TOXICOLOGICAL SCIENCES **131(1)**, 325–335 (2013)
doi:10.1093/toxsci/kfs283
Advance Access publication September 17, 2012

Purinergic Receptor Antagonist A438079 Protects Against Acetaminophen-Induced Liver Injury by Inhibiting P450 Isoenzymes, Not by Inflammasome Activation

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Received August 22, 2012; accepted September 12, 2012

Acetaminophen (APAP) overdose is the most frequent cause of acute liver failure in the western world. Controversy exists regarding the hypothesis that the hepatocyte injury is amplified by a sterile inflammatory response, rather than being the result of intracellular mechanisms alone. A recent study suggested that the purinergic receptor antagonist A438079 protects against APAP-induced liver injury by preventing the activation of the Nalp3 inflammasome in Kupffer cells and thereby preventing inflammatory injury. To test the hypothesis that A438079 actually affects the intracellular signaling events in hepatocytes, C57Bl/6 mice were treated with APAP (300 mg/kg) and A438079 (80 mg/kg) or saline and GSH depletion, protein adduct formation, c-jun-N-terminal kinase (JNK) activation, oxidant stress, and liver cell necrosis were determined between 0 and 6 h after APAP administration. APAP caused rapid GSH depletion, extensive protein adduct formation in liver homogenates and in mitochondria, JNK phosphorylation and mitochondrial translocation of phospho-JNK within 2 h, oxidant stress, and extensive centrilobular necrosis at 6 h. A438079 significantly attenuated GSH depletion, which resulted in a 50% reduction of total liver and mitochondrial protein adducts and substantial reduction of JNK activation, mitochondrial P-JNK translocation, oxidant stress, and liver injury. The same results were obtained using primary mouse hepatocytes. A438079 did not directly affect JNK activation induced by *tert*-butyl hydroperoxide and GSH depletion. However, A438079 dose-dependently inhibited hepatic P450 enzyme activity. Thus, the protective effect of A438079 against APAP hepatotoxicity *in vivo* can be explained by its effect on metabolic activation and cell death pathways in hepatocytes without involvement of the Nalp3 inflammasome.

Key Words: **acetaminophen; hepatotoxicity; purinergic receptor P2X7; protein adducts; mitochondria; c-jun-N-terminal kinase.**

Acetaminophen (APAP) is an effective and safe analgesic and antipyretic drug when used at therapeutic doses. However, an overdose can cause liver injury and even liver failure (Larson, 2007). Although the mechanisms of APAP-induced liver cell death are not completely understood, extensive progress has been made during the last several decades (Cohen *et al.,* 1997; Hinson *et al*., 2004; Jaeschke and Bajt, 2006; Jaeschke *et al*., 2012a; Nelson, 1990). Key events in the pathophysiology include the formation of a reactive metabolite (N-acetyl-*p*-benzoquinone imine, NAPQI). At low doses, this metabolite can be detoxified by glutathione (GSH), but after an overdose the excess binds to cellular proteins, especially mitochondrial proteins, and initiates a mitochondrial oxidant stress (Cohen *et al.,* 1997; Nelson, 1990). This initial reactive oxygen formation causes activation of c-jun-N-terminal kinase (JNK) which, upon translocation to the mitochondria, further amplifies the mitochondrial oxidant stress and finally triggers the mitochondrial permeability transition pore opening and cell necrosis (Jaeschke *et al*., 2012a).

In addition to the intracellular mechanisms of cell death, a potential contribution of innate immune cells to the pathophysiology has received increasing attention during the last few years (Jaeschke *et al*., 2012b). It is known that damage-associated molecular patterns released from necrotic cells can activate toll-like receptors on macrophages and induce the transcriptional activation of cytokine gene expression (Martin-Murphy *et al*., 2010). One cytokine that was hypothesized to be critical for this sterile inflammatory response in the case of APAP hepatotoxicity is interleukin-1β (IL-1β; Imaeda *et al*., 2009). A unique feature of IL-1β is that a pro-form of the protein needs to be cleaved by activated caspase-1 to yield the active, soluble proinflammatory cytokine (Dinarello, 2011). The Nalp3 (NACHT, LRR, and pyrin domain-containing protein 3) inflammasome is the protein scaffold that activates caspase-1 (Gross *et al*., 2011). Recently, it was postulated that ATP released from necrotic cells binds to the purinergic receptor P2X7 on macrophages and activates the Nalp3 inflammasome and caspase-1, which then promotes IL-1β maturation and

subsequently the activation of neutrophils (Hoque *et al*., 2012). This concept was primarily based on the observation that the selective P2X7 inhibitor A438079 strongly protected against APAP hepatotoxicity (Hoque *et al*., 2012). However, there are several concerns with this interpretation. First, recent data argue against a critical role of IL-1β in the pathophysiology (Williams *et al*., 2010b), did not show any involvement of the Nalp3 inflammasome in APAP hepatotoxicity (Williams *et al*., 2011), and found no evidence for activation or actual cytotoxicity of neutrophils after APAP overdose (Cover *et al*., 2006; James *et al*., 2003; Lawson *et al.,* 2000; Williams *et al*., 2010a). Second, as the concept of sterile inflammation is based on an initial necrotic cell death, which is amplified by inflammation, even an effective elimination of the amplification process should still leave the initial injury. However, the P2X7 inhibitor A438079 appeared to be close to 100% effective in preventing any cell death after APAP overdose. These observations and the fact that P2X7 is also expressed on hepatocytes (Emmett *et al*., 2008; Gonzales *et al.,* 2007) raise the possibility that P2X7 signaling may directly affect cell death mechanisms in the hepatic parenchyma rather than modulate cytokine formation in macrophages. Thus, the aim of this investigation was to study the potential mechanisms of protection by A438079 against APAP toxicity in isolated hepatocytes and *in vivo*. Because key mechanisms of cell death are similar between humans and mice (McGill *et al*., 2011, 2012), a better understanding of these hepatoprotective mechanisms of the P2X7 receptor antagonist could identify a promising therapeutic strategy against APAP toxicity for humans.

