cells using another A_{2A} receptor agonist, MRE0094 (1 μM (Victor-Vega *et al*., 2002)) similarly suppressed MMP-14 mRNA expression by real-time PCR

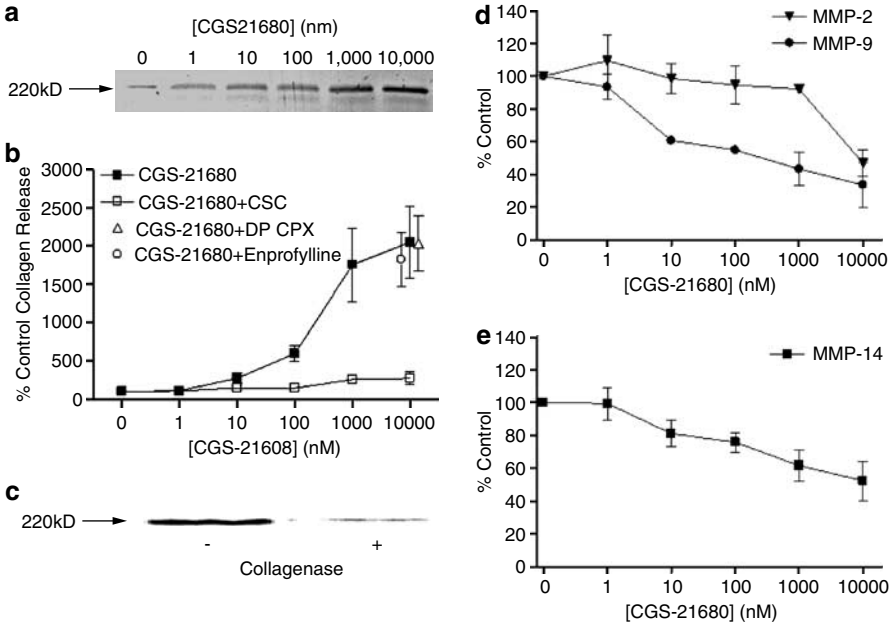

Figure 2 Adenosine A_{2A} receptor occupancy stimulates collagen production by hepatic stellate cells. (a) Phosphorimager detection of high-molecular weight ^{14}C band identified as collagen in supernates of LX-2 cells (human hepatic stellate cell line). (b) Adenosine A_{2A} receptor agonist, CGS-21680, promotes collagen production by rat hepatic stellate cells. Stellate cell lines were treated sequentially with ascorbic acid (50 $\mu g\,ml^{-1}$), β-aminoproprionitrile (50 $\mu g\,ml^{-1}$), ^{14}C-proline (2 $\mu Ci\,ml^{-1}$), CGS-21680 (16 or 24 h for rat or human hepatic cell lines, respectively) in the presence or absence of the adenosine A_{2A} receptor antagonist, CSC (10 μM). Maximal inhibition of the effect of CGS-21680 on collagen production was 86% (from 2045 ± 470 to 271 ± 83% control value, two-way ANOVA, $n=9$, $P<0.001$). The suppressive effect of CSC on CGS-21680-induced collagen production was not seen with the adenosine A_1 receptor antagonist, DPCPX (10 μM), or A_{2B} receptor antagonist, enprofylline (10 μM). Percentage control values for collagen production with CGS-21680 alone vs CGS-21680 with DPCPX vs CGS-21680 with enprofylline were 2045 ± 470 vs 2033 ± 364 vs 1820 ± 351%, respectively (NS, one-way ANOVA, $n=9$, 6, 5, respectively, for CGS-21680 alone vs CGS-21680 with DPCPX vs CGS-21680 with enprofylline, $P=0.93$). (c) Collagen was identified as a high-molecular-weight protein (>220 kDa) that was cleavable by collagenase, and measured by phosphoimager quantification of ^{14}C after adjustment to relative density of protein in Coomassie Blue-stained gels. (d) CGS-21680 treatment of LX-2 cells for 24 h modulated activities of MMP-9 and MMP-2 by gelatin zymography. (e) CGS-21680 treatment of LX-2 cells for 24 h modulated expression of MMP-14 by Western blotting.

by 31 ± 17% ($n=3$, $P<0.001$). These data suggest that A_{2A} receptor agonists promote collagen production by hepatic stellate cells at least in part by suppressing metalloprotease activity and diminishing collagen degradation.

