

Acetaminophen-Induced Oxidant Stress and Cell Injury in Cultured Mouse Hepatocytes: Protection by *N*-Acetyl Cysteine

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The increase in cellular and mitochondrial glutathione disulfide (GSSG) levels and the GSSG:GSH ratio after acetaminophen (AAP) overdose suggest the involvement of an oxidant stress in the pathophysiology. However, the initial severe depletion of hepatocellular glutathione makes quantitative assessment of the oxidant stress difficult. Therefore, we tested the hypothesis that oxidant stress precedes the onset of cell injury in a cell culture model using 2',7'-dichlorofluorescein (DCF) fluorescence as a marker for intracellular oxidant stress. Cultured primary murine hepatocytes were exposed to 5 mM AAP. DCF fluorescence, XTT reduction, lactate dehydrogenase (LDH) release, and trypan blue uptake were determined from 0 to 12 h. After glutathione depletion at 3 h, DCF fluorescence increased by 16-fold and was maintained at that level up to 12 h. At 1.5 h after AAP, a significant decrease of the cellular XTT reduction capacity was observed, which continued to decline until 9 h. Cell necrosis (LDH release, trypan blue uptake) was detectable in 20% of cells at 6 h, with a significant further increase at later time points. Pretreatment with 20 mM *N*-acetylcysteine (NAC) 1 h before AAP enhanced cellular glutathione content, prevented or attenuated the AAP-induced decrease of GSH levels and XTT reduction capacity, respectively, and reduced the loss of cell viability. Additionally, treatment with NAC 2 h after AAP exposure prevented further deterioration of XTT reduction at 3 h and later, and attenuated cell necrosis. Thus, AAP-induced oxidant stress precedes cell necrosis and, in cultured hepatocytes, the oxidant stress is involved in the propagation of cell injury.

Key Words: acetaminophen; hepatotoxicity; oxidant stress; *N*-acetylcysteine; cultured hepatocytes.

Acetaminophen overdose is the most frequent cause of drug-induced liver injury in the U.S. and the U.K. (Lee, 2003). Despite substantial progress in our understanding of the mechanism of hepatocellular injury during the last 30 years, many aspects of the pathophysiology are still unknown or controversial (Jaeschke *et al.*, 2003; Nelson and Bruschi, 2003). There is general

consensus that the formation of an electrophilic metabolite by the P450 system, presumably *N*-acetyl-*p*-benzoquinone imine (NAPQI), is a prerequisite for the injury (Nelson and Bruschi, 2003). NAPQI is effectively detoxified by glutathione (Mitchell *et al.*, 1973). However, after the cellular glutathione stores are depleted, the reactive metabolite covalently binds to a substantial number of cytosolic and mitochondrial proteins (Cohen *et al.*, 1997). As a consequence, impaired mitochondrial respiration (Meyers *et al.*, 1988; Ramsay *et al.*, 1989), depletion of hepatocellular ATP levels (Jaeschke, 1990; Tirmenstein and Nelson, 1990), the opening of the mitochondrial membrane permeability transition pore (Kon *et al.*, 2003) and release of cytochrome *c* from the mitochondria (Adams *et al.*, 2001; Knight and Jaeschke, 2002) are observed.

During the recovery phase of the cellular glutathione content, substantial increases in the cellular and especially mitochondrial levels of glutathione disulfide (GSSG) are found (Jaeschke, 1990; Knight *et al.*, 2001). These data have been interpreted as evidence for a mitochondrial oxidant stress during acetaminophen hepatotoxicity. However, these conclusions are not without criticism. Although GSSG formation is generally considered a reliable and specific indicator for cellular oxidant stress (Jaeschke *et al.*, 1988; Lauterburg *et al.*, 1984; Smith, 1991), an interfering factor is the severe initial depletion of cytosolic and mitochondrial GSH after AAP overdose. Because of these low GSH levels, only a small shift of the GSSG-to-GSH ratio to a more oxidized state can be observed (Knight *et al.*, 2001). It takes at least 4 h or longer until enough GSH is re-synthesized to measure GSSG levels higher than baseline, which would be considered solid evidence for an intracellular oxidant stress (Jaeschke, 1990; Knight *et al.*, 2001). However, at that time, the release of cytosolic liver enzymes indicates cell injury. Thus, Smith and coworkers argue that this oxidant stress is most likely a consequence of cell injury rather than an early event, which might be relevant for the mechanism of cell death (Rogers *et al.*, 2000; Smith *et al.*, 1985;). Since the depletion of GSH is a prerequisite for the injury (Mitchell *et al.*, 1973), measurement of GSSG formation cannot unequivocally answer the question whether the oxidant stress precedes cell injury or is only a late

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event of the injury. Therefore, we used 2',7'-dichlorofluorescein (DCF) fluorescence as a marker of oxidant stress in a cell culture model to evaluate the time course of reactive oxygen formation in relationship to cell viability.

