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Peroxynitrite Is a Critical Mediator of Acetaminophen Hepatotoxicity in Murine Livers: Protection by Glutathione

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ABSTRACT

Acetaminophen (AAP) overdose causes formation of nitrotyrosine, a footprint of peroxynitrite, in centrilobular hepatocytes. The importance of peroxynitrite for the pathophysiology, however, is unclear. C3Heb/FeJ mice were treated with 300 mg/kg AAP. To accelerate the restoration of hepatic glutathione (GSH) levels as potential endogenous scavengers of peroxynitrite, some groups of animals received 200 mg of GSH/kg i.v. at different time points after AAP. AAP induced severe liver cell damage at 6 h. Total liver and mitochondrial glutathione levels decreased by >90% at 1 h but recovered to 75 and 45%, respectively, of untreated values at 6 h after AAP. In addition, the hepatic and mitochondrial glutathione disulfide (GSSG) content was significantly increased over baseline, suggesting a mitochondrial oxidant stress. Moreover, centrilobular hepatocytes stained for nitrotyrosine. Treatment with GSH at $t = 0$ restored hepatic GSH levels and completely prevented the mitochondrial oxidant stress, peroxynitrite formation, and liver cell injury. In contrast, treatment at 1.5 and 2.25 h restored hepatic and mitochondrial GSH levels but did not prevent the increase in GSSG formation. Nitrotyrosine adduct formation and liver injury, however, was substantially reduced. GSH treatment at 3 h after AAP was ineffective. Similar results were obtained when these experiments were repeated with glutathione peroxidase-deficient animals. Our data suggest that early GSH treatment ($t = 0$) prevented cell injury by improving the detoxification of the reactive metabolite of AAP. Delayed GSH treatment enhanced hepatic GSH levels, which scavenged peroxynitrite in a spontaneous reaction. Thus, peroxynitrite is an important mediator of AAP-induced liver cell necrosis.

Overdose of the widely used analgesic drug acetaminophen (AAP) causes hepatotoxicity, which can in severe cases lead to liver failure in experimental animals and humans (Thomas, 1993). Although a large percentage of the dose of AAP is directly conjugated with glucuronic acid or sulfate and excreted, a significant amount of AAP is metabolized by the cytochrome P450 system (Nelson, 1990). This leads to the formation of a reactive metabolite, presumably *N*-acetyl-*p*-benzoquinone imine (NAPQI), which reacts rapidly with glutathione (GSH) (Nelson, 1990). Thus, AAP metabolism causes dramatic depletion of cellular glutathione levels in the liver (Mitchell et al., 1973). If the formation of the reactive metabolite exceeds the capacity of hepatocellular glutathione, NAPQI will covalently bind to cellular proteins (Jollow et al., 1973). During the last decade, a large number of these proteins were identified (Cohen and Khairallah, 1997; Qiu et al., 1998). However, the marginal inactivation of the function of these proteins cannot explain the severe cell necrosis during AAP overdose. Thus, the mechanism of cell injury after the initial NAPQI formation, glutathione depletion, and covalent binding to proteins is still unclear. The moderate extent of covalent binding and the lack of highly vulnerable target proteins suggest that covalent binding may be an initiating event that requires amplification to cause cell death.

Reactive oxygen and reactive nitrogen species (i.e., peroxynitrite) emerged as potential secondary mediators involved in hepatocyte cell death. Peroxynitrite is generated by the spontaneous, diffusion-limited reaction of nitric oxide and superoxide (Squadrito and Pryor, 1998). It is an aggressive oxidant, which can cause nitration of proteins (e.g., nitrotyrosine formation) and induce oxidative damage to all types of cellular macromolecules (Beckman, 1996; Squadrito and Pryor, 1998). Increased levels of plasma nitrite and nitrotyrosine formation indicated that nitric oxide and peroxynitrite, respectively, are indeed formed during AAP hep-

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ABBREVIATIONS: AAP, acetaminophen; NAPQI, *N*-acetyl-*p*-benzoquinone imine; GSH, reduced glutathione; iNOS, inducible nitric-oxide synthase; Gpx1, glutathione peroxidase-1; ALT, alanine aminotransferase; NEM, *N*-ethylmaleimide; GSSG, glutathione disulfide; KPP, potassium phosphate buffer; BSA, bovine serum albumin; NT, nitrotyrosine.

atotoxicity (Gardner et al., 1998; Hinson et al., 1998). There is evidence for Kupffer cell activation (Laskin and Pilaro, 1986), vascular nitrotyrosine staining (Knight et al., 2001), and involvement of these macrophages in the injury process (Laskin et al., 1995). However, mice deficient in NADPH oxidase activity had similar nitrotyrosine staining as wild-type animals and were not protected against AAP hepatotoxicity (James et al., 2002). Although neutrophils accumulate in the hepatic vasculature during AAP-induced liver injury, antibodies against β_2 integrins, which prevent a neutrophil-derived oxidant stress (Jaeschke et al., 1993), did not attenuate AAP-induced liver injury (Lawson et al., 2000). These data suggest that neither Kupffer cells nor neutrophils are the main source of superoxide and peroxynitrite formation. On the other hand, AAP causes mitochondrial dysfunction (Meyers et al., 1988; Ramsay et al., 1989), which leads to mitochondrial oxidant stress (Jaeschke, 1990) and peroxynitrite formation (Knight et al., 2001). Despite the clear evidence for the generation of peroxynitrite during AAP hepatotoxicity, the pathophysiological importance of this reactive metabolite is still unclear. Gardner et al. (1999) reported that mice deficient in the inducible nitric-oxide synthase (iNOS) were moderately protected against AAP-induced cell injury. On the other hand, Michael et al. (2001) did not find a relevant reduction of AAP-induced liver injury in these mice. In addition, there are conflicting reports on the effect of NOS inhibitors in AAP hepatotoxicity. One study reported protection with the iNOS inhibitor aminoguanidine in rats (Gardner et al., 1998). Hinson et al. (2002), however, found no protective effects with several inhibitors in mice. These data and the fact that mitochondrial dysfunction per se could lead to cell death suggest the possibility that peroxynitrite may be an epiphenomenon and may not be relevant for the injury mechanism. Therefore, the objective of this investigation was to test the hypothesis that peroxynitrite is a critical mediator of AAP-induced cell injury. Our approach was to treat animals intravenously with glutathione to enhance glutathione resynthesis in the liver at a time when acetaminophen metabolism is completed and peroxynitrite formation is in progress.