Adenosine A_{2A} receptor expression is increased in cirrhotic murine livers

To investigate whether adenosine A_{2A} receptor expression is altered in cirrhosis, we studied the expression of adenosine A_{2A} receptors by RT–PCR in whole murine liver homogenates (Figure 3a). Adenosine A_{2A} receptor expression was increased by 3.5-fold in thioacetamide-induced cirrhotic murine liver compared with normal liver ($n=2$) (Figure 3b, normalized to GAPDH).

Thioacetamide or CCl_4 treatment induces hepatic adenosine release ex vivo

Models of hepatic fibrosis in mice involve administration of toxins such as thioacetamide and CCl_4, which may, like other cellular insults, promote adenosine release. We therefore determined whether treatment of mice with thioacetamide or CCl_4 at doses that lead to hepatic fibrosis might promote adenosine release from *ex vivo* cultured murine liver slices harvested after treatment of mice with these hepatotoxins. Treatment of the mice with a single dose of either thioacet-

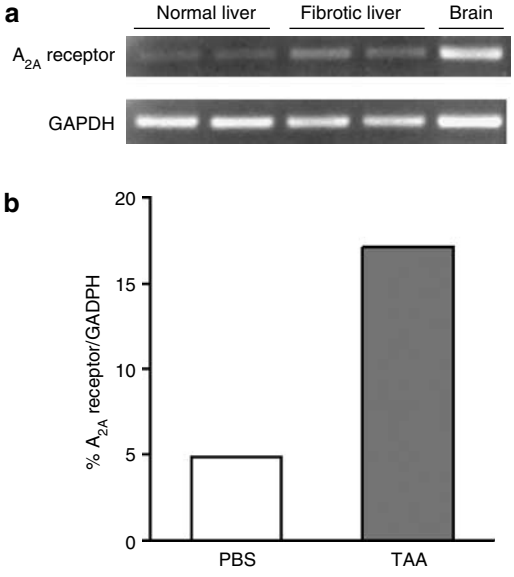

Figure 3 There is increased expression of adenosine A_{2A} receptors in fibrotic liver. (a) Expression of adenosine A_{2A} receptor was increased in homogenates from fibrotic murine livers compared with normal murine livers by RT–PCR. Expression in murine brain tissue was shown as a positive control. (b) Expression of adenosine A_{2A} receptor normalized to GAPDH was increased in liver homogenates from thioacetamide-treated animals ($n=2$ animals each for control and fibrotic mice).

amide or CCl_4 led to increased adenosine concentrations in supernates of their cultured liver slices (Figure 4). Although the concentration of adenosine in the supernates of liver slices from thioacetamide- and CCl_4-treated mice differed significantly from that of supernates of control livers ($P<0.002$ for each), they did not differ significantly from each other. The observed increases in adenosine concentration are within the range over which adenosine A_{2A} receptors are optimally activated.

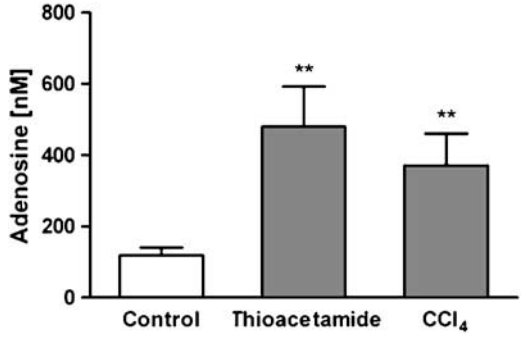

Figure 4 Agents that promote hepatic fibrosis increase hepatic adenosine release. *Ex vivo* treatment of murine liver slices with thioacetamide or CCl_4 significantly increased the release of adenosine into supernate (from 119 ± 21 to 480 ± 113 or 371 ± 89 nM adenosine, control vs thioacetamide vs CCl_4, $n=11$, 6 and 5, respectively, $^{**}P<0.001$, ANOVA).