MATERIALS AND METHODS

Animals. Male C57Bl/6 mice (8 weeks old) were acquired from Jackson Laboratories for the experiments. Animals were housed in a controlled environment with a 12-h light/dark cycle and free access to food and water. Animals were acclimatized for at least 3 days and fasted overnight before experiments. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

Experimental design. Mice were treated ip with A438079 (Santa Cruz Biotechnology, Santa Cruz, CA), which is a competitive P2X7 antagonist, at 2 mg/mouse (80 mg/kg) 1 h before APAP treatment (Hoque *et al*., 2012). The corresponding control groups were treated with 8 ml/kg saline. APAP (Sigma, St Louis, MO) was dissolved in warm saline and a dose of 300 mg/kg was injected ip into the mice, which were fasted overnight. Mice were sacrificed 10 min, 20 min, 30 min, 2 h, or 6 h after APAP injection. For phorone/*tert*-butylhydroperoxide (tBHP) treatment, animals were pretreated with 2 mg A438079 per mouse, and control groups were treated with saline. One h later, 100 mg/kg of phorone (Sigma) was injected ip and tBHP (Sigma) was given 1 h after phorone. Mice were sacrificed 1 h after tBHP treatment. Blood was drawn from the vena cava into heparinized syringes for the determination of alanine aminotransferase (ALT) activity using an ALT reagent kit (Pointe Scientific, MI). The liver was excised and rinsed in saline before being divided for histological staining and mitochondrial isolation. The histology fractions were fixed in 10% phosphate-buffered formalin. The rest of the liver was snap frozen in liquid nitrogen and subsequently stored at −80°C.

Histology. Formalin-fixed tissue samples were embedded in paraffin and 5 µm sections were cut. Replicate sections were stained with hematoxylin and eosin (H&E) to assess necrosis. Additionally, some liver sections were stained with the In Situ Cell Death Detection Kit, AP (Roche Diagnostics, Indianapolis, IN) for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay as described previously (Gujral *et al*., 2002).

Isolation of subcellular fractions and Western blotting. Mitochondria and cytosolic fractions were isolated using differential centrifugation. In short, the freshly excised liver was homogenized in ice-cold isolation buffer (pH 7.4) containing 22mM mannitol, 70mM sucrose, 2.5mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10mM EDTA, 1mM ethylene glycol tetraacetic acid, and 0.1% bovine serum albumin. Mitochondria were isolated by differential centrifugation (20,000 × g) and washed with 2 ml of isolation buffer. The supernatant after 20,000 × g centrifugation was saved as the cytosolic fraction, and both the cytosolic and the mitochondrial fractions were subjected to Western blotting. Western blot was performed as described in detail (Bajt *et al*., 2000), using rabbit anti-JNK and anti-phospho-JNK antibodies (Cell Signaling Technology, Danvers, MA). A horseradish peroxidase-coupled donkey anti-rabbit IgG (Santa Cruz) was used as the secondary antibody. Proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech., Inc., Piscataway, NJ).

Measurement of GSH and glutathione disulfide. Total soluble GSH and glutathione disulfide (GSSG) were determined in the liver homogenate using a modified Tietze assay (Jaeschke, 1990). In brief, the frozen tissue was homogenized at 0°C in 3% sulfosalicylic acid containing 0.1mM EDTA. To quantify GSH, the homogenate was centrifuged after dilution with 0.01 N HCl, and the supernatant was further diluted with 100mM potassium phosphate buffer (KPP), pH 7.4. To measure GSSG, GSH in the homogenate was trapped through reaction with 1M N-ethylmaleimide leaving only GSSG in solution. The samples were then assayed using dithionitrobenzoic acid.

APAP protein adducts and P450 activity. APAP-cysteine (APAP-CYS) in liver tissue was measured using high pressure liquid chromatography with electrochemical detection (HPLC-ECD) according to the method of Muldrew *et al*. (2002) with previously described modifications (Ni *et al*., 2012). APAP-CYS in primary mouse hepatocytes was measured as described (McGill *et al*., 2011) but with electrochemical detection rather than mass spectrometry. For determination of cytochrome P450 activity, the 14,000 × g supernatant from liver homogenates were used in the 7-ethoxy-4-trifluoromethylcoumarin (7EFC) deethylase assay, which measures at least two P450 isoenzymes (cytochrome P4501a2 and 2e1) (Buters *et al*., 1993), as described (Ramachandran *et al*., 2011).

Mouse primary hepatocyte isolation and cell viability by lactate dehydrogenase release. Primary hepatocytes were isolated by a two-step isolation procedure as described previously in detail (Bajt *et al*., 2004). Generally, cell viability of each isolation was more than 90%, and cell purity was > 95% hepatocytes. After isolation, primary hepatocytes were plated in six-well plates (BioCoat collagen I cellware plates; Becton Dickinson, Franklin Lakes, NJ) with a concentration of 6×10^5 per well. Cells were treated with 1, 10, or 100µM of A438079 (Santa Cruz) 1 h before APAP treatment. APAP was dissolved in warm Williams E medium to 5mM. Control groups were treated with saline at the corresponding time points. All cells were harvested 9 h after APAP treatment for LDH and JNK/P-JNK measurement or 1 and 3 h after APAP for protein adduct determination.

After removing medium, cells were lysed with cell lysis buffer containing 25mM HEPES, 5mM EDTA, 0.1% CHAPS, and 1 mg/ml each of pepstatin, leupeptin, and aprotinin for 5 min. The lysates were sonicated and centrifuged for 20 min at 20,000 × g at 4°C. LDH levels in the medium or in the cells were measured by incubation with KPP containing pyruvate and NADH. The decline of absorbance at 340 nm reflected the level of LDH.

Statistics. All results were expressed as mean ± SE. Comparisons between multiple groups were performed with one-way ANOVA followed by a *post hoc* Bonferroni test. If the data were not normally distributed, we used the Kruskal-Wallis test (nonparametric ANOVA) followed by Dunn's Multiple Comparisons Test. $P < 0.05$ was considered significant.

RESULTS

Effect of A438079 on Liver Injury

Treatment of mice with a dose of APAP of 300 mg/kg resulted in injury by 6 h, as indicated by the extensive increase of plasma ALT levels and histological evidence of centrilobular necrosis (Figs. 1A and B). In contrast, at 2 h after APAP there was only a minor ALT activity increase in plasma (Fig. 1A) or cell necrosis (data not shown). Pretreatment with the P2X7 receptor antagonist A438079 effectively attenuated APAP-induced liver injury as indicated by the 77% lower plasma ALT activities (Fig. 1A) and the dramatically reduced areas of necrosis (Fig. 1B). As there was no obvious injury at 2 h, A438079 had no significant effect on plasma ALT levels (Fig. 1A) or histology (data not shown) at that time.