Materials and Methods

Animals. Male C3Heb/FeJ mice with an average weight of 18 to 20 g were purchased from The Jackson Laboratory (Bar Harbor, ME) and used in most in vivo experiments. To test if glutathione peroxidase-1 (Gpx1) could be involved in peroxynitrite detoxification (Sies et al., 1997), Gpx1 gene knockout (Gpx1−/−) mice were used in selected experiments. Construction of the Gpx1−/− mice (129SV/B6 background) has been described (Ho et al., 1997). The animals lack mRNA for Gpx1 as assessed by Northern blotting and, compared with wild-type animals, have less than 0.5% of the Gpx1 activity in cytosol and no detectable Gpx1 activity in mitochondria (Esworthy et al., 1997; Ho et al., 1997). Ten- to 14-week old male mice were used in these studies. All animals were housed in an environmentally controlled room with a 12-h light/dark cycle and allowed free access to food (certified rodent diet 8640; Harlan, Indianapolis, IN) and water. The experimental protocols followed the criteria of University of Arkansas for Medical Sciences and the National Research Council for the care and use of laboratory animals in research. All animals were fasted overnight before the experiments. Animals received an intraperitoneal injection of 300 mg/kg AAP (Sigma-Aldrich, St. Louis, MO) between 8 and 9 AM. AAP was dissolved in warm saline (15 mg/ml). Some groups of animals were treated intravenously with 200 mg/kg GSH (0.65 mmol/kg) between 0 to 3 h after AAP administration. GSH was dissolved in phosphate-buffered saline (25 mg/ml).

Experimental Protocols. At selected times after AAP treatment, the animals were killed by cervical dislocation. Blood was drawn from the vena cava into heparinized syringes and centrifuged. The plasma was used for determination of alanine aminotransferase (ALT) activities. Immediately after collecting the blood, the livers were excised and rinsed in saline. A small section from each liver was placed in 10% phosphate-buffered formalin to be used in immunohistochemical analysis. A portion of the remaining liver was homogenized for isolation of mitochondria or frozen in liquid nitrogen and stored at −80°C for later analysis of glutathione.

Isolation of Mitochondria. The detailed protocol has been described previously (Knight et al., 2001). Briefly, the liver was homogenized in ice-cold isolation buffer (pH 7.4) containing 220 mM mannitol, 70 mM sucrose, 2.5 mM HEPES, 10 mM EDTA, 1 mM EGTA, and 0.1% bovine serum albumin. Mitochondria were isolated by differential centrifugation and washed with 2 ml of isolation buffer. The mitochondrial pellet was resuspended in 3% sulfosalicylic acid containing 0.1 mM EDTA, vigorously vortexed, and centrifuged to sediment the precipitated protein. A part of the supernatant was diluted in 100 mM potassium phosphate buffer (KPP) (pH 6.5) for the determination of total glutathione [GSH + glutathione disulfide (GSSG)], and another part was added to 10 mM *N*-ethylmaleimide (NEM) in potassium phosphate buffer for the determination of GSSG.

Methods. Plasma ALT activities were determined with the test kit DG 159-UV (Sigma-Aldrich) and expressed as international units per liter. Protein concentrations were assayed using the bicinchoninic acid kit (Pierce chemical, Rockford, IL). Total soluble GSH and GSSG were measured in the liver homogenate and mitochondrial homogenate with a modified method of Tietze, as described in detail by Jaeschke and Mitchell (1990). Briefly, the frozen tissue or isolated mitochondria were homogenized at 0°C in 3% sulfosalicylic acid containing 0.1 mM EDTA. An aliquot of the homogenate was added to 10 mM NEM in potassium phosphate buffer, and another aliquot was added to 0.01 N HCl. The NEM-KPP sample was centrifuged, and the supernatant was passed through a C_{18} cartridge to remove free NEM and NEM-GSH adducts (Sep-Pak; Waters, Milford, MA). The HCl sample was centrifuged, and the supernatant was diluted with KPP. All samples were assayed using dithionitrobenzoic acid. All data are expressed in GSH-equivalents.

Histology and Immunohistochemistry. Formalin-fixed tissue samples were embedded in paraffin, and 5-μm sections were cut. Replicate sections were stained with H&E for evaluation of necrosis (Gujral et al., 2001). All sections were obtained from the left lateral lobe. Preliminary studies using several livers showed no difference in necrosis or nitrotyrosine staining between the different lobes of the liver in this model. The percentage of necrosis was estimated by evaluating the number of microscopic fields with necrosis compared with the entire cross-section. In general, necrosis was estimated at low power (100×); questionable areas were evaluated at higher magnification (200× or 400×) All histological evaluations were done in a blinded fashion by the pathologist (A.F.). Nitrotyrosine staining was assessed by immunohistochemistry with the DAKO LSAB peroxidase kit (K684; DAKO, Carpinteria, CA), which was used according to the manufacturer's instructions. The anti-nitrotyrosine antibody was obtained from Molecular Probes (Eugene, OR).