Adenosine A_{2A} receptor-deficient mice are resistant to thioacetamide-induced hepatic fibrosis

To determine whether the *in vitro* effects of A_{2A} receptor ligation on collagen production are relevant to the development of hepatic fibrosis, we examined toxin-induced hepatic fibrosis/cirrhosis due to thioacetamide in adenosine A_{2A} receptor-deficient mice and their otherwise genetically identical wild-type littermate controls, as well as adenosine A_3 receptor-deficient mice. Severe hepatic fibrosis/cirrhosis developed in wild-type mice as well as the adenosine A_3 receptor-deficient mice treated with thioacetamide. In contrast, animals lacking adenosine A_{2A} receptors were protected from the development of hepatic fibrosis (Figure 5). There were modest elevations in

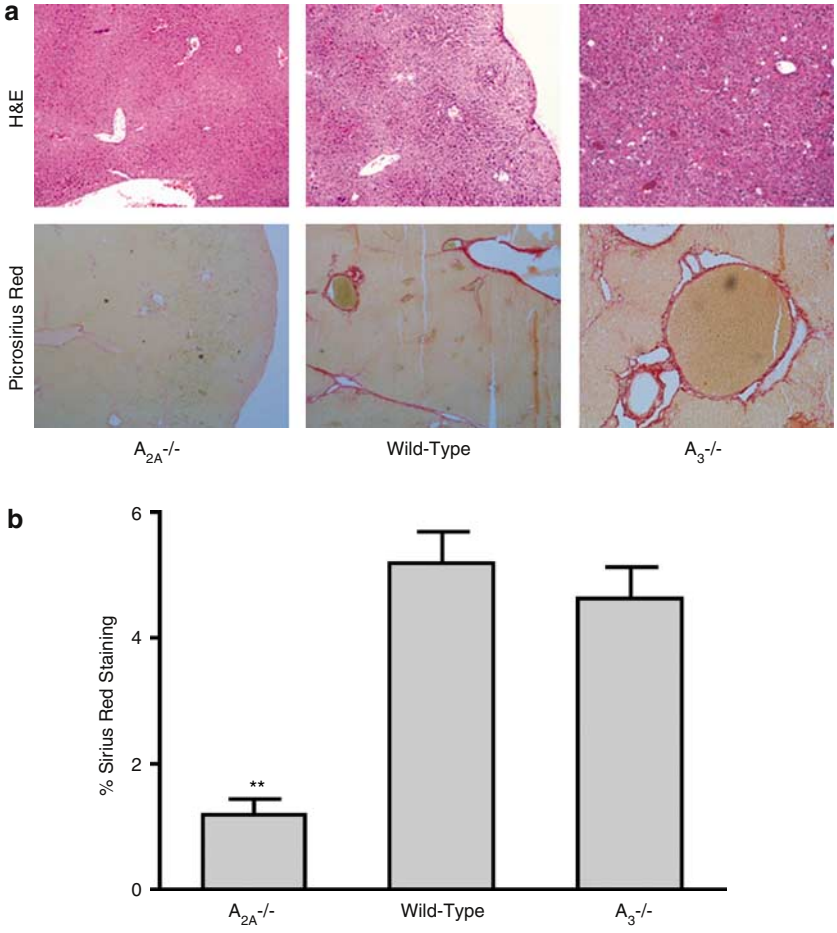

Figure 5 Adenosine A_{2A} receptor-deficient mice are protected from CCl_4-induced hepatic fibrosis. (a) Adenosine A_{2A} receptor- or A_3 receptor-deficient mice were treated with the hepatic toxin CCl_4 (0.05 ml in oil, 50 : 50 v : v, subcutaneously, twice weekly for 6 weeks). Hepatic sections were stained with picrosirius red and H&E. (b) Quantification of picrosirius red staining was performed digitally using SigmaScan Pro v.5.0.0, and data are presented as the percentage of total liver area stained by picrosirius red (one-way ANOVA, $P<0.001$). Percentage hepatic area stained with picrosirius red in CCl_4-treated animals were 5.2 ± 1.7 and 4.6 ± 0.7% for wild-type control mice and A_3 receptor-deficient mice, respectively, whereas hepatic slices from A_{2A} receptor-deficient mice showed markedly less fibrosis with 1.2 ± 0.3% picrosirius red staining ($n=28$, 12, 4 for A_{2A} receptor-deficient mice, wild-type controls and A_3 receptor-deficient mice, respectively; one-way ANOVA, $^{**}P<0.001$).