MATERIALS AND METHODS

Animals. Male C3Heb/FeJ mice with an average weight of 18 to 20 g were purchased from Jackson Laboratory (Bar Harbor, Maine). All animals were housed in an environmentally controlled room with 12-h light/dark cycle and allowed free access to food (certified rodent diet no. 8640, Harlan Teklad, Indianapolis, IN) and water. All experimental protocols followed the criteria of the University of Arizona and the National Research Council for the care and use of laboratory animals in research.

Mouse hepatocyte isolation. Primary hepatocytes were isolated from mice anesthetized with pentobarbital sodium solution (Nembutal, Abbott Laboratories, North Chicago; 50 mg/kg ip) as previously described (Hatano *et al.*, 2000; Shen *et al.*, 1991) with some modifications. Briefly, the inferior vena cava was cannulated and the liver was first perfused *in situ* with an oxygenated Hanks' buffer salt solution (HBSS) containing 100 U/ml penicillin/streptomycin (Gibco, Grand Island, New York), pH 7.4 (8 ml/min, 37°C for 10 min), followed by perfusion with oxygenated HBSS containing 1 mM Ca²⁺ and Mg²⁺, penicillin/streptomycin (100 U/ml), and 0.04% collagenase D (Roche Molecular Biochemicals, Mannheim, Germany), pH 7.4 for 10 min. The liver was removed and then gently minced in HBSS containing 1 mM Ca²⁺ and Mg²⁺, penicillin/streptomycin (100 U/ml), and 1 × 10⁻⁷ M insulin (Sigma), pH 7.4. The liver cell suspension was then filtered with Falcon cell strainers (40, 70, and 100 µm; Becton Dickinson, Bedford, MA) and centrifuged at 50 × g for 2 min. From the isolation of one mouse liver, a typical yield was about 50–60 × 10⁶ hepatocytes. Cell viability, as determined by trypan blue exclusion, was generally >90%, and cell purity was >95% hepatocytes. Cells were plated on 6-well plates (6 × 10⁵ cells/well) or 24-well plates (8 × 10⁴) (Biotec collagen I cellware plates; Becton Dickinson) in Williams' Medium E (Gibco) containing 10% fetal bovine serum (Gibco), 100 U/ml penicillin/streptomycin, and 1 × 10⁻⁷ M insulin and cultured at 37°C with 5% CO₂. After an initial 4-h attachment period, cultures were washed with phosphate-buffered saline (PBS) and then plain culture medium (controls) or media containing various concentrations of AAP were added. Preliminary experiments indicated that concentrations of 5–10 mM AAP caused a dose-dependent cell injury in cultured mouse hepatocytes within 6–12 h. We selected 5 mM AAP for all further experiments. In some experiments, cells were treated with 20 mM *N*-acetylcysteine (dissolved in 10X PBS, pH 7.4) (Harman and Self, 1986) either 1 h before or 2 h after AAP administration.

Cell viability. Cell viability was assessed by trypan blue uptake and LDH release. After removal of the cell medium, hepatocytes were incubated with 0.8% trypan blue solution for 3 min at room temperature. Trypan blue-positive cells were counted in 4 different fields (×10; a total of approximately 1500 cells). For LDH release measurements, medium was removed from cells and lysis buffer containing 25 mM HEPES, 5 mM EDTA, 0.1% CHAPS, and 1 mg/ml each of pepstatin, leupeptin, and aprotinin, pH 7.5, was added to the hepatocytes for 5 min. Cells were removed from wells with a cell scraper and placed into a test tube. After sonication, cells were centrifuged for 20 min at 15,000 rpm at 4°C. Aliquots of the cell lysate or medium added to a reaction mixture in potassium phosphate buffer (60 mM, pH 7.5) containing 0.72 mM pyruvate and 216 mM NADH. The kinetics of absorbance decrease at 340 nm was measured with a spectrophotometer (UV-1601PC, Shimadzu Scientific Instruments, Columbia, MD).