Nitration of BSA in Vitro. The nitration of proteins by peroxynitrite (Upstate Biotechnology, Lake Placid, NY) was determined spectrophotometrically at 438 nm as the intensely yellow phenolate of nitrotyrosine (Knight et al., 2001). Briefly, bovine serum albumin (BSA) (Sigma-Aldrich) was added to 60 mM carbonate buffer (pH 9.6) with a final concentration of 2 mg/ml. In some samples, GSH (1 mM) was added to the BSA-carbonate buffer. Peroxynitrite was then added to each solution (a final concentration of 500 μM) to nitrate BSA. A spectrum was recorded and the amount of nitrotyrosine in

the peroxynitrite-treated BSA was determined at the absorbance maximum of the phenolate ion at 438 nm. To evaluate the potential of peroxynitrite and hydrogen peroxide to oxidize glutathione spontaneously, hydrogen peroxide or peroxynitrite (a final concentration of 100 or 500 μM) were added to a 100 μM GSH solution in air-saturated 5 mM potassium phosphate buffer (pH 7.4) and incubated at 37°C for 10 min. The reaction was stopped by adding 140 U of catalase to the hydrogen peroxide sample and pipetting an aliquot into NEM. Total glutathione and GSSG levels were determined as described above. Data are given as the mean of four separate incubations.

Statistics. All results were expressed as mean ± S.E. Comparisons between multiple groups were performed with one-way analysis of variance followed by a Bonferroni t test. If the data were not normally distributed, we used the Kruskal-Wallis test (nonparametric analysis of variance) followed by Dunn's multiple comparisons test. A P value <0.05 was considered significant.

Results

In agreement with previous articles (Lawson et al., 2000; Knight et al., 2001), a dose of 300 mg/kg AAP caused significant liver injury in C3Heb/FeJ mice as indicated by the substantial increase in plasma ALT activities (Fig. 1A).

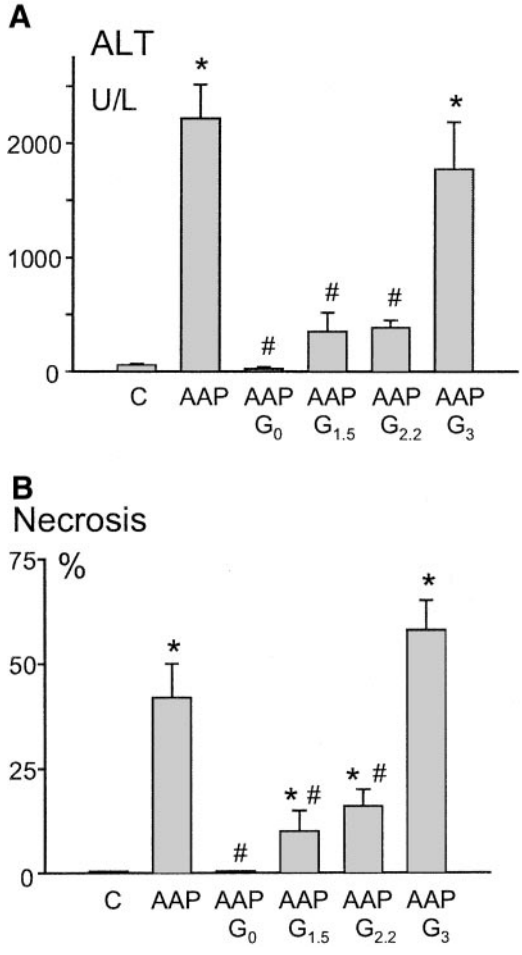

Fig. 1. Plasma ALT activities (A) and hepatocellular necrosis (B) were determined in control C3Heb/FeJ mice and 6 h after i.p. injection of acetaminophen (300 mg of AAP/kg, i.p.). Some of the animals were treated with a single bolus dose (i.v.) of 200 mg/kg glutathione (G) at t = 0, 1.5, 2.25, or 3 h after AAP. Data represent means ± S.E. of n = 5 animals/group. ⋆, $P < 0.05$ [compared with controls (C)]; #, $P < 0.05$ (compared with AAP).

Intravenous injection of glutathione results in a rapid degradation in the kidney, reabsorption of the individual amino acids, and resynthesis of glutathione in the liver of fasted mice (Wendel and Jaeschke, 1982). Therefore, 200 mg/kg GSH was injected i.v. at the time of AAP administration (t = 0) or 1.5, 2.25, and 3 h after AAP. The expectation was that injection at t = 0 would protect by scavenging NAPQI, but later treatment would enhance tissue GSH levels after most AAP was metabolized and peroxynitrite formation was initiated (Knight et al., 2001). Although treatment with GSH at t = 0 was most effective (i.e., reducing plasma ALT values to baseline), treatment at 1.5 and 2.25 h after AAP was also protective, as indicated by the 83 to 86% reduction of ALT activities (Fig. 1A). The beneficial effect was lost with GSH administration at 3 h after AAP. Blinded histological evaluation of the injury by the pathologist (A.F.) confirmed the ALT data. AAP caused severe centrilobular necrosis, which was completely prevented in animals treated with GSH at time 0 (Fig. 1B). Delayed injection of GSH at 1.5 or 2.25 h attenuated necrosis by 77 or 62%, respectively. Treatment with GSH at 3 h had no effect on AAP-induced necrosis (Fig. 1B).

AAP treatment caused depletion of hepatic glutathione levels by 90 to 95% at 1 to 2 h (data not shown). The glutathione levels recovered to values 75% of untreated controls at 6 h (Fig. 2A). Tissue GSSG levels and the percentage of GSSG of the total glutathione content, however, were significantly elevated compared with untreated controls (Fig. 2, B and C). This indicates an intracellular oxidant stress in these livers. Administration of GSH resulted in significantly higher glutathione levels in those groups treated at 1.5 h or later (Fig. 2A). However, GSSG levels and percentage of GSSG remained at baseline values with GSH injection at t = 0 (Fig. 2, B and C) suggesting that this treatment regimen prevented the intracellular oxidant stress. On the other hand, GSH injection at 1.5 h or later resulted in significantly higher GSSG levels with a similar percentage of GSSG compared with AAP alone (Fig. 2, B and C).