AST, ALT and alkaline phosphatase in both wild-type and knockout mice (Table 1) and modified Knodell scores were similar for all groups of mice tested (aggregate scores of 3–4 for all groups). These results indicate that adenosine A_{2A} receptor-deficient mice are protected from thioacetamide-induced hepatic fibrosis without any discernible difference in the level of hepatocellular injury or inflammation, as reflected by serum levels of AST, ALT, alkaline phosphatase and Knodell scoring.

Adenosine A_{2A} receptor-deficient mice are resistant to CCl_4-induced hepatic fibrosis

To further elucidate the *in vitro* effects of A_{2A} receptor ligation on hepatic fibrosis, we also studied a mechanistically distinct model of toxin-induced hepatic fibrosis/cirrhosis induced by CCl_4. Severe hepatic fibrosis/cirrhosis developed in wild-type mice as well as adenosine A_3 receptor-deficient mice treated with CCl_4, but animals lacking adenosine A_{2A} receptors were protected from the development of hepatic fibrosis (Figure 6). The extent of hepatic fibrosis that developed in CCl_4-treated animals was similar to that observed with thioacetamide treatment. Despite the absence of fibrosis in the A_{2A} knockout animals, CCl_4 induced greater release of the enzymes AST, ALT and alkaline phosphatase (Table 2). Nonetheless, modified Knodell scores showed no significant differences between groups with respect to necrosis or inflammation (scores for all livers ranged from 3 to 4). These results confirm the finding in thioacetamide-treated mice that loss of adenosine A_{2A} receptors leads to diminished hepatic fibrosis following CCl_4 treatment and that the observed protection from fibrosis was not associated with an obvious decrease in toxin-induced hepatocellular injury.

Adenosine A_{2A} receptor antagonism prevents the development of hepatic fibrosis in vivo

To further characterize the role of adenosine A_{2A} receptors in the development of toxin-induced fibrosis, we assessed the effect of adenosine receptor antagonists on the development of hepatic fibrosis. C57BL/6 mice were treated with thioacetamide or CCl_4 in the presence or absence of the selective