Glutathione. For cell glutathione measurements, media was removed and 0.5 ml of 3% sulfosalicylic acid was added to the cells. Each well was scraped with a cell scraper and the precipitated proteins centrifuged for 5 min. The acidic supernatant was diluted in 100 mM potassium phosphate buffer (KPP), pH 7.4, and assayed with a modified Tietze assay as described (Jaeschke and Mitchell,

1990). A 10% SDS solution was added to the pellet to solubilize the proteins. Protein concentrations were assayed using the bicinchoninic acid kit (Pierce, Rockford, IL). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise.

Detection of reactive oxygen intermediates. Cultured hepatocytes were treated with 5 mM AAP for 1.5, 3, 6, 9, or 12 h. At the indicated time points, hepatocytes were rinsed in phosphate-buffered saline (PBS). Cells were loaded with 1 mM 5- and 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA; Molecular Probes, Inc., Eugene, OR) for 30 min. CM-H₂DCFDA, a cell-permeable indicator for reactive oxygen species, is non-fluorescent until hydrolyzed by intracellular esterases and oxidized by intracellular reactive oxygen species. After loading the cells, the linear increase in fluorescence intensity was monitored in a thermostated fluorescence microplate reader (Spectra Max Gemini EM, Molecular Devices, Corp., Sunnyvale, CA) at 37°C for 1 h, using an excitation wavelength of 490 nm and an emission wavelength of 530 nm. The slope of the curve was used to calculate the change in fluorescence intensity per min.

XTT assay. Cell viability was also determined using the 2,3-bis[2-Methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxyanilide inner salt (XTT) system according to the manufacturer's instructions (Sigma). The XTT assay measures the activity of mitochondrial and extramitochondrial dehydrogenases (Bernas and Dobrucki, 2002; Huet *et al.*, 1992) and therefore provides an indicator of overall functional cell viability. The tetrazolium ring of XTT is cleaved by dehydrogenases of viable cells to produce soluble orange formazan, which can be detected spectrophotometrically. After adding XTT, the cells were incubated for 2 h and the increase in formazan absorbance was read at a wavelength of 450 nm on a microplate reader (SpectraMax 190, Molecular Devices, Corp., Sunnyvale, CA).

Statistics. All results were expressed as mean ± SE. Comparisons between multiple groups were performed with one-way ANOVA or, where appropriate, by two-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

Measurement of DCF fluorescence as indicator of reactive oxygen formation after exposure to AAP showed no significant change at 1.5 h (Fig. 1). However, between 3.5 and 12.5 h after AAP treatment, DCF fluorescence was increased >10-fold above values of untreated cells (Fig. 1). The oxidant stress was first detectable when hepatocellular GSH levels reached their lowest values at 3 h after AAP exposure (data not shown). Evaluation of LDH release and trypan blue uptake as indicators of cell injury in these experiments showed no significant increase in cell membrane permeability up to 3 h after AAP exposure (Fig. 2). At 6 h, about 20% of cells were trypan blue-positive (Fig. 2A), which correlated well with the cellular LDH release of 17% above untreated cells (Fig. 2B). Between 6 and 12 h, cell injury progressed rapidly. More than 90% of all cells were trypan blue-positive and 65% of the entire cellular LDH content was released into the cell culture medium at 12 h after AAP treatment (Fig. 2).

Since trypan blue uptake and LDH release assess the permeability of the cell membrane, we used the XTT assay to evaluate cell viability with a functional parameter. This assay depends on cellular respiration, i.e., the activity of mitochondrial and