Measurement of GSH and GSSG in mitochondria isolated from animals treated with AAP revealed that the total glutathione levels were still reduced by 55% at 6 h compared with mitochondria from untreated controls (Fig. 3A). GSSG levels, however, were slightly elevated, which resulted in a significant increase of the percentage of GSSG from 5.9 to 16.1% (Fig. 3, B and C). Treatment with GSH at 1.5 h induced a complete recovery of mitochondrial glutathione content, a more than 4-fold increase in GSSG levels and the percentage of GSSG (Fig. 3, B and C). These data suggest that treatment with GSH at 1.5 h did not prevent the AAP-induced mitochondrial oxidant stress. Mitochondrial superoxide is thought to be responsible for peroxynitrite formation during AAP toxicity (Knight et al., 2001). To verify peroxynitrite formation, tissue sections were stained for nitrotyrosine residues. Livers from AAP-treated animals showed nitrotyrosine staining in all cells of the centrilobular areas (Fig. 4B). Although GSH administration at t = 0 completely prevented nitrotyrosine staining in these livers (Fig. 4C), treatment at 1.5 h (data not shown) and at 2.25 h (Fig. 4D) substantially attenuated nitrotyrosine accumulation. Treatment at 3 h after AAP had no effect on nitrotyrosine adduct formation (data not shown).

Since treatment with GSH at 1.5 h did not prevent the

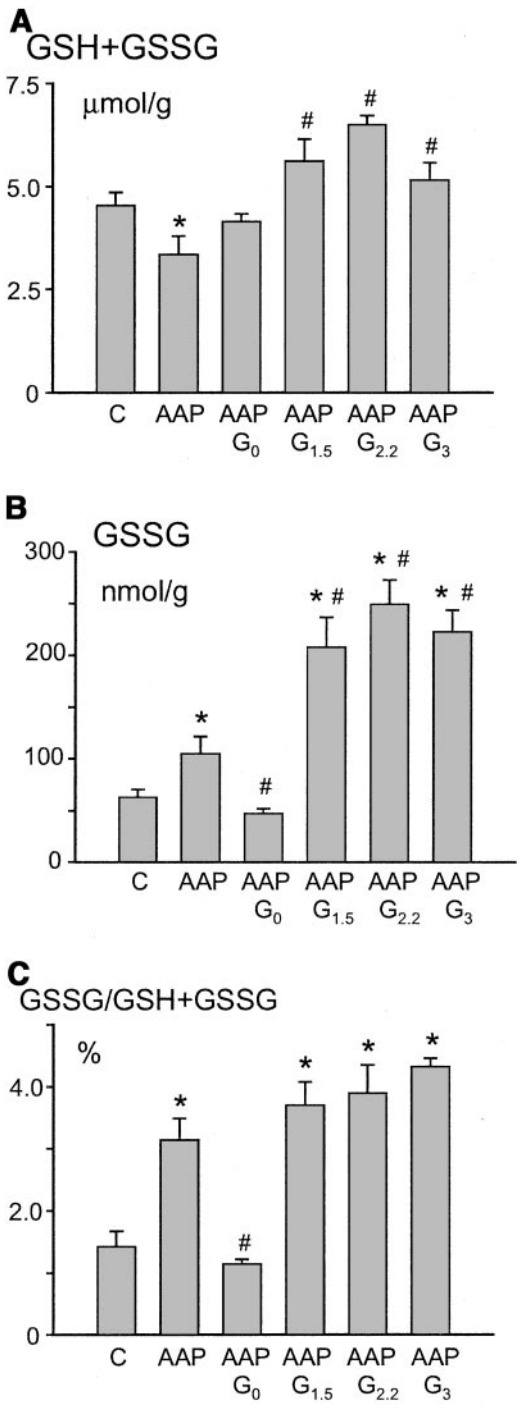

Fig. 2. Liver glutathione content in controls and 6 h after i.p. injection of acetaminophen (300 mg of AAP/kg, i.p.). Some of the animals were treated with a single bolus dose (i.v.) of 200 mg/kg glutathione (G) at t = 0, 1.5, 2.25, or 3 h after AAP. A, total glutathione (GSH + GSSG); B, GSSG; C, percentage of GSSG of total glutathione. All data are given in GSH-equivalents. Data represent means ± S.E. of n = 5 animals/group. ⋆, $P < 0.05$ [compared with controls (C)]; #, $P < 0.05$ (compared with AAP).