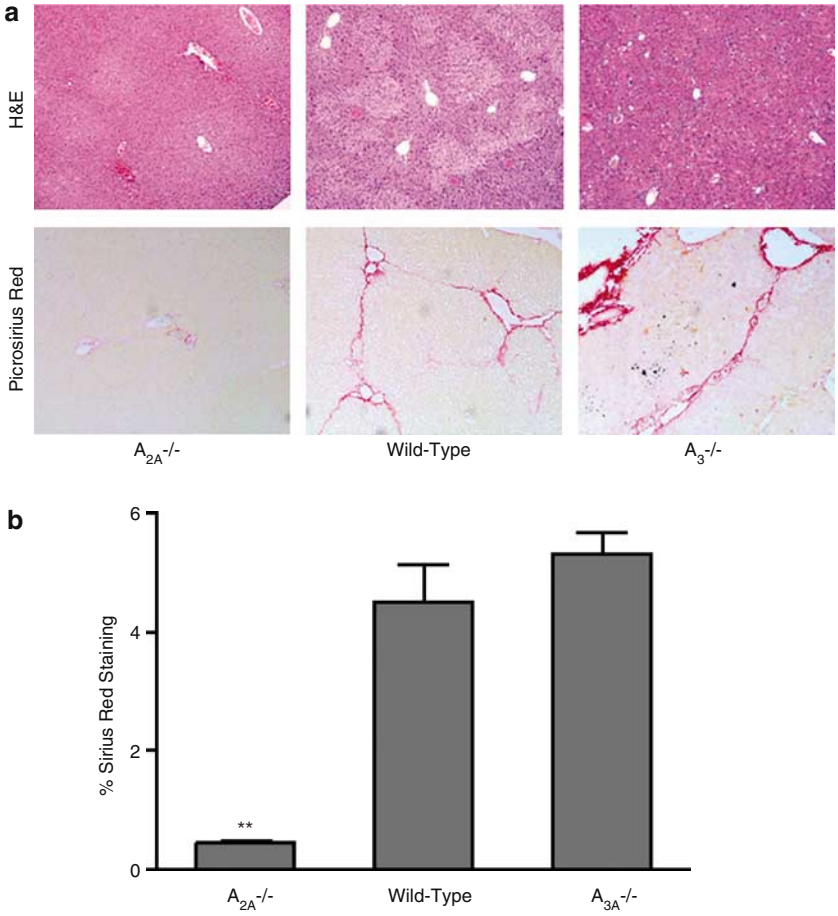

Figure 6 Adenosine A_{2A} receptor-deficient mice are protected from thioacetamide-induced hepatic fibrosis. (a) Adenosine A_{2A} receptor- or A_3 receptor-deficient mice were treated with thioacetamide (100 mg kg^{-1}, intraperitoneally, three times weekly for 9 weeks). Hepatic sections were stained with picrosirius red. (b) Quantification of picrosirius red staining was performed digitally using SigmaScan Pro v.5.0.0, and data are presented as the percentage of total liver area stained by picrosirius red. Percentage hepatic area stained with picrosirius red in thioacetamide-treated animals were 9.0 ± 2.7 and $10.7 \pm 2.8\%$ for wild-type control mice and A_3 receptor-deficient mice, respectively, whereas hepatic slices from A_{2A} receptor-deficient mice showed markedly less fibrosis with $0.9 \pm 0.4\%$ picrosirius red staining ($n = 28$, 12, 4 for A_{2A} receptor-deficient mice, wild-type controls and A_3 receptor-deficient mice, respectively; one-way ANOVA, $^{**}P < 0.001$).

adenosine receptor antagonists DPCPX (A_1 receptor), enprofylline (A_{2B} receptor), ZM-241385 (A_{2A} receptor) or vehicle. Neither the A_1-selective antagonist (DPCPX) nor the A_{2B} receptor antagonist enprofylline prevented the development of hepatic fibrosis in response to thioacetamide or CCl_4. In contrast, the A_{2A} receptor-specific antagonist, ZM-241385, almost completely blocked the development of fibrosis following treatment with thioacetamide or CCl_4 (Figure 7). Epidemiological studies suggest that coffee consumption protects against the development of hepatic fibrosis in human populations although the mechanism for this protection has not previously been elucidated (Klatsky *et al*., 1993; Corrao *et al*., 1994; 2001; Sharp *et al*., 1999; Gallus *et al*., 2002; Ruhl & Everhart, 2005). As caffeine is present in pharmacologically relevant concentrations in coffee and caffeine's best-documented pharmacological action is as a nonselective adenosine receptor antagonist, we determined whether caffeine prevents the development of hepatic fibrosis in the two murine models of hepatic injury and fibrosis. Caffeine conferred substantial protection against thioacetamide- or CCl_4-induced hepatic fibrosis, although protection was not as great as observed with ZM-241385 in either model. Of all of the agents tested, only the adenosine A_{2A} receptor antagonist ZM-241385 increased serum levels of ALT and AST in the CCl_4-treated animals (but not the thioacetamide-treated animals, Tables 1 and 2).