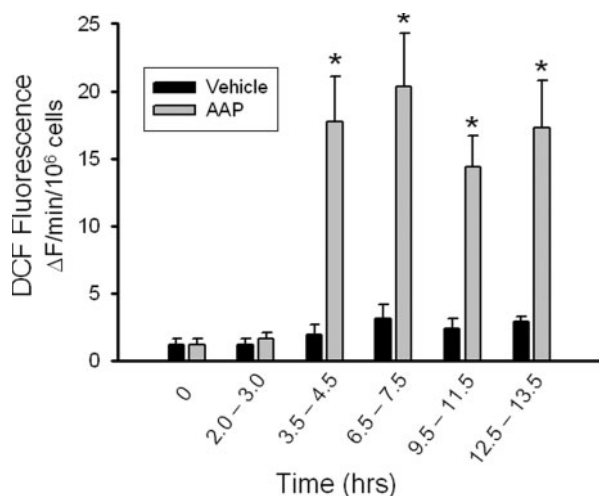


FIG. 1. Time course of 2',7'-dichlorofluorescein (DCF) fluorescence in cultured murine hepatocytes exposed to 5 mM acetaminophen (AAP) or vehicle (culture media) for 1.5, 3, 6, 9, or 12 h: After removal of the cell culture medium, cells were loaded with 5- and 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (dissolved in PBS) for 30 min, and the increase in fluorescence was monitored for 60 min. Data are given as mean \pm SE for each time period of 4 separate time-course experiments; * $p < 0.05$ (compared to vehicle control at each time period).

extramitochondrial dehydrogenases. At various times after AAP treatment, XTT was added to the cell culture medium, and the cells were incubated for an additional 2 h. Compared to time-matched control cells, the capacity to reduce XTT was unchanged during the assay period between 0.75 and 2.75 h after AAP treatment (Fig. 3). However, cellular respiration was reduced by 35% between 1.5 and 3.5 h, by 55% between 3 and 5 h, by 67% between 6 and 8 h, and by 74% between 9 and 11 h. There was no significant further decline of the respiration at 12 h (Fig. 3). Taken together, these time course experiments indicate that the onset of a functional deterioration of the cell correlates with the nadir of cellular glutathione levels, which is followed by increases in intracellular oxidant stress and later by cell necrosis as indicated by the permeability increases of the cell membrane and cell content release.

Since our data showed that the oxidant stress preceded loss of cell viability, we assessed whether enhanced recovery of cellular glutathione levels could reduce cell injury, as previously demonstrated *in vivo* (Bajt *et al.*, 2003; Knight *et al.*, 2002). We added *N*-acetylcysteine (final conc. 20 mM NAC) to cells either 1 h before or 2 h after AAP. Compared to control cells (15.9 ± 1.1 nmol GSH-equivalents/mg protein) (Fig. 4A), cells pretreated with NAC for 1 h had significantly higher hepatocellular GSH levels (32.0 ± 1.5 nmol GSH-equivalents/mg protein) (Fig. 4B). Exposure of hepatocytes to AAP resulted in a time-dependent decline of GSH levels in controls (Fig. 4A) but not in NAC-treated cells (Fig. 4B). In contrast, cells treated with NAC at 2 h after AAP (Fig. 4B) showed the initial decline up to 1.5 h and then a rapid recovery to levels similar to untreated controls (Fig. 4A) or NAC-pretreated cells (Fig. 4B). Thus, both

