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LIPID PEROXIDATION AND PROTEIN THIOL DEPLETION ARE NOT INVOLVED IN THE CYTOTOXICITY OF *N*-HYDROXY-2-ACETYLAMINOFLUORENE IN ISOLATED RAT HEPATOCYTES

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Abstract—Freshly isolated rat hepatocytes were used to study the mechanism of cell death induced by *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF). Exposure to 1.0 mM N-OH-AAF resulted in more than 90% cell death (as measured by LDH leakage) of hepatocytes isolated from male rats within 6 hr. Only 36% of the hepatocytes isolated from female rats died within this period. When inorganic sulfate was omitted from the incubation medium, a 6 hr exposure to 1.0 mM N-OH-AAF resulted in only 40% cell death of male hepatocytes. These findings are in accordance with the sex difference and sulfation dependence of N-OH-AAF hepatotoxicity observed in the rat *in vivo*. N-OH-AAF decreased glutathione (GSH) in male hepatocytes in a concentration-dependent manner. This GSH consumption was only partly dependent on the presence of inorganic sulfate. No lipid peroxidation was observed during N-OH-AAF exposure; N-OH-AAF even prevented endogenous and diethyl maleate (DEM)-induced lipid peroxidation. No reduction of free protein thiol groups was found after exposure to N-OH-AAF, even after 75% cell death had occurred. A reduction of protein thiols after N-OH-AAF exposure was observed in GSH depleted hepatocytes (obtained by DEM plus vitamin E pretreatment). Under these conditions N-OH-AAF-induced cell death occurred earlier. Therefore, GSH protects against protein thiol depletion by N-OH-AAF in control cells. N-OH-AAF-induced cell death was preceded by a loss of intracellular ATP. It is concluded, therefore, that neither lipid peroxidation nor depletion of protein thiols, but possibly loss of intracellular ATP, is involved in the sulfation-dependent cytotoxic mechanism of N-OH-AAF in isolated rat hepatocytes.

One of the acute biological effects of the carcinogen *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF‡) in male rats *in vivo* is the induction of hepatotoxicity, characterized by necrosis of periportal hepatocytes [1]. For several reasons, this hepatotoxicity is relevant to the process of hepatocarcinogenesis by this compound. Firstly, it induces a regenerative mitogenic stimulus that may enhance both the process of initiation and promotion [2–4]. Secondly, it plays a role in the formation of (pre)neoplastic cells: the outgrowth of initiated cells during N-OH-AAF exposure is thought to result from their resistance towards its cytotoxic effects [5–10].

The endogenous thiol, glutathione (GSH), protects against cytotoxicity of many chemicals [11, 12]. For instance, metabolism of acetaminophen and bromobenzene leads to the formation of reactive electrophilic metabolites, which are responsible for hepatotoxicity due to their covalent binding to critical macromolecules in the cell. GSH, however, may trap and thereby detoxify these electrophiles [13, 14]. Also, GSH is an essential cosubstrate in the GSH–peroxidase catalyzed detoxification of reactive oxygen species, generated by hepatotoxic compounds such as menadione and adriamycin [15, 16], and only after depletion of GSH will these compounds elicit cytotoxicity.

The role of GSH in N-OH-AAF-induced cytotoxicity is less clear. We showed previously that N,O-sulfation leads to the formation of an unstable, reactive *N*-sulfate ester that covalently binds to nucleophilic groups in macromolecules [17–22]. Hepatic GSH was transiently decreased and biliary GSH-conjugates of N-OH-AAF were identified after administration of the carcinogen to male rats *in vivo* [23, 24]. The use of the sulfotransferase inhibitor pentachlorophenol showed that a major part of these GSH-conjugates stemmed from metabolites generated by the sulfation pathway [24]. However, pretreatment with diethylmaleate to deplete the intracellular GSH pool did not result in the anticipated increase in macromolecular binding of N-OH-AAF in rat liver *in vivo* [23]. Moreover, N-OH-AAF binds to hepatic macromolecules and elicits hepatotoxicity at doses far below those required to deplete hepatic GSH [24]. Thus, GSH does not seem to be very effective in protecting liver cells against the acute cytotoxic effects of N-OH-AAF.