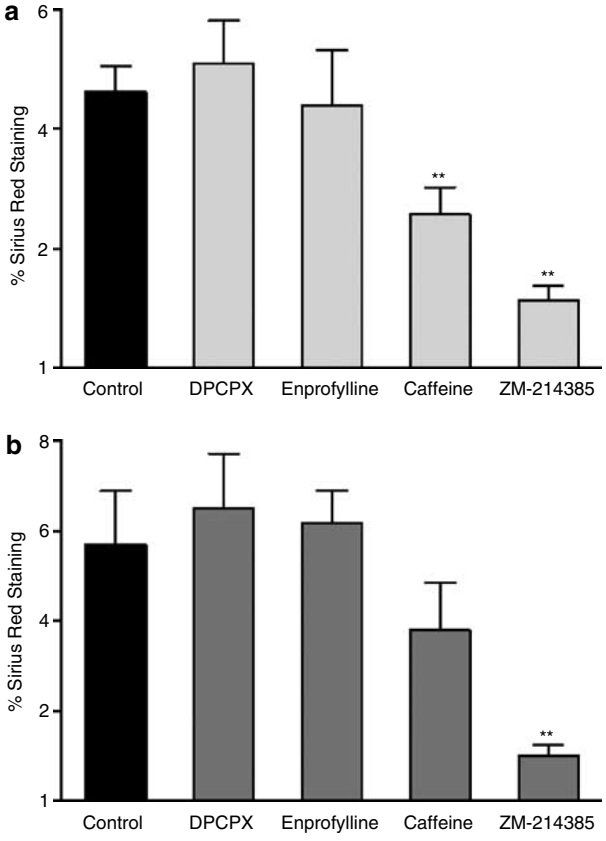

Figure 7 Adenosine A_{2A} receptor antagonism protects mice from chemical-induced hepatic fibrosis. C57BL/6 mice were treated with the hepatic toxins CCl_4 (0.05 ml in oil, 50 : 50 v : v, subcutaneously, twice weekly for 6 weeks) or thioacetamide (100 mg kg^{-1}, intraperitoneally, three times weekly for 7 weeks) in the presence or absence of adenosine receptor antagonists DPCPX (A_1 receptor, 50 mg kg^{-1} day^{-1} orally), enprofylline (A_{2B} receptor, 50 mg kg^{-1} day^{-1} orally), caffeine (nonselective, 50 mg kg^{-1} day^{-1} orally) or ZM-241385 (A_{2A} receptor, 25 mg kg^{-1} twice daily intraperitoneally in vehicle consisting of 15% Cremophor EL, 15% DMSO, 70% water. All mice also received intraperitoneal injections of vehicle in addition to any other treatments received). Hepatic sections were stained with picrosirius red. Quantification of picrosirius red staining was performed digitally. (a) Percentage area of picrosirius red-stained hepatic tissue in CCl_4-treated mice were 4.6 ± 0.5, 5.1 ± 0.7, 4.4 ± 0.9, 2.6 ± 0.4 and 1.2 ± 0.2% for control, DPCPX, enprofylline, caffeine and ZM-241385-treated mice, respectively ($n = 12$, 8, 8, 16, 8 for control, DPCPX, enprofylline, caffeine and ZM-241385-treated mice, respectively, one-way ANOVA, $P < 0.001$). (b) Percentage area of picrosirius red-stained hepatic tissue in thioacetamide-treated mice were 5.7 ± 1.2, 6.5 ± 1.2, 6.2 ± 0.7, 3.8 ± 1.1 and 1.0 ± 0.2% for control, DPCPX, enprofylline, caffeine and ZM-241385-treated mice, respectively ($n = 11$, 5, 9, 12, 13 for control, DPCPX, enprofylline, caffeine and ZM-241385-treated mice, respectively, one-way ANOVA, $^{**}P < 0.001$).

Discussion

The results reported here demonstrate that endogenously released adenosine, acting at A_{2A} receptors, plays an important role in the pathogenesis of hepatic fibrosis in response to hepatotoxins. First, the livers of hepatotoxin-treated mice release significantly more adenosine into the extracellular milieu, a finding mirrored *in vitro* by a toxin-treated hepatoma cell line. Secondly, mice lacking adenosine A_{2A} receptors are protected from developing hepatic fibrosis in two different hepatic fibrosis models despite suffering similar hepatocellular injury as their otherwise genetically identical wild-type littermates or A_3 receptor knockouts. Thirdly, selective adenosine A_{2A}, but not A_1 or A_{2B}, receptor antagonists, prevent hepatic fibrosis from developing in mice treated with hepatotoxins. The capacity of extracellular adenosine to induce fibrosis *in vivo* appears to correlate with A_{2A} receptor-mediated enhancement of collagen production by LX-2 hepatic stellate cells.

Inflammation, tissue repair and scarring are closely linked events, and strong evidence indicates that endogenously released adenosine, acting at the A_{2A} receptor, suppresses inflammation (Cronstein *et al*., 1983a, b; 1985; 1986; Linden, 2001). In the liver, A_{2A} receptor activation by endogenously released adenosine is responsible for the suppression of acute hepatic inflammation (Ohta & Sitkovsky, 2001) and, as reported here, hepatic fibrosis. Similar to the observations reported here, adenosine A_{2A} receptor occupancy increases matrix formation in healing wounds (Montesinos *et al*., 1997) in the skin and high levels of adenosine in the lungs of mice lacking adenosine deaminase lead to pulmonary fibrosis as well (Blackburn *et al*., 2003), although the adenosine receptor involved in this phenomenon is not yet established.