Effect of A438079 on Intracellular Signaling Events in APAP Toxicity in vivo

To evaluate the mechanism of action of A438079, various intracellular events known to be critical in APAP-induced cell death were investigated. First, hepatic levels of GSH and GSSG were determined. As indicated (Fig. 2A), GSH + GSSG levels were reduced by 90% at 2 h and partially recovered by 6 h. Similarly, GSSG levels declined during the first 2 h after APAP but then increased significantly above baseline values by 6 h (Fig. 2B). The GSSG-to-GSH ratio was similar to controls at 2 h but showed a 7.3-fold increase at 6 h (Fig. 2C). Both the increase of GSSG and the GSSG-to-GSH ratio reflected an increased oxidant stress at that time (Figs. 2B and C). A438079 treatment did not prevent the initial decline of the GSH + GSSG levels but substantially improved the

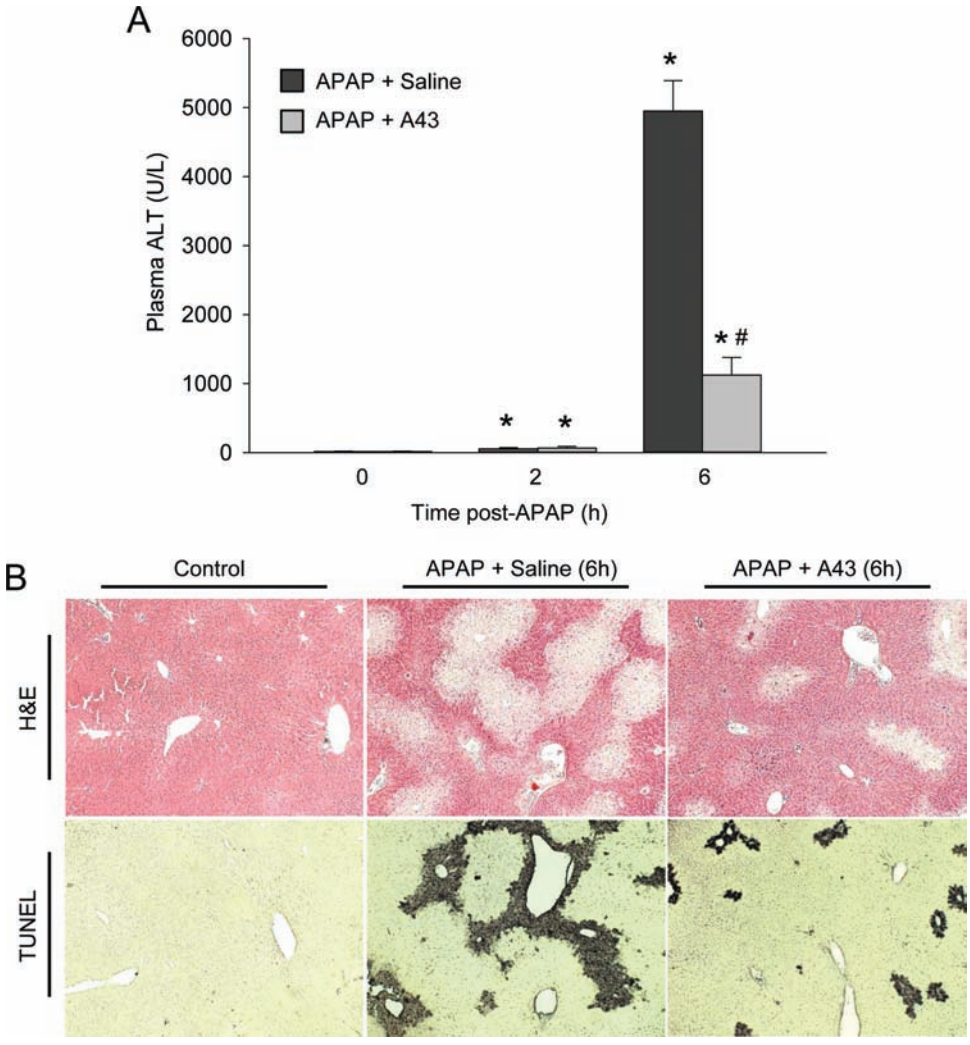

FIG. 1. Acetaminophen-induced liver injury in C57Bl/6 mice with or without A438079. Animals were pretreated with 2 mg/mouse A438079 or saline and then 1 h later with 300 mg/kg APAP or vehicle control. (A) Plasma ALT at 0, 2, and 6 h. (B) Representative H&E-stained liver sections (×50 magnification) and TUNEL staining (×50 magnification) are shown for controls and animals treated with APAP for 6 h. Data represent means ± SE of $n = 6$ animals per group. *$p < 0.05$ (compared with controls, $t = 0$). #$p < 0.05$ (compared with APAP/saline).