In the present study we investigated the mechanism

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‡ Abbreviations: BSA, bovine serum albumin; LDH, lactate dehydrogenase; GSH, glutathione (reduced); GSSG, glutathione disulfide; MDA, malondialdehyde; DEM, diethylmaleate; vit.E, vitamin E (α-tocopherol); N-OH-AAF, *N*-hydroxy-2-acetylaminofluorene.

of N-OH-AAF hepatotoxicity and the role of GSH in freshly isolated rat hepatocytes. With this *in vitro* system, parameters relevant to GSH status and cytotoxicity, such as lipid peroxidation [16, 25, 26] and protein thiols [15, 16, 27], can easily be monitored. In addition to these, we also monitored intracellular ATP, since the loss of it has been associated with the hepatotoxicity of various chemicals [28–30].

MATERIALS AND METHODS

Chemicals. Collagenase was from Boehringer, Mannheim, F.R.G. N-OH-AAF was synthesized as described previously [21]. Bovine serum albumin (BSA) type V, α-tocopherol succinate (vit.E) and 5,5′-dithiobis(2-nitrobenzoic acid) (Ellmann's reagent) were from the Sigma Chemical Co., St Louis, MO, U.S.A. Diethyl maleate (DEM) was from Merck, Darmstadt, F.R.G. All other chemicals (analytical grade) were from J. F. Baker Chemicals, Deventer, The Netherlands.

Isolation and incubation of hepatocytes. SPF Wistar rats (230–270 g) of the Sylvius Laboratories, University of Leiden, outbred with the strain of CPB-Harlan, Zeist, The Netherlands, were used. Unless indicated otherwise, male rats were used. They were housed in macrolon cages with a standard hardwood bedding and had free access to food (MRH-B, Hope Farms, Woerden, The Netherlands) and tap water. An alternating 12 hr light and dark cycle was maintained in the animal rooms.

Rats were anesthetized with sodium pentobarbital (60 mg/kg; i.p.). Hepatocytes were isolated (between 10 and 12 a.m.) according to a collagenase perfusion method originally described by Seglen [31], with some modifications [32]. Thus, magnesium sulfate was omitted from the isolation buffers and replaced by an equimolar amount of $MgCl_2$. Routinely, more than 95% of the freshly isolated hepatocytes excluded Trypan Blue.

The cell suspension obtained was diluted to a final concentration of about 2×10^6 hepatocytes/mL in pre-oxygenated Hanks'–HEPES buffer, supplemented with 2.5% (w/v) BSA, pH 7.4. Calcium was omitted from the incubation buffer for reasons described previously [16]. This buffer contained either 80 mM $MgSO_4$ (sulfate-containing incubations) or 80 mM $MgCl_2$ (sulfate-free incubations). Cells were incubated in polyethylene vials on a rotary shaker (Adolf Kühner, AG, Switzerland) at 200 rpm and 37°. The oxygenation of hepatocytes with 95% CO_2/5% O_2 was performed as described [32].

Hepatocytes were preincubated for 30 min before the addition of the N-OH-AAF solution. Due to its slow rate of dissolution, this compound was dissolved in aqueous 0.25 N NaOH solution and aqueous 8 N HCl solution was added until N-OH-AAF precipitated. Aqueous 2 N NaOH solution was added then until N-OH-AAF was just dissolved; the volume was adjusted with water as required. No breakdown of N-OH-AAF occurred under these conditions [21]. The cells were exposed to N-OH-AAF (at 0.2, 1.0 and 2.5 mM) for 6 hr. Both DEM and vit.E (dissolved in ethanol; final concentration 5 mM and 75 mM respectively) were added to the incubation vials. After evaporation of ethanol, the hepatocyte suspension was added and incubated for 30 min prior to exposure to N-OH-AAF.

Analysis. Cell viability was determined by measuring LDH leakage from cells [31] as described previously [33]. GSH was determined according to the colorimetric method of Saville [34] as described elsewhere [33]. As an index for lipid peroxidation, the amount of malondialdehyde (MDA) production was determined by the thiobarbituric acid assay [33, 35]. Cellular protein thiols were determined with a method using Ellman's reagent as described by DiMonte *et al.* [15] and performed as in Dogterom *et al.* [36]. N-OH-AAF did not interfere with the GSH and protein thiol assay. Cellular protein was determined by the method of Lowry *et al.* [37], using BSA as standard. The ATP measurement was based on the bioluminescence assay as originally described by Kimmich *et al.* [38] and performed as described in Boogaard *et al.* [39].