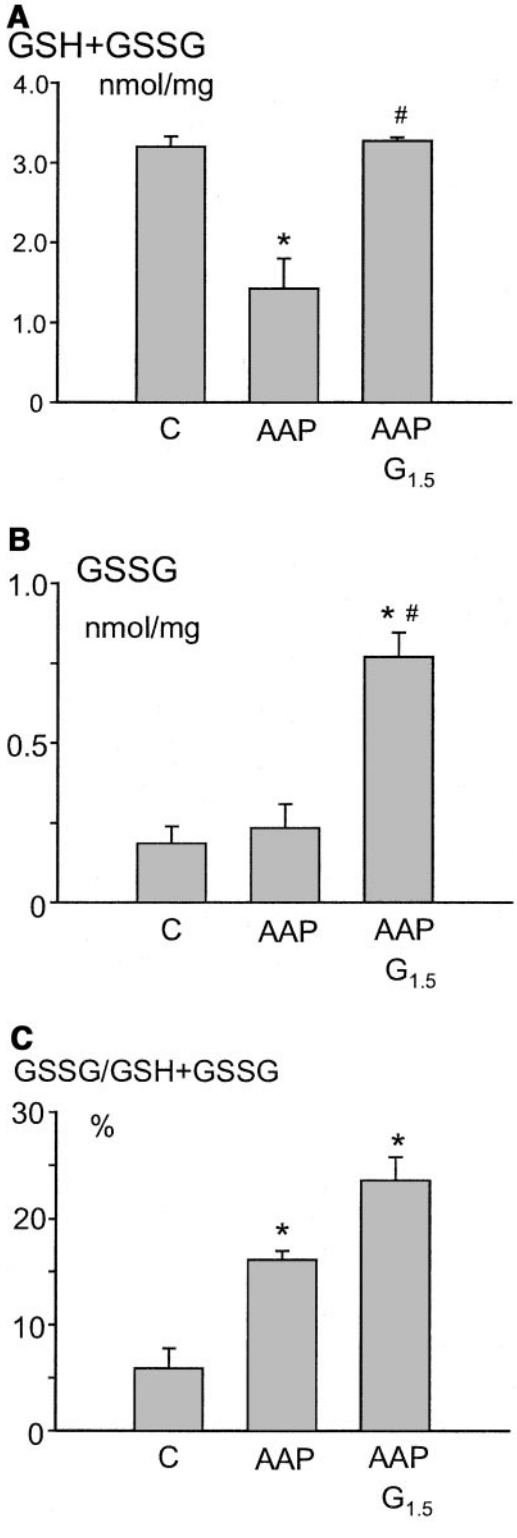

Fig. 3. Mitochondrial content of glutathione in controls or 6 h after a single i.p. dose of acetaminophen (300 mg of AAP/kg). A, total glutathione (GSH + GSSG); B, GSSG; C, percentage of GSSG of total glutathione. All results are given in GSH-equivalents. Some animals received a single bolus dose (i.v.) of 200 mg/kg GSH at 1.5 h after acetaminophen. Data represent means ± S.E. of n = 4 animals/group. ⋆, $P < 0.05$ [compared with controls (C)]. #, $P < 0.05$ (compared with AAP).

mitochondrial oxidant stress but attenuated nitrotyrosine formation and protected against AAP-induced liver injury, our data suggest that the newly synthesized GSH detoxified peroxynitrite. To investigate whether glutathione peroxidase was involved in this process, the experiments were repeated with Gpx1−/− mice. A dose of 300 mg/kg AAP caused significant liver injury in both wild-type and Gpx1−/− mice (Fig. 5). The overall cell damage was slightly higher in wild-type animals compared with Gpx1−/− mice, as indicated by

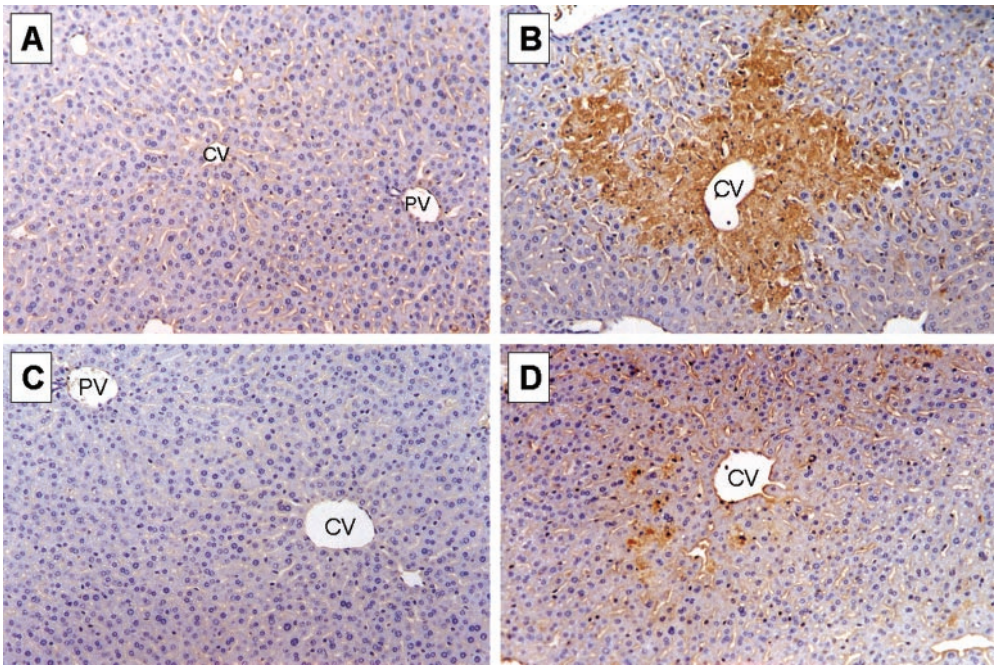

Fig. 4. Immunohistochemical staining of liver sections for nitrotyrosine (NT) protein adducts in controls and 6 h after 300 mg/kg AAP. A, control: the liver was histologically normal with no evidence of NT staining or hepatocyte injury. B, 6-h AAP: confluent hepatocellular staining for NT in centrilobular areas. C, 6-h AAP/0-h GSH: the liver was histologically normal with no evidence of NT staining or hepatocyte injury. D, 6-h AAP/2.2-h GSH: only minor staining for NT was observed. Magnification is 200× for all micrographs. CV, central vein; PV, portal vein.