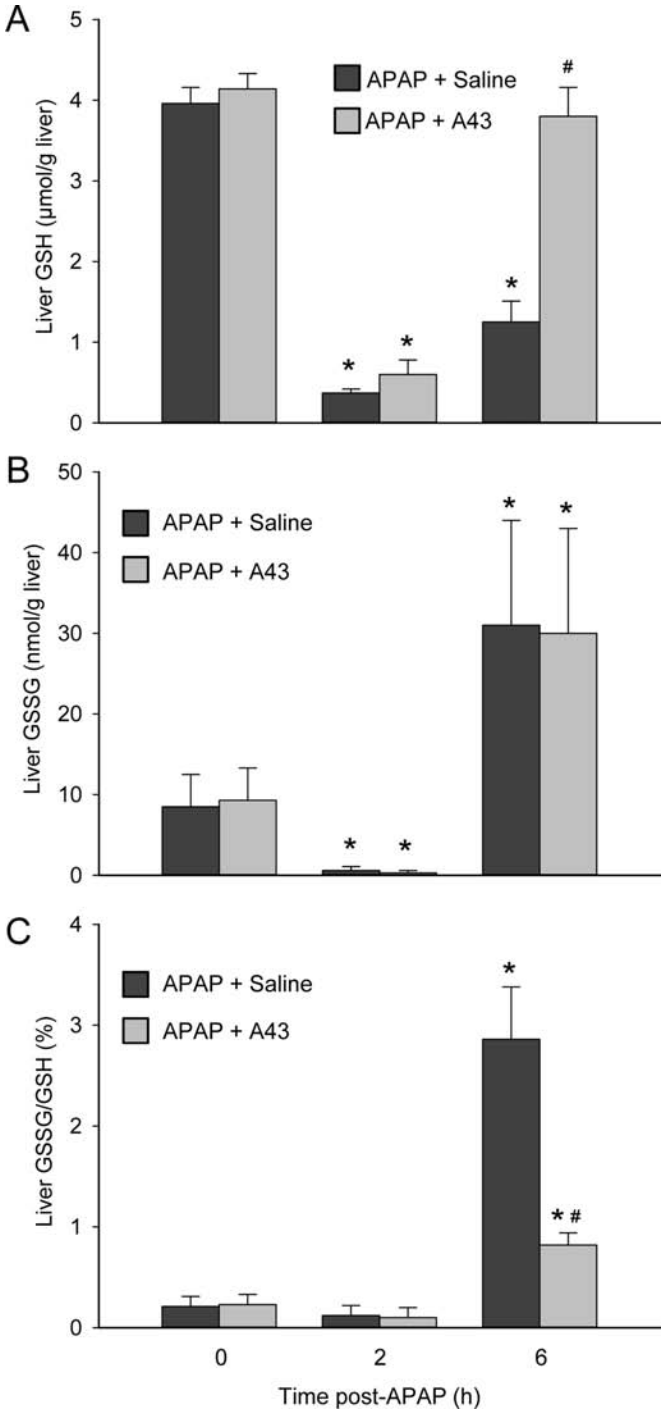

FIG. 2. Liver GSH and GSSG. Animals were pretreated with 2 mg/mouse A438079 or saline and then 1 h later with 300 mg/kg APAP or vehicle control. Total GSH was measured in liver tissue homogenate at 0, 2, and 6 h post-APAP (A). GSSG was measured at the same times (B) and the ratio of GSSG to total GSH is shown (C). Data represent means ± SE of $n = 6$ animals per group. *$p < 0.05$ (compared with control, $t = 0$). #$p < 0.05$ (compared with APAP/saline).

recovery at 6 h (Fig. 2A). Similarly, the decline of the GSSG-to-GSH ratio at 2 h compared with controls was not affected by A438079 but the receptor antagonist attenuated the increase by 6 h reflecting a significantly attenuated oxidant stress (Fig. 2C).

APAP overdose induced JNK activation (phosphorylation) in the cytosol as early as 2 h, and this activation status was maintained up to 6 h (Figs. 3A and C). Some of the activated P-JNK translocated to the mitochondria (Figs. 3B and D). A438079 treatment largely prevented JNK activation in the cytosol at 2 and 6 h (Figs. 3A and C) and drastically reduced P-JNK translocation to the mitochondria (Figs. 3B and D). It is generally hypothesized that protein binding, especially binding to mitochondrial proteins, is responsible for the initial mitochondrial oxidant stress, which triggers JNK activation. Therefore, we investigated the possibility that the protein-binding upstream of JNK activation was affected. Measurement of APAP protein adducts in liver homogenates indicated significant adducts at 0.5 h, peak levels of adducts at 2 h, and a decline at 6 h after APAP overdose (Fig. 4A). At all time points, adducts in the mitochondria accounted for 33–43% of the total adducts in the homogenates (Fig. 4B). Treatment with A438079 significantly attenuated adducts formation in the homogenates by 79% (0.5 h), 51% (2 h), and 33% (6 h) (Fig. 4A). Likewise, mitochondrial adduct formation was completely prevented at 0.5 h and reduced by 40% (2 h) and 50% (6 h) (Fig. 4B). Our data clearly demonstrate that A438079 reduced mitochondrial protein adduct formation, which is the likely reason for the drastically reduced JNK activation, oxidant stress, and cell death. In order to evaluate if the reduced protein binding was caused by inhibition of NAPQI formation, the GSH depletion kinetics during the first 30 min was determined. At this very early time, conjugation of NAPQI with GSH is thought to be the main reason for the exponential GSH depletion (Jaeschke, 1990). Interestingly, the GSH loss was slightly delayed in the A438079/APAP group compared with the APAP/saline group resulting in significantly higher GSH levels during the first 30 min after APAP treatment (Fig. 5A). The data indicate that there was a moderate reduction in NAPQI formation in the presence of A438079. In order to test if A438079 has the potential to directly inhibit P450 enzyme activities, 7EFC deethylase assay was used. The inhibitory activity of A438079 was compared with the general P450 inhibitor piperonyl butoxide (Fig. 5B). Based on the 7EFC deethylase assay, A438079 dose-dependently inhibited P450 activities. A438079 was even more potent than piperonyl butoxide (Fig. 5B).

Effect of A438079 on APAP Toxicity in Primary Mouse Hepatocytes

In order to substantiate the *in vivo* effects of A438079 and to provide evidence for the direct impact of this drug on hepatocytes, experiments were performed with primary murine hepatocytes. Exposure of these cells to 5mM APAP for 9 h caused extensive necrotic cell death (40 ± 8%) as indicated by the release of LDH (Fig. 6A). Pretreatment with A438079 (100μM) for 1 h resulted in a complete protection against APAP-induced cell death (Fig. 6A). Lower concentrations of A438079 (1 and 10μM) were ineffective (data not shown). The reduced cell death after