Adenosine A_{2A} receptors are expressed widely in cells and tissues outside of the central nervous system. In the liver prior studies have demonstrated A_{2A} receptors on Kupffer cells and hepatocytes where they modulate TNF production and hepatic glycogen metabolism, respectively (Reinstein *et al*., 1994; Gonzalez-Benitez *et al*., 2002; Ohta & Sitkovsky, 2001). A_2 receptors, most likely A_{2A} receptors, are also expressed on hepatic stellate cells where they appear to modulate endothelin receptor function (Reinehr *et al*., 2002). Adenosine A_{2A} receptors are present on both venous and microvascular endothelium where they stimulate angiogenesis (Montesinos *et al*., 1997; 2002; Nguyen *et al*., 2003) although it is not known

whether they are expressed on hepatic endothelium. The precise effects of adenosine A_{2A} receptors on hepatocytes, stellate cells and Kupffer cells that contribute to hepatic fibrosis are not known but the prior demonstration that adenosine A_{2A} receptor agonists promote increased matrix formation in healing wounds is consistent with our finding that these receptors play a role in hepatic fibrosis. Nonetheless, it is possible that absence or blockade of A_{2A} receptors diminishes toxic injury to the liver which indirectly contributes to diminished fibrosis. As we were unable to detect any significant differences between either A_{2A} receptor knockout vs wild-type animals, or A_{2A} receptor antagonist-treated vs control animals in their levels of transaminases, or in the necrosis and inflammation components of Knodell scoring, it seems less likely that loss of the A_{2A} receptor confers hepatoprotective effects.

Hernandez-Munoz *et al*. (2001) have observed that intraperitoneal administration of adenosine blocked CCl_4-induced hepatic fibrogenesis in rats. It is doubtful that the observed hepatoprotective effects are in fact due to adenosine itself, administered at a dose of 200 mg kg^{-1} body weight three times weekly, as the half-life of adenosine in blood and other body fluids is measured in seconds (Moser *et al*., 1989). The brief half-life of adenosine in physiologic fluids is due to rapid cellular uptake and to the ubiquitous presence of adenosine deaminase which catalyzes the hydrolysis of adenosine to inosine, a purine which is inactive at adenosine receptors but which may be converted intracellularly to adenine nucleotides following cellular uptake. Indeed, adenosine deaminase levels increase in patients with cirrhosis (Aguado *et al*., 1990; Fernandez *et al*., 2000; Burgess *et al*., 2001). Additionally, there is very rapid uptake of adenosine by erythrocytes and other cells (Moser *et al*., 1989). Hernandez-Munoz hypothesized that the vasodilating properties of adenosine are hepatoprotective by enhancing oxygenation in hepatic tissues rather than by diminishing fibrosis although it is hard to understand how several minutes of vasodilation per week could protect the liver from permanent injury. Alternatively, changes in the hepatocyte redox state, increased intracellular ATP, induced by adenosine treatment may be beneficial and these adenosine-induced metabolic alterations provide a more attractive explanation for the hepatoprotective effects of exogenous adenosine (Hernandez-Munoz *et al*., 1990; 1994).

The fibrogenic effects of hepatic toxins often depend on the generation of harmful intermediates, and in the case of CCl_4, metabolism by a cytochrome *P*450 enzyme isoform (CYP2E1) is a key factor in the development of hepatotoxicity (Wong *et al*., 1998). It is therefore possible that changes in the expression of these enzymes in adenosine A_{2A} receptor-deficient mice may be responsible for some of the observed differences in susceptibility to toxin-induced fibrogenesis. Nonetheless, we did not observe any evidence that either CCl_4 or thioacetamide were any less hepatotoxic in the knockout mice or mice treated with adenosine receptor antagonists. Indeed, adenosine A_{2A} receptor knockout mice had higher levels of AST and ALT than their wild-type controls following CCl_4 but not thioacetamide exposure, consistent with previous findings of Ohta & Sitkovsky (2001) that the adenosine A_{2A} receptor may have a different role in hepatic inflammatory responses than it does in fibrosis. Despite these observations, we cannot be certain that reduction of liver injury in A_{2A} receptor-deficient mice or in A_{2A} receptors antagonist-treated mice is explained by diminution of fibrosis alone and cannot rule out other mechanisms that could contribute to the hepatic preservation. Moreover, the wild-type littermate control mice are genetically identical except for the adenosine receptor that has been deleted and must otherwise share susceptibility to the hepatotoxin with the wild-type littermate control mice.