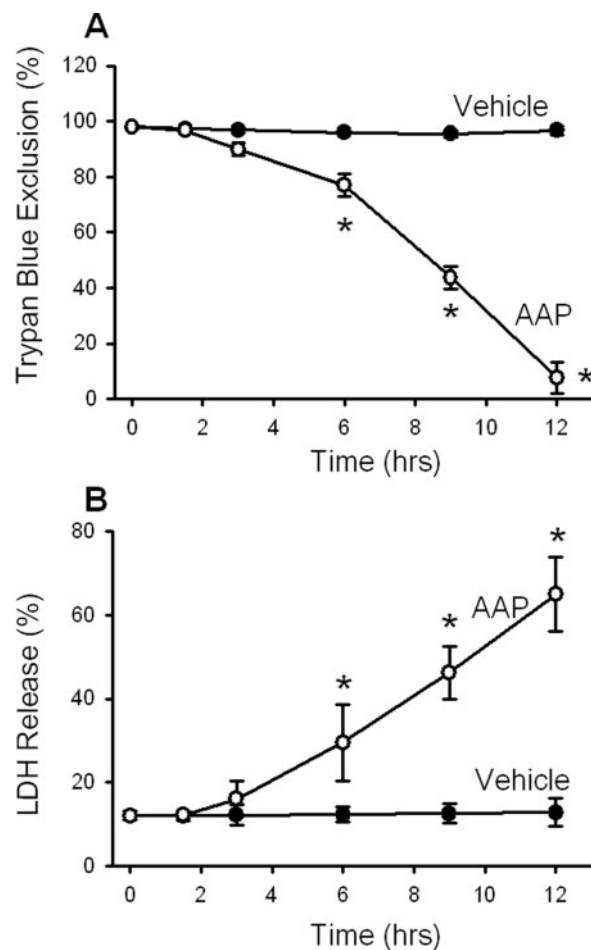


FIG. 2. Time course of cell injury as indicated by trypan blue exclusion (A) and release of lactate dehydrogenase (LDH) (B) in cultured murine hepatocytes exposed to 5 mM acetaminophen (AAP) or vehicle (culture media) for 0.75–12 h. The percent of viable (trypan blue-negative) cells are shown for each time point. LDH was measured in the culture supernatant and after lysis of the cells. Results are expressed as the percentage of LDH released into the supernatant of the total LDH. Data are given as mean \pm SE for each time point of 4 separate time-course experiments; * $p < 0.05$ (compared to vehicle control at each time point).

NAC-treated groups had substantially higher GSH levels at 3–6 h when compared to AAP alone. Consistent with the higher detoxification capacity for reactive oxygen species, significantly less DCF fluorescence was measured in AAP + NAC-treated cells at 6 h compared to AAP alone (Fig. 5). The XTT assay demonstrated significantly improved respiration of cells pretreated with NAC at all time points except the initial 2-h period after AAP addition (Fig. 6). In addition, the further deterioration of the cellular respiration between 3 and 6 h after AAP was prevented in cells treated with NAC at 2 h after AAP (Fig. 6). As a result, both NAC-treated groups had significantly less trypan blue-positive cells at 9 and 12 h compared to AAP alone (Fig. 7). Since we observed some loss of cell viability, even in cells pretreated with NAC at 12 h after AAP, compared to

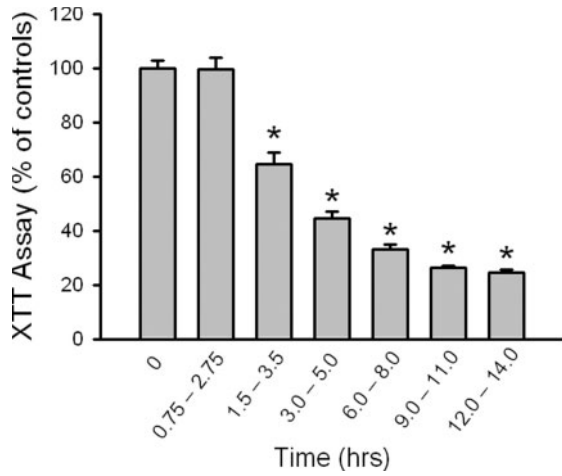


FIG. 3. Time course of functional viability (XTT assay) of cultured murine hepatocytes exposed to 5 mM acetaminophen (AAP) or vehicle (culture media) for 0.75–12 h: After removal of the cell culture medium, cells were incubated with XTT for 2 h and the increase in absorbance was measured. Results are shown as the XTT absorbance of AAP-treated cells expressed in percent of controls at each time point. Data are given as mean ± SE of 4 separate time-course experiments; **p* < 0.05 (compared to *t* = 0).