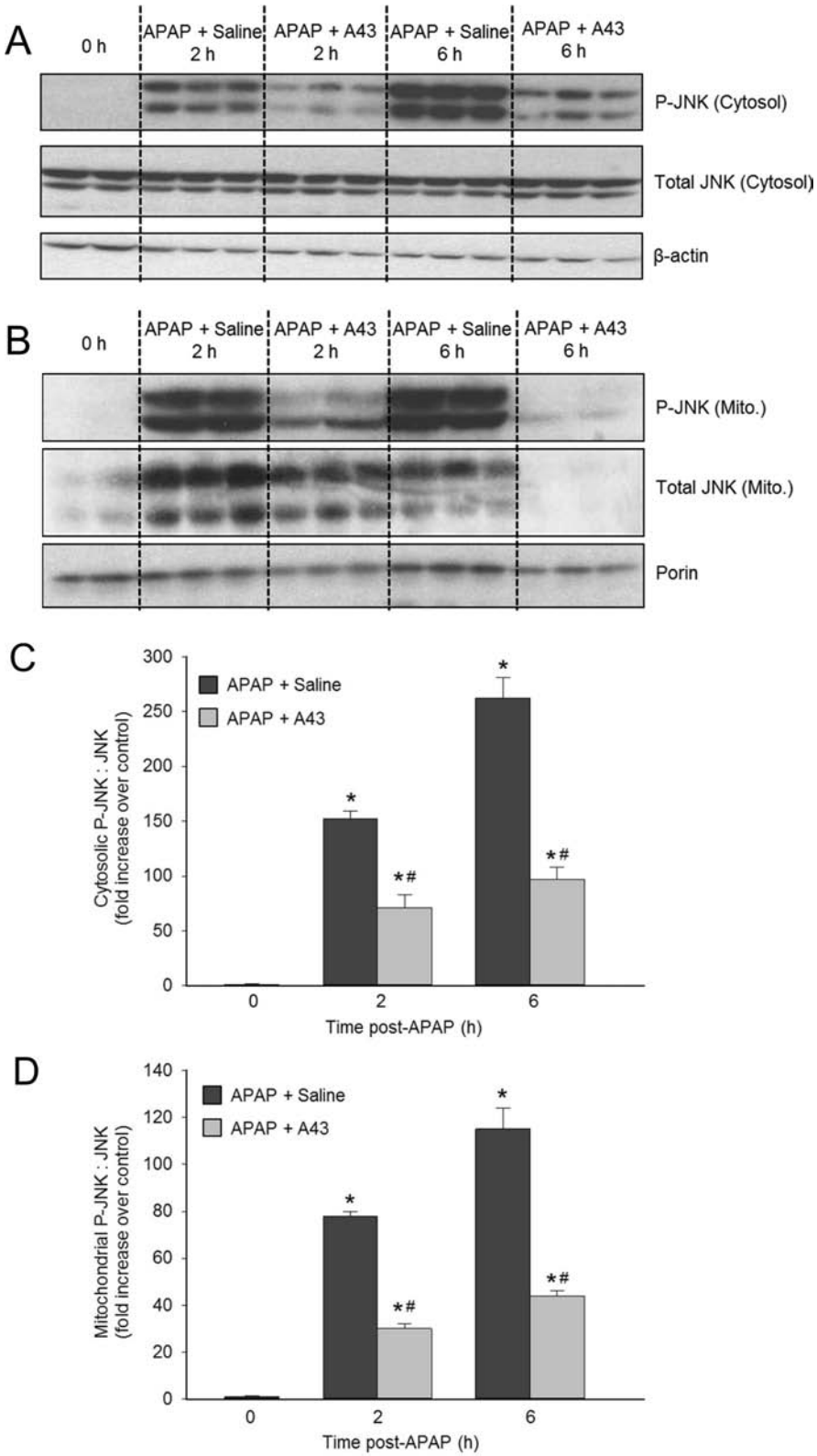

FIG. 3. JNK phosphorylation after APAP with and without A438079 pretreatment. Animals were pretreated with 2 mg/mouse A438079 or saline and then 1 h later with 300 mg/kg APAP or vehicle control. At 0, 2, and 6 h after APAP, cytosolic factions were subjected to Western blotting for phosphorylated JNK, total JNK, and beta-actin (A). Western blotting was also performed on the isolated mitochondrial fraction for phosphorylated JNK, total JNK, and porin (B). Densitometry was performed on these blots and the P-JNK-to-JNK ratio was calculated for the different time points in the cytosol (C) and in the mitochondria (D). *$p < 0.05$ (compared with control, $t = 0$). #$p < 0.05$ (compared with APAP/saline).

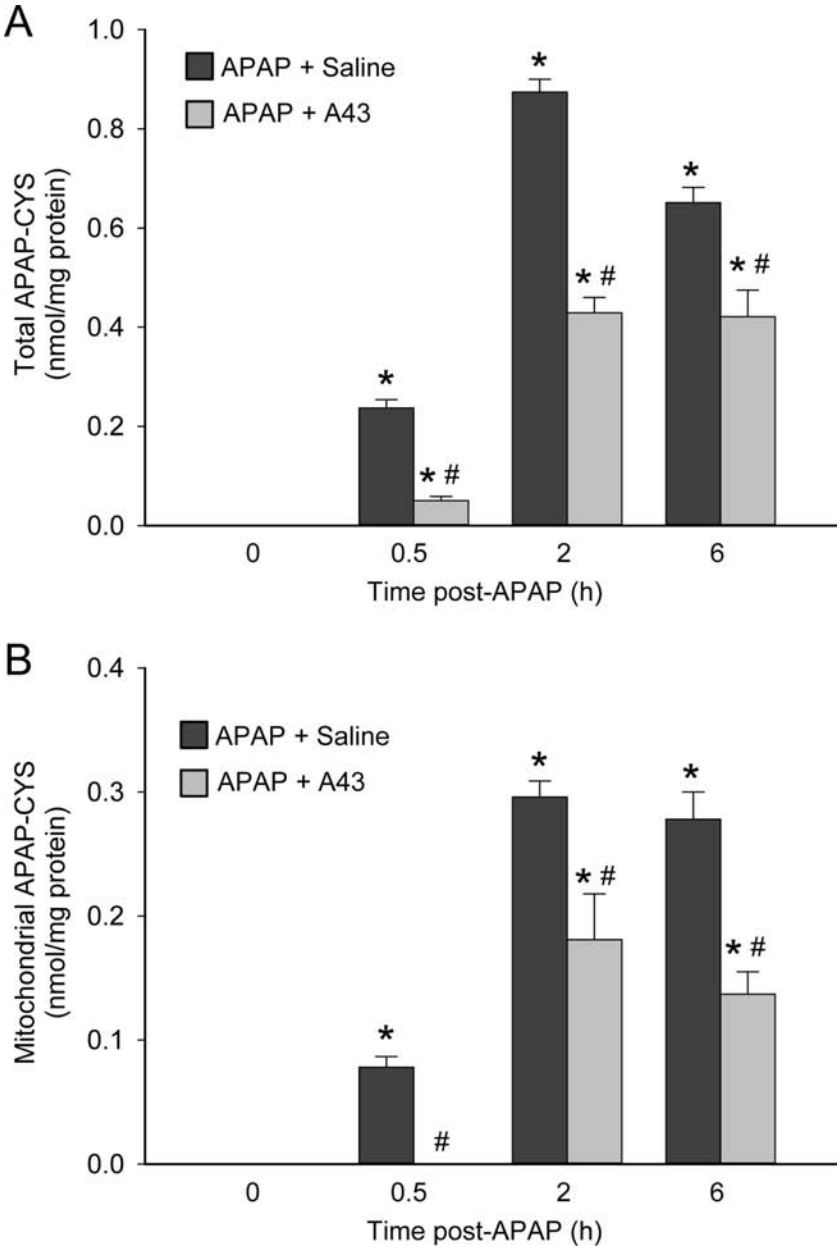

FIG. 4. Effects of A438079 on APAP-protein adduct formation. Animals were pretreated with 2 mg/mouse A438079 or saline and then 1 h later with 300 mg/kg APAP or vehicle control. APAP-cysteine adducts were quantified by HPLC-ECD in liver homogenate (A) and in the mitochondrial fraction (B) at 0, 0.5, 2, and 6 h post-APAP. Data represent means ± SE of $n = 3$–6 animals per group. *$p < 0.05$ (compared with control, $t = 0$). #$p < 0.05$ (compared with APAP/saline).

100μM A438079 was preceded by reduced protein adduct formation at 1 and 3 h after APAP exposure (Fig. 6B) and correlated with reduced JNK activation at 9 h (Figs. 6C and D). These data are consistent with the findings *in vivo* and support the hypothesis that the main effect of A438079 occurs in hepatocytes.

Alternative Mechanisms of Protection by A438079

Data from the literature indicate that the purinergic receptor antagonists can directly affect JNK activation (Humphreys *et al.*, 2000). To test this hypothesis in the liver, JNK activation was induced by GSH depletion (phorone) and oxidant stress (tBHP) (Saito *et al.*, 2010). As shown in Figures 7A and B, treatment with phorone and tBHP caused significant JNK activation at 1 h with no relevant changes of total JNK expression. However, pretreatment with A438079 did not significantly affect total JNK expression or P-JNK formation after phorone/tBHP, suggesting that A438079 had no direct effect on JNK activation (Fig. 7).