To date, there has been no direct *in vivo* validation that toxins, including ethanol, increase adenosine levels in the liver. However Puig's observations that alcohol increases purine nucleotide degradation, enhances turnover of the adenine nucleotide pool and increases serum and urinary oxypurines in human subjects are highly suggestive that alcohol ingestion leads to release of at least some purine as adenine nucleotides or adenosine (Puig & Fox, 1984). In addition, studies by Miyamoto & French (1988) on liver extracts from ethanol-fed mice strongly support the notion that chronic ethanol feeding increases adenosine levels in the liver. We have now demonstrated that direct treatment of mice with thioacetamide or CCl_4 released three-fold more adenosine from freshly harvested liver slices compared to control.

Ethanol is a common cause of hepatic fibrosis in Western societies and adenosine, which contributed to the hepatic fibrosis induced experimentally here, mediates many of the central nervous system effects of ethanol ingestion. Adenosine A_{2A} receptor-deficient mice display reduced sensitivity towards both the sedative and hypothermic effects of ethanol consumption (Naassila *et al*., 2002), adenosine receptor antagonists reverse the CNS effects of alcohol and adenosine receptor agonists reverse alcohol withdrawal (Kaplan *et al*., 1999; Dunwiddie & Masino, 2001; El Yacoubi *et al*., 2001). The observation that extracellular adenosine and its cellular receptors play a role in the development of hepatic fibrosis in response to CCl_4 and thioacetamide suggests that adenosine may also play a role in the development of cirrhosis in response to chronic ethanol ingestion.

The hypothesis that adenosine A_{2A} receptors play a critical role in the pathogenesis of hepatic fibrosis is compatible with pathogenic mechanisms responsible for hepatic fibrosis postulated by others. Thus, it has been suggested that increased uptake of endotoxin from the gastrointestinal tract in alcoholics leads to increased hepatic inflammatory changes associated with stellate cell activation and fibrosis (French, 2000; Quiroz *et al*., 2001). Treatment of various cell types with either LPS (Bshesh *et al*., 2002) or the inflammatory cytokines TNFα or IL-1 (Khoa *et al*., 2001; Nguyen *et al*., 2003) leads to increased expression and function of adenosine A_{2A} receptors. The two NFκB regulatory sites in the promoter region of the adenosine A_{2A} receptor are probably responsible for the increased expression of adenosine A_{2A} receptors following stimulation with LPS, TNFα or IL-1 (Khoa *et al*., 2001). In accord with this hypothesis, we observed an increase in adenosine A_{2A} receptor mRNA in fibrotic livers. A role for toxic oxygen radicals in the pathogenesis of alcoholic liver injury has also been suggested and the demonstration that patients with cirrhosis are more likely to generate greater amounts of oxygen radicals following ethanol exposure as a result of genetic polymorphisms supports this hypothesis (Naassila *et al*., 2002). Toxic oxygen radicals such as H_2O_2 also promote increased adenosine release from cells (Morabito *et al*., 1998). Our results suggest that the toxin- (or cytokine-) mediated increase in A_{2A} receptor responsiveness to adenosine leads to hepatic fibrosis, and our demonstration that both

CCl_4 and thioacetamide stimulate hepatic adenosine release at concentrations known to activate the A_{2A} receptor *in vivo* lends further support in favor of this hypothesis.

In summary, our findings suggest a novel pathway for the pathogenesis of toxin-induced hepatic fibrosis/cirrhosis. Toxins such as ethanol or methotrexate directly stimulate adenosine release from hepatocytes, which may in turn promote hepatic stellate cell production of collagen through A_{2A} receptors and may further alter liver injury. The demonstration of a critical role for the A_{2A} receptor in hepatic fibrosis suggests a new strategy for the treatment and prevention of cirrhosis through selective antagonism of adenosine A_{2A} receptors. Furthermore, adenosine A_{2A} receptor antagonism may find application in other conditions leading to hepatic fibrosis such as infections (viruses and schistosomiasis) or primary biliary cirrhosis occurring *de novo* or as a result of other autoimmune diseases such as systemic sclerosis.

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