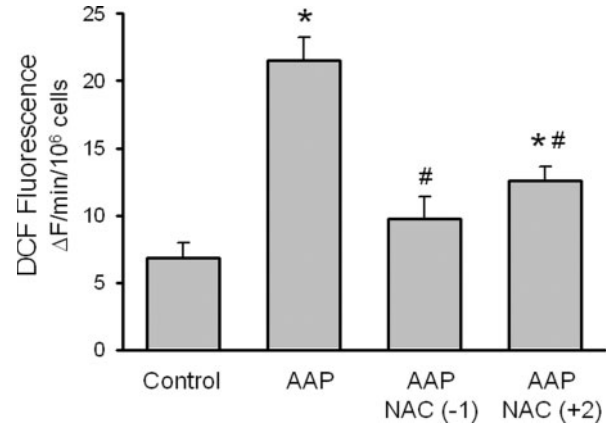


FIG. 5. Reactive oxygen formation as indicated by 2',7'-dichlorofluorescein (DCF) fluorescence in cultured murine hepatocytes exposed to 5 mM acetaminophen (AAP) or vehicle (control) for 6 h: Some cells were treated with *N*-acetylcysteine (20 mM final concentration) 1 h before AAP (NAC-1) or 2 h after AAP (NAC + 2). After removal of the cell culture medium, cells were loaded with 5-and 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate for 30 min; and then the increase in fluorescence was monitored for 60 min. Data are given as mean ± SE for each time point of 4 experiments; **p* < 0.05 (compared to vehicle control); #*p* < 0.05 (compared to AAP alone).

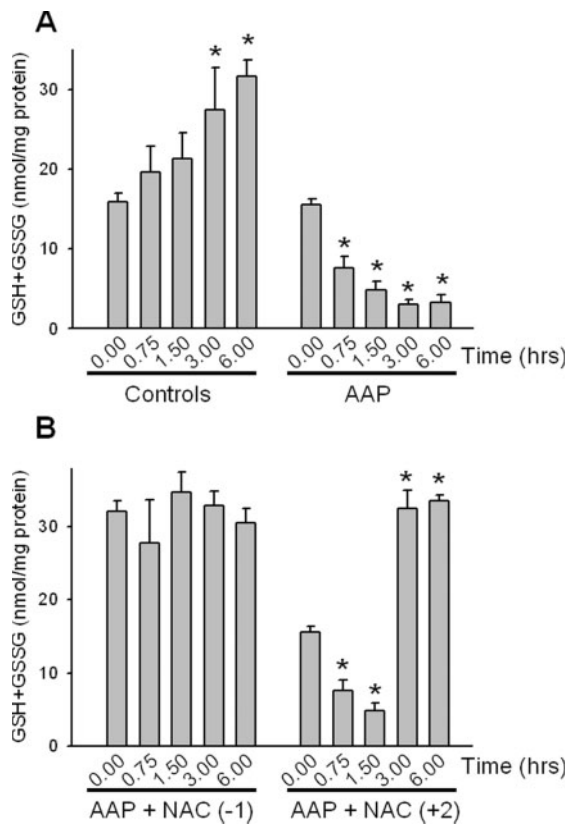


FIG. 4. Time course of changes in intracellular glutathione (GSH + GSSG) levels in cultured murine hepatocytes: Cells were incubated with vehicle or 5 mM acetaminophen AAP for 0.75–6 h (A). Some cells were treated with *N*-acetylcysteine (20 mM final concentration) 1 h before AAP or 2 h after AAP (B). Data are given as mean ± SE for each time point of 4 separate time course experiments; **p* < 0.05 (compared to *t* = 0).

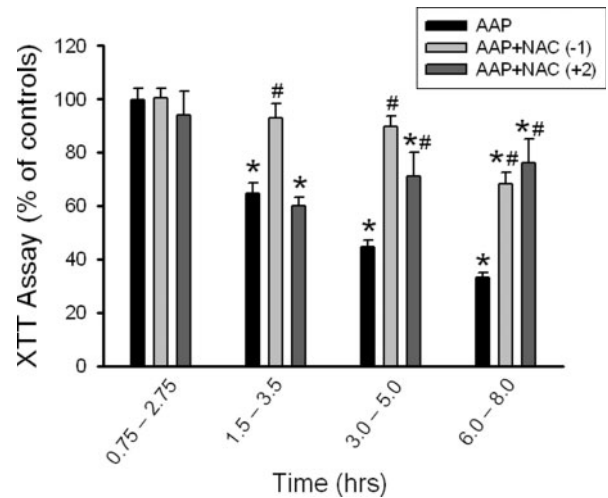


FIG. 6. Time course of functional viability (XTT assay) of cultured murine hepatocytes exposed to 5 mM acetaminophen (AAP) or vehicle (culture medium) for 0.75–6 h: Some cells were treated with *N*-acetylcysteine (20 mM final concentration) for 1 h before AAP (-1) or 2 h after AAP (+2). After removal of the cell culture medium, cells were incubated with XTT for 2 h, and the increase in absorbance was measured. Results are shown as the XTT absorbance of AAP-treated cells expressed in percent of vehicle-treated controls at each time point. Data are given as mean ± SE of 3 separate time course experiments; **p* < 0.05 (compared to *t* = 0); #*p* < 0.05 (compared to AAP alone).

untreated cells (Fig. 7), we evaluated trypan blue-uptake at 24 h. Whereas cell viability in AAP-treated cells further decreased at 24 h (11 ± 9%), there was no further loss in cell viability in NAC-treated cells compared to the 12-h time point (data not shown).