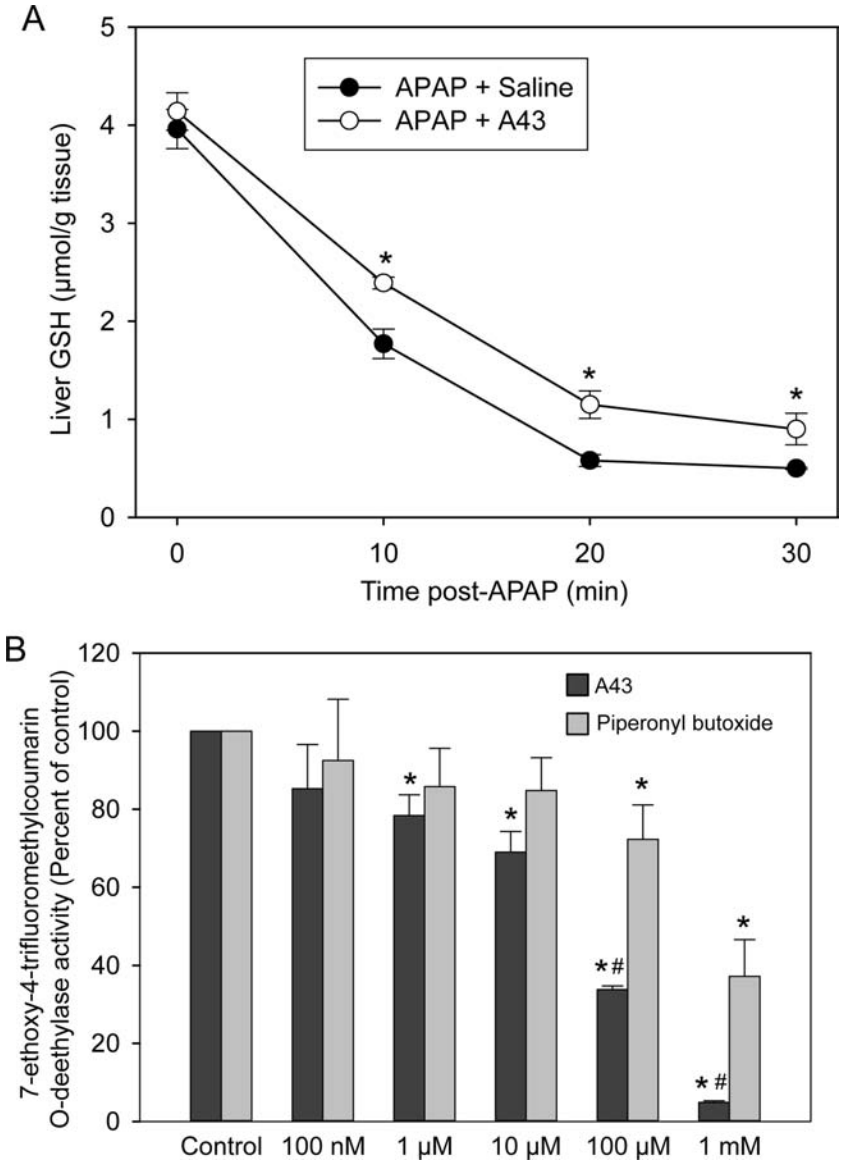

FIG. 5. Effect of A438079 on GSH depletion kinetics *in vivo* and on P450 activities. Animals were pretreated with 2 mg/mouse A438079 or saline and then 1 h later with 300 mg/kg APAP or vehicle control. (A) Total liver GSH was quantified at very early times (0, 10, 20, and 30 min post-APAP). Data represent means ± SE of $n = 3$–5 animals per group. *$p < 0.05$ (compared with APAP/saline). (B) Cytochrome P450 activities were measured in the 14,000 × g supernatant of mouse liver homogenate using the 7EFC deethylase assay. Various concentrations of A438079 or the classical P450 inhibitor piperonyl butoxide were added. Data are expressed in percent compared with untreated control samples (100%); data represent means ± SE of $n = 3$ samples per concentration. *$p < 0.05$ (compared with control). #$p < 0.05$ (compared with piperonyl butoxide).

DISCUSSION

The objective of this study was to evaluate the mechanisms of protection of A438079, a competitive antagonist of the P2X7 receptor, in a model of APAP hepatotoxicity. Authors of a recent study concluded that A438079 protected because it prevented the ATP-mediated activation of the Nalp3 inflammasome in Kupffer cells and therefore attenuated the aggravation of liver injury by a sterile inflammatory response (Hoque *et al.*, 2012). Our data clearly indicate that the inhibitor acts upstream in the pathophysiology by moderately reducing the metabolic activation of APAP and substantially attenuating mitochondrial protein adduct formation, JNK activation, and oxidant stress, resulting in extensive protection against APAP-induced cell death *in vivo* and in isolated hepatocytes.

Effect of A438079 on Metabolic Activation and Protein Adduct Formation

It is well established that APAP toxicity depends on the formation of a reactive metabolite (NAPQI), which reacts with GSH and with cysteine residues on proteins (Cohen *et al.*, 1997;