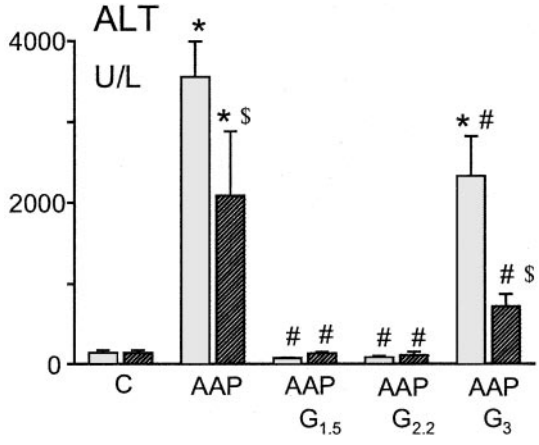

Fig. 5. Acetaminophen hepatotoxicity in wild-type (open bars) and Gpx1−/− mice (striped bars). Plasma ALT activities were measured in controls or 6 h after a single i.p. dose of 300 mg/kg AAP. Some of the animals were treated with a single bolus dose (i.v.) of 200 mg/kg glutathione (G) at t = 1.5, 2.25, or 3 h after acetaminophen. Data represent means ± S.E. of n = 5 animals/group. ⋆, $P < 0.05$ [compared with controls (C)]; #, $P < 0.05$ (compared with AAP); $, $P < 0.05$ (compared with wild-type).

higher plasma ALT activities (Fig. 5). In both strains of mice, injection of GSH at 1.5 or 2.25 h proved to be equally protective. Treatment at 3 h after AAP was only moderately effective (Fig. 5). Liver glutathione levels remained substantially depleted in both groups of animals at 6 h after AAP (Fig. 6A). Although the absolute GSSG concentrations did not significantly increase, the percentage of GSSG was 8- to 9-fold higher in wild-type and in Gpx1−/− mice compared with untreated controls (Fig. 6, B and C). Treatment with GSH at 1.5 h after AAP injection restored tissue GSH levels to values above the fasted control values (Fig. 6A) and completely prevented the increase in the percentage of GSSG in both wild-type and Gpx1−/− mice (Fig. 6C). In contrast, treatment with GSH at 2.25 h restored hepatic GSH levels and, although protective, increased the GSSG content and the GSSG-to-GSH ratio by 5- to-7-fold (Fig. 6, B and C). Treatment with GSH at 3 h had similar effects on the hepatic GSH and GSSG levels but was only marginally protective. The nitrotyrosine staining pattern in a liver section of wild-type and Gpx1−/− was similar to C3Heb/FeJ mice. AAP caused centrilobular nitrotyrosine staining, which was prevented by GSH treatment at 1.5 and 2.25 h but only partially attenuated with treatment at 3 h (data not shown).

Since late GSH administration protected against peroxynitrite toxicity in both wild-type and Gpx1−/− mice, the data are consistent with a spontaneous reaction of GSH with peroxynitrite. To evaluate this hypothesis, peroxynitrite or hydrogen peroxide was added to a GSH solution in vitro. A hydrogen peroxide dose dependently oxidized GSH to the disulfide (Fig. 7A). In contrast, peroxynitrite caused only minimal GSSG formation. Based on the observation that more than 70% of the glutathione was not recovered as GSH or GSSG, it can be concluded that peroxynitrite oxidized the sulfhydryl group to higher oxidation states (Fig. 7A). When peroxynitrite was added to a BSA solution, nitration of the protein could be detected spectrophotometrically through the absorbance of the phenolate anion of nitrotyrosine (Fig. 7B). Addition of 1 mM GSH completely prevented protein nitration (Fig. 7B).

Discussion

The objective of this investigation was to test the hypothesis that peroxynitrite is an important mediator of liver cell injury after AAP overdose. Our approach was to use intravenous GSH injection as a tool to accelerate the recovery of cytosolic and mitochondrial GSH levels at a critical time of peroxynitrite formation. Sulfhydryl reagents are known to be potent peroxynitrite scavengers (Radi et al., 1991; Kirsch et al., 2001). Using an in vitro system, we could confirm this. In contrast to hydrogen peroxide, peroxynitrite did not cause oxidation of GSH to the disulfide (GSSG). Nevertheless, that GSH was consumed and nitrotyrosine formation, a footprint of peroxynitrite (Beckman, 1996), was prevented in vitro

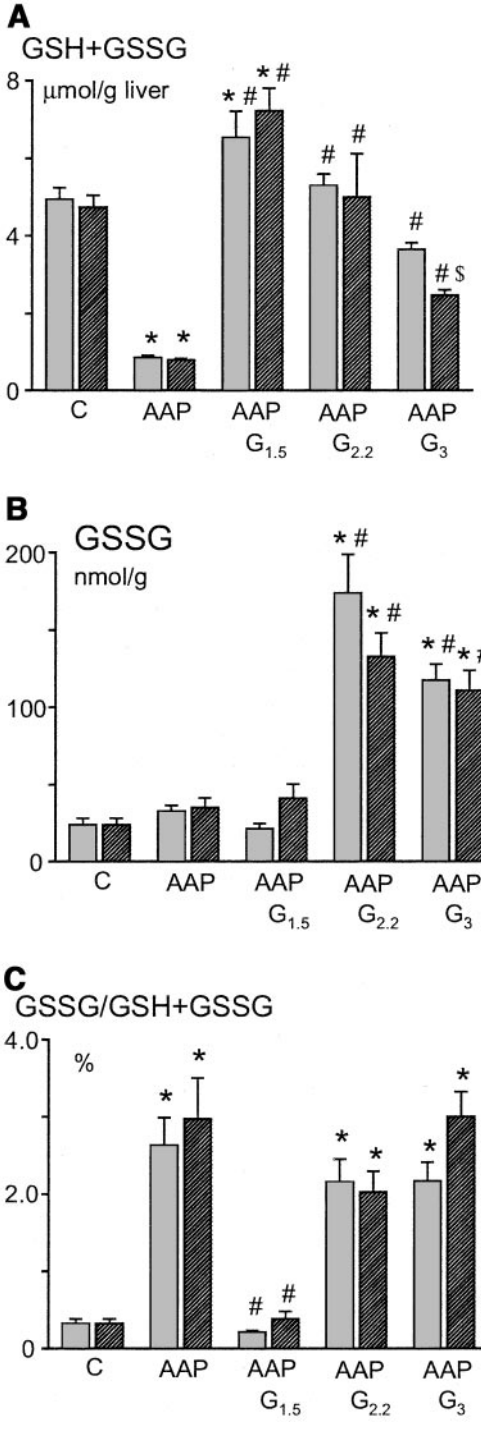

Fig. 6. Liver glutathione content in wild-type animals (open bars) or Gpx1−/− mice (striped bars). Animals were either untreated or received acetaminophen (300 mg of AAP/kg). A, total glutathione (GSH + GSSG); B, GSSG; C, percentage of GSSG of total glutathione. All data are given in GSH-equivalents. Data represent means ± S.E. of $n = 5$ animals/group. ⋆, $P < 0.05$ [compared with controls (C)]; #, $P < 0.05$ (compared with AAP); $, $P < 0.05$ (compared with wild-type).