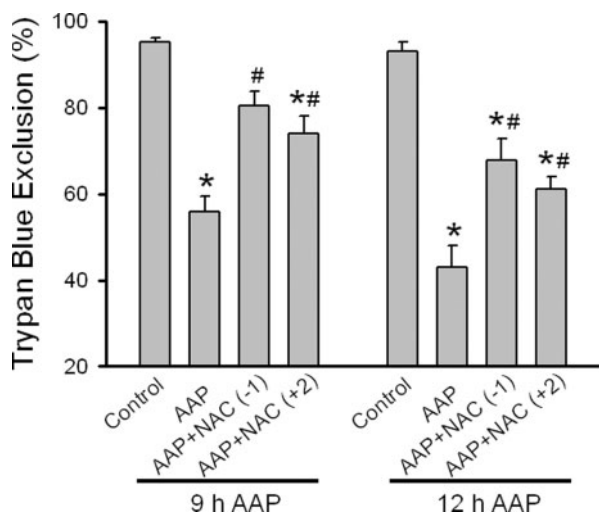


FIG. 7. Cell injury as indicated by trypan blue exclusion in cultured murine hepatocytes exposed to 5 mM acetaminophen (AAP) or vehicle (culture medium) for 9 or 12 h. Some cells were treated with *N*-acetylcysteine (20 mM final concentration) 1 h before AAP (-1) or -2 h after AAP (+2). The percent of viable (trypan blue-negative) cells are shown for each time point. Data are given as mean \pm SE for each time point of 4 separate experiments; * p < 0.05 (compared to vehicle control at each time point); # p < 0.05 (compared to AAP alone).

DISCUSSION

The main objective of this investigation was to test the hypothesis that oxidant stress precedes cell injury in murine hepatocytes exposed to AAP. Using 2',7'-dichlorodihydrofluorescein, which reacts with a number of oxidants including H_2O_2 and peroxynitrite, our data showed a rapid onset of an intracellular oxidant stress in cultured hepatocytes at the time of maximal GSH depletion. At the same time, the XTT assay indicated substantial functional deterioration of the cell. The XTT assay measures the oxidation of an artificial substrate by mitochondrial and extramitochondrial dehydrogenases and, therefore, reflects overall functional cell viability (Bernas and Dobrucki, 2002; Huet *et al.*, 1992). Thus, AAP caused a progressive impairment of dehydrogenase activity at the time of severe cellular GSH depletion but well in advance of increases in cell membrane permeability as indicated by trypan blue uptake and LDH release. These findings are consistent with observations *in vivo*, where the depletion of cytosolic and mitochondrial GSH during the first 30 min after AAP overdose is followed by an increase of the GSSG-to-GSH ratio (Knight *et al.*, 2001) and impairment of the mitochondrial respiration (Donnelly *et al.*, 1994; Meyers *et al.*, 1988) and ATP depletion (Jaeschke, 1990; Tirmenstein and Nelson, 1990) during the second h. Mitochondrial proteins are preferred targets of NAPQI (Cohen *et al.*, 1997). The importance of mitochondria is supported by the fact that AAP and the nonhepatotoxic isomer 3'-hydroxyacetanilide show similar overall protein binding, but there is substantially more covalent binding of mitochondrial proteins after

AAP (Qiu *et al.*, 2001). In addition, exposure of mouse hepatocytes to NAPQI simulated the mitochondrial dysfunction observed with AAP (Burcham and Harman, 1991). Although mitochondrial glutathione depletion and increased Ca^{2+} uptake may also contribute to mitochondrial dysfunction, these data suggest that covalent binding of NAPQI to mitochondrial proteins may play an important role in causing impaired mitochondrial respiration. As our present data show, the impaired mitochondrial function may cause the release of reactive oxygen. Moreover, early ultrastructural changes in mitochondria are observed with hepatotoxic doses of AAP (Placke *et al.*, 1987; Ruepp *et al.*, 2002), although some minor changes also are seen with subtoxic doses (Ruepp *et al.*, 2002). Most importantly, all these events occur several h before the onset of cell membrane permeability indicated by trypan blue uptake and LDH release.