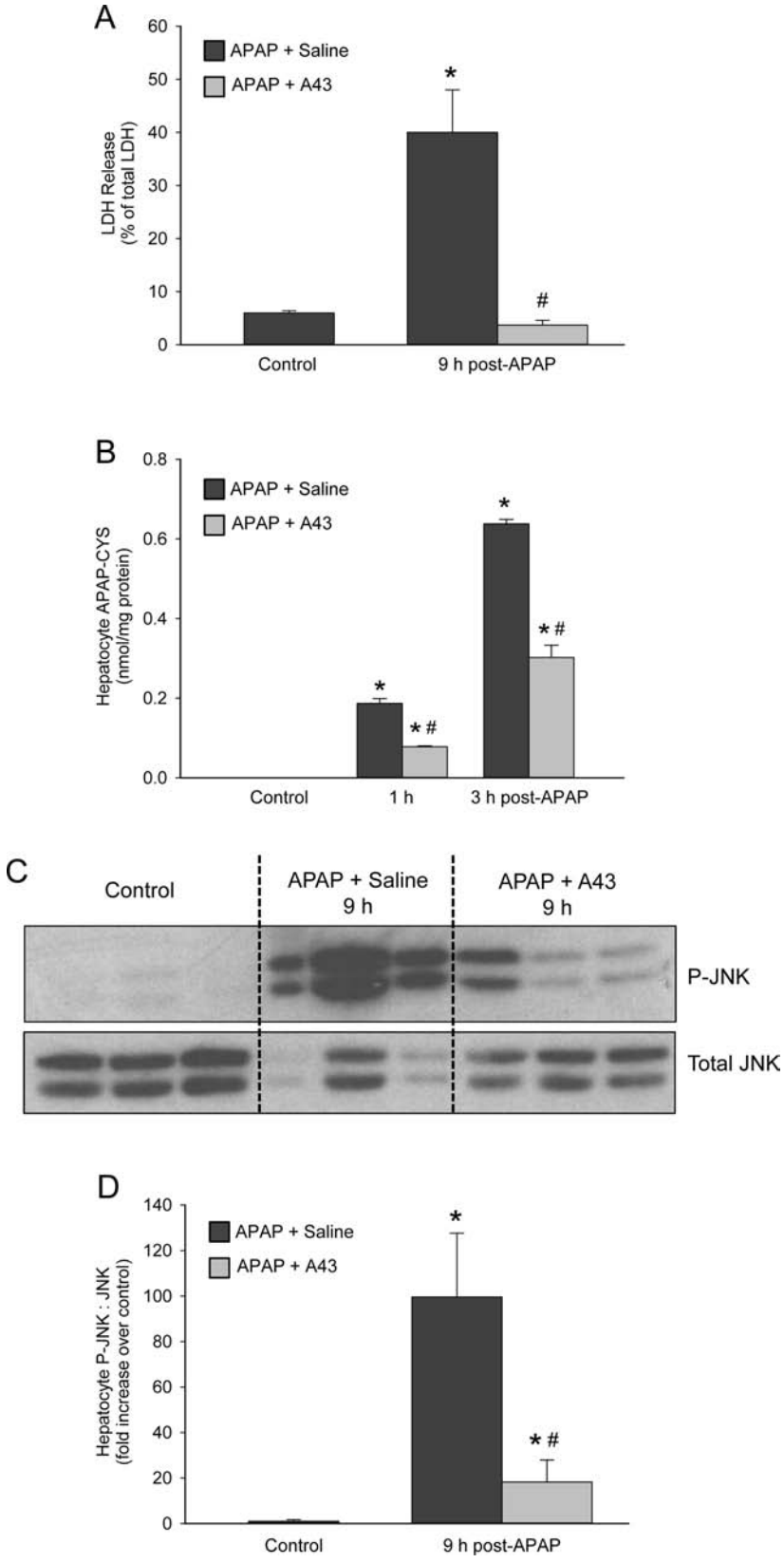

FIG. 6. Effects of A438079 on APAP toxicity in mouse hepatocytes. Primary mouse hepatocytes were pretreated for 1 h with A438079 (100μM) and then APAP was added (5mM). (A) Cell death was measured by LDH release at 9 h. (B) APAP protein adducts were determined at 1 and 3 h after APAP. (C) P-JNK and JNK protein expression was evaluated by Western blotting in untreated cells and at 9 h. (D) Densitometric analysis of the Western blots and calculation of the P-JNK-to-JNK ratio. Data represent means ± SE of $n = 3–5$ separate experiments. *$p < 0.05$ (compared with control). #$p < 0.05$ (compared with APAP/saline).

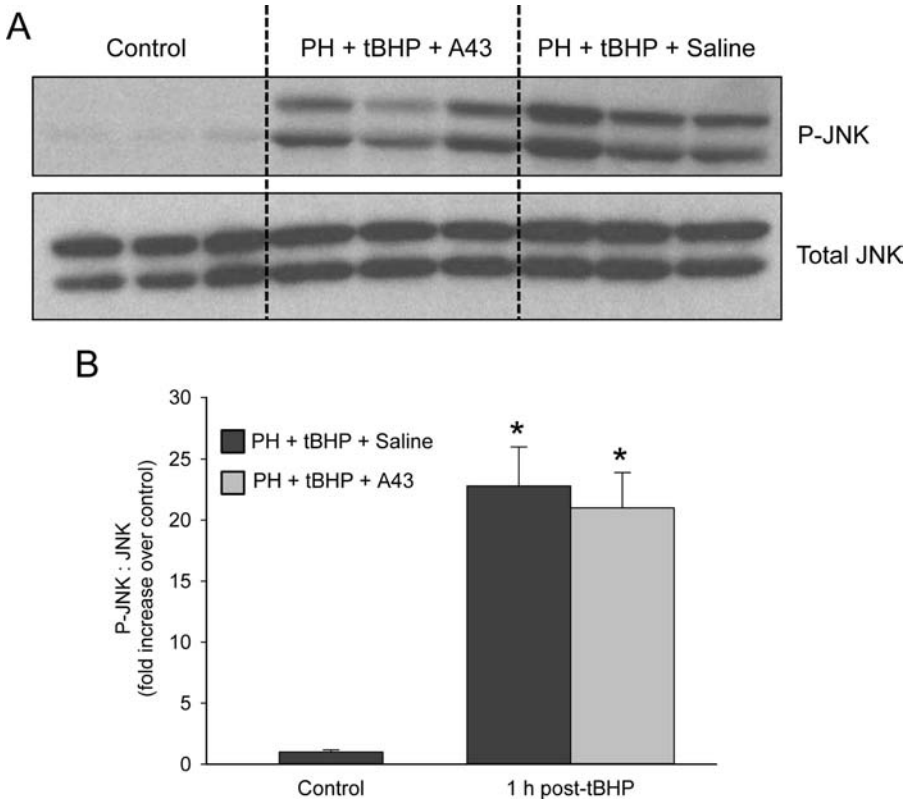

FIG. 7. Effect of A438079 on JNK phosphorylation. Mice were treated with phorone (PH) and tBHP to cause GSH depletion and oxidant stress, respectively. Animals were either pretreated with 2 mg/mouse of A438079 or saline. One hour after PH/tBHP, JNK phosphorylation was assessed by Western blotting (A) and the P-JNK-to-JNK was ratio was calculated (B). Data represent means ± SE of $n = 3$ animals per group. *$p < 0.05$ (compared with vehicle control).

Nelson, 1990). Thus, initial GSH depletion kinetics (0–20 min) provides a reasonable estimate of NAPQI formation (Jaeschke, 1990). Based on the kinetics, a significant difference in the loss of GSH between animals treated with APAP and saline or A438079 was observed during the first 30 min after APAP overdose suggesting that the drug had a significant impact on the metabolic activation of APAP. Using the 7EFC deethylase assay, which involves at least Cyp 1a2 and 2e1 (Buters *et al.*, 1993), demonstrated that A438079 can directly inhibit P450 enzyme activities, especially at concentrations of ≥ 100µM. These effects correlate well with results obtained with isolated hepatocytes where 100µM of A438079 was required for protection. As A438079 has only a 19% bioavailability *in vivo* after ip administration, is 84% bound to plasma proteins, and has a very short half-life of 1 h (McGaraughty *et al.*, 2007), the estimated free blood levels after a dose of 80 mg/kg could be between 10 and 100 µM. Thus, the protective effect of A438079 *in vivo* is most likely caused by the direct, albeit modest inhibition of P450 enzymes.