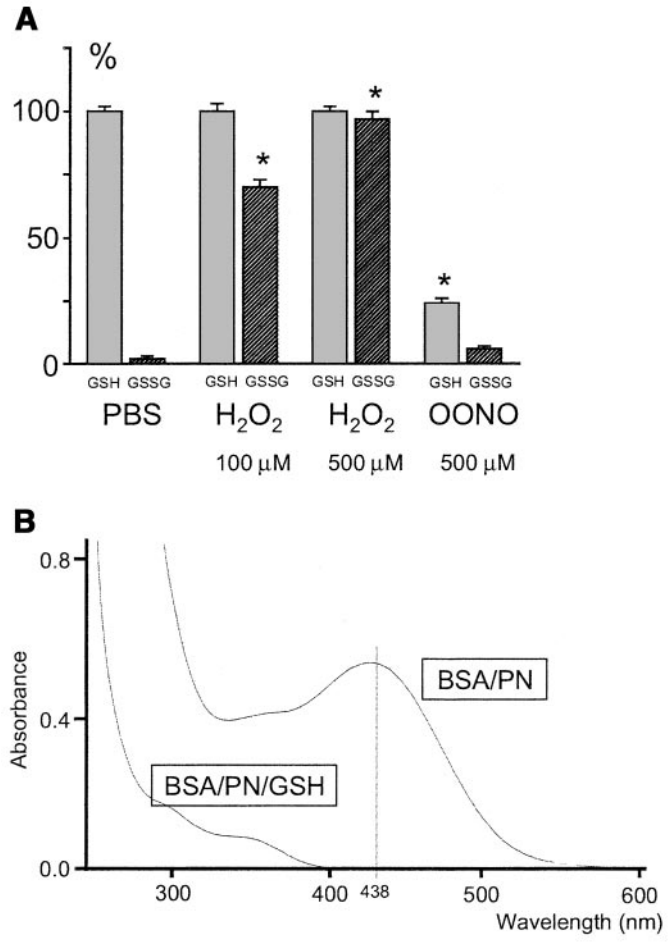

Fig. 7. A, oxidation of GSH in 10 mM phosphate-buffered saline (PBS) (pH 7.4; 37°C) by hydrogen peroxide (H_2O_2) or peroxynitrite (ONOO). Total glutathione (GSH + GSSG) and GSSG were determined 10 min after addition of the oxidant. Data are given as the percentage of the GSH concentration at $t = 0$ min (100 μM). Data represent means ± S.E. of $n = 3$ separate incubations. ⋆, $P < 0.05$ (compared with PBS controls). B, effect of GSH on protein nitration by peroxynitrite (PN). Nitration of BSA was determined spectrophotometrically as the intensely yellow phenolate of nitrotyrosine at 438 nm. PN (0.5 mM) was added to BSA-carbonate buffer (final concentration of 2 mg/ml; pH 9.6) in the presence or absence of 1 mM GSH. A spectrum was recorded, and the amount of nitrotyrosine in the peroxynitrite-treated BSA was calculated by reading the absorbance maximum of the phenolate ion at 438 nm.

suggested that GSH scavenged peroxynitrite. Since peroxynitrite did not cause relevant GSSG formation, all GSSG measured in the total liver or mitochondrial compartment had to be generated by hydrogen peroxide through spontaneous oxidation or enzymatically via Gpx1. As shown previously, AAP toxicity causes increased GSSG formation, particularly in mitochondria, which is indicative of increased reactive oxygen formation (Jaeschke, 1990; Knight et al., 2001). The mitochondrial oxidant stress after AAP administration is one of the consequences of mitochondrial dysfunction, also reflected by reduced ATP levels (Tirmenstein and Nelson, 1989; Jaeschke, 1990; Knight et al., 2001), mitochondrial cytochrome *c* release (Knight and Jaeschke, 2002), and impaired respiration (Meyers et al., 1988; Ramsay et al., 1989). Mitochondrial dysfunction per se, however, may be responsible for hepatocellular cell death without direct involvement of peroxynitrite.

To investigate the role of peroxynitrite formation in AAP hepatotoxicity, we used administration of pharmacological doses of GSH. It was previously shown that intravenously injected GSH is rapidly degraded in the kidney with a half-life in plasma of less than 5 min in starved animals (Wendel and Jaeschke, 1982). The amino acids are reabsorbed and used in the liver and other organs to resynthesize GSH