A critical question remains: do this early oxidant stress and mitochondrial dysfunction actually contribute to cell death in these hepatocytes? To address this question, we enhanced cellular GSH levels before AAP or stimulated GSH synthesis, after exposure to AAP as previously shown *in vivo* (Bajt *et al.*, 2003; Knight *et al.*, 2002). In contrast to the *in vivo* situation, where intravenously supplied GSH is degraded in the kidney and the amino acids can be taken up by hepatocytes for *de novo* glutathione synthesis (Wendel and Jaeschke, 1982), cells in culture have to be supplied with precursor cysteine in the form of the nontoxic *N*-acetylcysteine (NAC). Treatment with NAC before exposure to AAP has been shown previously to attenuate covalent binding and cell injury *in vivo* (Corcoran *et al.*, 1985) and in isolated hepatocytes (Harman and Self, 1986). Our data demonstrate the enhanced baseline GSH levels after NAC pretreatment, which resulted in reduced loss of functional viability (XTT assay), less oxidant stress, and attenuated cell necrosis after AAP treatment. However, when NAC was supplied at 2 h after AAP exposure, the initial metabolic dysfunction had already occurred, as indicated by the XTT assay. Under these conditions, the rapid restoration of cellular glutathione levels within the next hour prevented the further deterioration of the cellular respiration and reduced the overall cell injury at 9, 12, and 24 h as indicated by less trypan blue uptake. These findings support the conclusion that NAC treatment 2 h after AAP exposure strengthened the detoxification potential of the GSH/glutathione peroxidase system as previously shown *in vivo* (Knight *et al.*, 2002). Consequently, these data suggest that the oxidant stress contributed to the progression of cell injury in this model.

A number of investigators concluded previously that reactive oxygen species might play a role in AAP-induced cell injury of cultured hepatocytes. Several studies showed a beneficial effect of treatment with vitamin E (Nagai *et al.*, 2002), the iron-chelator deferoxamine (Gerson *et al.*, 1985; Adamson and Harman, 1993) and catalase/superoxide dismutase (Kyle *et al.*, 1987). On the other hand, pretreatment with iron or inhibitors of glutathione peroxidase or glutathione reductase enhanced AAP toxicity in hepatocytes (Adamson and Harman, 1993; Gerson

et al., 1985; Kyle *et al.*, 1987). However, many of these findings obtained with cultured cells could not be reproduced *in vivo*. For example, neither the beneficial effect of iron chelation nor the enhanced injury with inhibition of glutathione reductase was observed after AAP overdose *in vivo* (Smith *et al.*, 1986; Smith and Mitchell, 1985). In addition, glutathione peroxidase gene-deficient mice were as susceptible to AAP as wild-type animals (Knight *et al.*, 2002). Moreover, despite positive effects against iron/allyl alcohol-induced lipid peroxidation and liver injury, treatment with α - or γ -tocopherol did not protect against AAP-induced liver injury in mice (Knight *et al.*, 2003). These discrepancies between the effects of antioxidants against liver cell damage *in vivo* versus cultured cells suggest a more prominent role of reactive oxygen species and lipid peroxidation in cell culture. As recently reviewed by Halliwell (2003), some of the higher oxidant stress in cultured cells may be due to the generally higher oxygen concentrations in incubators, compared to the oxygen levels hepatocytes are exposed to *in vivo*. However, while these experimental design issues may affect the exact mechanism of injury propagation, the answer to the most critical question addressed in this study, i.e., does the oxidant stress precede injury, should not have been affected.

In summary, our data demonstrate that AAP causes a rapid depletion of glutathione, a functional deterioration, and reactive oxygen formation in cultured hepatocytes during the first 3.5 h after exposure. Substantial cell necrosis indicated by the increase of cell membrane permeability occurs several h later. Treatment with NAC before or after AAP attenuates the functional deterioration of the cells and reduces cell necrosis. We conclude that AAP-induced oxidant stress precedes cell necrosis and that the oxidant stress is involved in the propagation of the cell injury in cultured primary mouse hepatocytes.

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