As a consequence of the reduced NAPQI formation, there was substantial reduction in overall protein adduct formation in whole liver and in mitochondria. Previous data, comparing APAP with its nontoxic analogue AMAP, indicated that there was no difference in total protein adduct formation but a substantial reduction in mitochondrial adducts with AMAP, which led to the conclusion that mitochondrial protein adducts are critical for initiating mitochondrial dysfunction (Qiu *et al.*, 2001; Tirmenstein and Nelson, 1989). It is well established that this leads to a sequence of events which amplify the early disturbance, eventually causing cell death (Jaeschke and Bajt, 2006). Based on these data, it appears reasonable to conclude that the reduced mitochondrial adduct formation is responsible for the protection against APAP toxicity.

It is interesting that the rather moderate difference in GSH depletion resulted in an ~50% reduction in mitochondrial protein adducts, which then had a profound effect on JNK activation and P-JNK translocation to the mitochondria, oxidant stress, and cell death. These findings emphasize three important aspects of the *in vivo* pathophysiology of APAP hepatotoxicity. First, it is critical to investigate a time course of the injury that includes early time points to discover off-target effects of a drug intervention. Unfortunately, many immunological studies use only a single, late time point. As a result, a critical early event may be missed leading to misinterpretation of the data. Second, measurement of GSH needs to be done very early (0–20 min) as subtle differences may not be detected at later time points (≥ 1 h). Unfortunately, even when GSH is measured during *in vivo* studies of APAP hepatotoxicity, the earliest time point that is generally used is 2 h. This study provides a clear example of why this is insufficient. Third, our study supports the concept

of an amplification cycle that is critical for the cell death. The initial mitochondrial protein adduct formation causes a minor oxidant stress, which triggers JNK activation (phosphorylation) in the cytosol (Hanawa *et al*., 2008; Saito *et al*., 2010). P-JNK translocates to the mitochondria (Hanawa *et al*., 2008), where it enhances the oxidant stress and peroxynitrite formation (Saito *et al*., 2010), which triggers the mitochondrial permeability transition pore opening with collapse of the membrane potential (Kon *et al*., 2004; LoGuidice and Boelsterli, 2011; Ramachandran *et al*., 2011) and ultimately results in necrotic cell death (Gujral *et al*., 2002). The current findings indicate that even a limited reduction of NAPQI formation and therefore of mitochondrial protein adduct formation can have a substantial beneficial effect on the intracellular signaling mechanisms and eventually cell death. It remains to be investigated if there is an overall threshold of protein adducts in the mitochondria that has to be exceeded or if specific proteins have to be adducted to trigger the initial oxidant stress.

APAP and Inflammasome Activation

A previous study has implicated IL-1β as a critical mediator of APAP hepatotoxicity (Imaeda *et al*., 2009). Transcriptional activation of IL-1β mRNA can be caused by damage-associated molecular patterns release by necrotic cells acting on toll-like receptors. However, the translation of IL-1β mRNA results in the synthesis of an inactive pro-form, which requires cleavage by Nalp3 inflammasome-activated caspase-1 to the active pro-inflammatory IL-1β (Gross *et al*., 2011). Imaeda *et al*. (2009) also suggested that the Nalp3 inflammasome is critical for APAP-induced liver injury, which led to the follow-up study on the assumed role of the purinergic receptor P2X7 in activating the inflammasome and causing a neutrophil-mediated liver injury (Hoque *et al*., 2012). Although we could directly confirm that IL-1β increases after APAP overdose in a caspase-1-dependent manner, the small amount of IL-1β endogenously produced has no impact on the pathophysiology (Williams *et al*., 2010b). Moreover, the addition of recombinant IL-1β did not aggravate APAP-induced liver injury (Williams *et al*., 2010b), caspase inhibitors did not protect (Lawson *et al*., 1999; Williams *et al*., 2010b), animals deficient in various components of the Nalp3 inflammasome were not protected (Williams *et al*., 2011), and a large number of interventions against neutrophils did not affect APAP-induced liver injury (Connolly *et al*., 2011; Cover *et al*., 2006; James *et al*., 2003; Lawson *et al*., 2000; Williams *et al*., 2010a). Thus, it was concluded that the sterile inflammatory response to the initial necrosis did not cause an aggravation of the injury by neutrophils (Jaeschke *et al*., 2012b). This study is consistent with these conclusions as the purinergic receptor antagonist clearly affected signaling events in hepatocytes, which are sufficient to explain its protective effect and the reduced inflammatory response. The postulated effect of A438079 on the inflammasome in Kupffer cells (Hoque *et al*., 2012) could be at best a secondary effect with no relevance for the overall injury.

Summary and Conclusions

Our data demonstrated that the protective effect of the purinergic receptor antagonist A438079 against APAP hepatotoxicity is caused by a moderate delay in GSH depletion and reduction in protein adduct formation, especially in mitochondria, which preclude the expected JNK activation and oxidant stress. This observation is supported by the fact that A438079 is a direct inhibitor of P450 enzymes including Cyp 2e1. The *in vivo* effects of A438079 are very early events and take place in hepatocytes. Although we cannot exclude that A438079 may also have an impact on inflammasome activation in Kupffer cells, the substantial effect of A438079 on protein adduct formation and JNK activation between 0.5 and 2 h after APAP overdose, that is, before any cell death occurs and any sterile inflammation could be initiated, strongly suggests that the critical effect of A438079 for its protection against APAP hepatotoxicity occurs in hepatocytes and not in Kupffer cells. Thus, our data further support the role of intracellular signaling events in APAP-induced cell death, including mitochondrial protein adduct formation, JNK activation, and oxidant stress in hepatocytes. We conclude that the protective effect of the purinergic receptor antagonist A438079 against APAP hepatotoxicity *in vivo* is mediated by affecting the metabolic activation of APAP and as a result the compound attenuates the amplification of the initial signal through cell death pathways in hepatocytes and does not involve modulation of the Nalp3 inflammasome.

FUNDING

National Institutes of Health (AA12916, DK070195); National Center for Research Resources (5P20RR021940-07); National Institute of General Medical Sciences (8 P20 GM103549-07); National Institute of Environmental Health Sciences (T32 ES007079-26A2 to M.R.M. and C.D.W.).

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