within 1 to 2 h (Wendel and Jaeschke, 1982). As a pretreatment, GSH administration restored the hepatic GSH content in fasted animals to the lowest levels observed in fed mice and protected against AAP hepatotoxicity (Wendel et al., 1982). Although hepatic GSH levels undergo diurnal variations that are caused by the nocturnal feeding habit of rodents, fasting eliminates this effect (Jaeschke and Wendel, 1985). GSH administration in fasted mice cause a similar increase of the hepatic GSH content to the lowest levels found in fed animals, independent of the time of day (Wendel and Jaeschke, 1982; Jaeschke and Wendel, 1985). Thus, diurnal variations in GSH levels could not be responsible for the differential protective effects of GSH injections at various times after AAP. Our study showed that GSH treatment even after AAP administration led to a faster recovery of the depleted hepatic GSH levels in hepatocytes. The intervention, however, was even more effective in restoring the mitochondrial GSH content. Since only nitrotyrosine, but not GSSG, formation was affected by GSH treatment at 1.5 or 2.25 h, these findings suggest that GSH administration at that time improved the scavenger capacity of the cell for reactive oxygen and in particular peroxynitrite. Despite ongoing mitochondrial oxidant stress, however, these livers show significantly less injury. Thus, our data suggest that peroxynitrite is actually an important mediator that significantly contributes to the hepatotoxicity of AAP. This is the first direct evidence for a role of peroxynitrite in AAP-induced cell injury. Previous attempts to address this question using iNOS gene knockout mice and NOS inhibitors yielded conflicting results. Gardner et al. (1999) showed hepatoprotection in iNOS gene knockout mice. These findings could not be confirmed by Hinson and coworkers (Michael et al., 2001). Similarly, the protective effect of the iNOS inhibitor aminoguanidine against AAP toxicity in rats (Gardner et al., 1998) was not found in the mouse model (Hinson et al., 2002). Thus, the source of intracellular NO formation is still controversial, and more studies are necessary to clarify this important issue.

Although hepatic GSH is a highly effective physiological scavenger of peroxynitrite, one relevant concern with using GSH is that it can also react with NAPQI. Previous time course studies of AAP metabolism in mice, however, indicated that the most extensive NAPQI formation occurs during the first 90 min after AAP injection (Roberts et al., 1991). By 30 min, GSH is completely depleted in mitochondria and the cytosol (Knight et al., 2001). AAP protein adduct formation reached a maximum at 1 h with no further significant increase during the second hour (Roberts et al., 1991). Thus, we expected that treatment with GSH at the time of AAP administration would improve scavenging of NAPQI and, therefore, would prevent toxicity. Indeed, injection of GSH with AAP prevented mitochondrial oxidant stress and peroxynitrite formation, which resulted in complete protection. These results were used as a control for an intervention that directly reacted with the reactive intermediate and eliminated the mitochondrial oxidant stress and all subsequent events. In contrast, the results were different when GSH was injected at 1.5 or 2.25 h after AAP administration. Here we saw no effect on the mitochondrial oxidant stress but a selective reduction of hepatic nitrotyrosine levels. These results suggest that later GSH administration selectively acted as a peroxynitrite scavenger without preventing the mitochondrial oxidant stress. If GSH was injected at 3 h, however, nitrotyrosine formation was not attenuated, and the protective effect was lost. This indicates that there is a critical window where peroxynitrite needs to be eliminated to attenuate cell injury. These results may also provide the explanation why *N*-acetylcysteine administration after AAP overdose in humans is at least partially effective in preventing injury when administered at later time points.

Recently, Sies and coworkers (1997) provided evidence that Gpx1 is able to metabolize peroxynitrite in vitro. Our results with Gpx1−/− mice, however, did not support the relevance of this mechanism in vivo. Gpx1−/− mice actually were not more susceptible to AAP than wild-type animals. Moreover, GSH administration protected similarly in wild-type/Gpx1−/− mice as in C3Heb/FeJ animals. The fact that the response to GSH injection at 1.5 h was the same as administration at time 0 in C3Heb/FeJ mice suggests a slower metabolism in wild-type/Gpx1−/− animals. Nevertheless, GSH injected at 2.25 h acted again as a peroxynitrite scavenger and protected against liver injury. These findings support the conclusion that peroxynitrite is scavenged by GSH in a spontaneous reaction. Furthermore, increased GSSG formation in Gpx1−/− mice also indicates that, in the absence of Gpx1, hydrogen peroxide reacted spontaneously with GSH. Thus, our study cannot exclude a potential deleterious effect of hydrogen peroxide and other reactive oxygen species. Hydroxyl radical formation and lipid peroxidation, however, only occur to a very limited degree in these livers (Mitchell et al., 1984; Michael et al., 2001). Therefore, we can conclude that the impact of reactive oxygen species on the injury is most likely considerably less than that of peroxynitrite. Despite the increasing evidence that supports a critical role of peroxynitrite in the pathophysiology of AAP-induced liver cell injury, the events following peroxynitrite formation are unclear. Peroxynitrite is a nitrating agent and a potent oxidant, which can cause oxidative damage to all types of cellular macromolecules (Squadrito and Pryor, 1998). Further studies are necessary to investigate the downstream mechanisms of AAP toxicity after peroxynitrite formation.

This study provides other important mechanistic information. Theoretically, NAPQI formation may affect a limited number of mitochondria. Peroxynitrite generated by these dysfunctional mitochondria could cause damage to additional mitochondria and thereby amplify the original insult (Cassina and Radi, 1996). Such an amplification mechanism involving mitochondria has recently been described for Fas receptor-mediated apoptosis in hepatocytes (Bajt et al., 2000, 2001). If peroxynitrite had been involved in amplifying the insult, however, GSH treatment should have attenuated the mitochondrial oxidant stress (Lizasoain et al., 1996). Yet, the mitochondrial oxidant stress was unaffected by late treatment with GSH. These results are not consistent with the original hypothesis and suggest that the mitochondrial oxidant stress does not appear to be amplified by peroxynitrite formation.

In summary, our results indicate that AAP induced mitochondrial oxidant stress, peroxynitrite formation, and hepatocellular necrosis. Early GSH administration completely prevented the mitochondrial oxidant stress, peroxynitrite formation, and liver cell injury by scavenging NAPQI. Administration of GSH at 1.5 or 2.25 h, however, enhanced the recovery of the cytosolic and mitochondrial GSH levels. The

drastic reduction of nitrotyrosine staining in combination with enhanced mitochondrial GSSG formation indicates that the newly synthesized GSH acted as a peroxynitrite scavenger without preventing the mitochondrial oxidant stress. These data support the conclusion that peroxynitrite is an important cytotoxic mediator of AAP-induced liver cell necrosis.

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