RESULTS

The role of sulfation in N-OH-AAF-induced cytotoxicity

The cytotoxicity of N-OH-AAF towards hepatocytes of male rats was concentration-dependent (Fig. 1A). At 0.2 mM, N-OH-AAF was only slightly toxic during a 6 hr incubation, while 1.0 mM N-OH-AAF resulted in over 90% cell death. At the highest concentration of N-OH-AAF (2.5 mM) cytotoxicity was observed within 2 hr, resulting in more than 90% cell death already after 4 hr exposure. Toxicity of the two lower N-OH-AAF concentrations (0.2 and 1.0 mM) was observed only in sulfate supplemented incubations, whereas 2.5 mM N-OH-AAF was also toxic in incubations without sulfate (Fig. 1B).

As N-OH-AAF is much less hepatotoxic towards female rats than towards males [17, 18], we also examined the cytotoxicity of N-OH-AAF towards hepatocytes isolated from females (in the presence of sulfate). At 0.2 mM N-OH-AAF, no toxicity was found during the 6 hr exposure, while 1.0 mM N-OH-AAF was only slightly toxic (Fig. 2); the toxic response was very similar to hepatocytes isolated from males and incubated without sulfate (Figs 1B and 2). However, 2.5 mM N-OH-AAF was clearly toxic and no difference in the cytotoxic response of female and male hepatocytes was observed (Figs 1A and 2).

N-OH-AAF-induced changes in GSH status

N-OH-AAF reduced GSH concentrations in hepatocytes in a concentration-dependent manner, whether they were supplemented with sulfate or not: the decrease was slightly more rapid in the presence of sulfate (Fig. 3). The effect of GSH depletion on N-OH-AAF cytotoxicity was investigated using DEM, which resulted in a more than 90% reduction of GSH levels within 30 min [40] (Fig. 4A). DEM itself is cytotoxic [33] (Fig. 4B), but this cytotoxicity can be completely prevented by inclusion of vit.E in the incubation medium without affecting the GSH depletion (Fig. 4A). The DEM/vit.E pretreatment

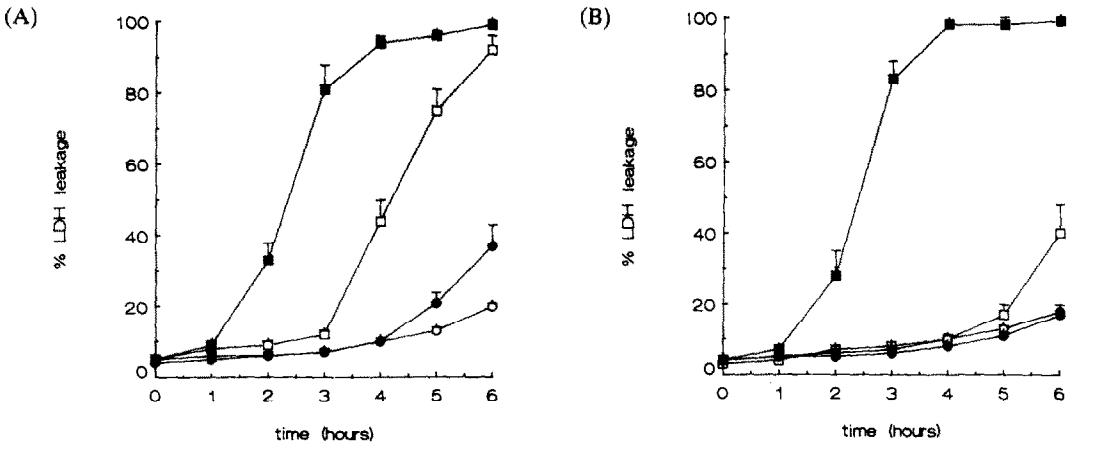

Fig. 1. N-OH-AAF-induced cell death in hepatocytes isolated from male rats. Hepatocytes were incubated and cell viability was determined as described in Materials and Methods. (A) Normal (sulfate supplemented) incubation. (B) Sulfate-deficient incubation. (○) Control; (●) 0.2 mM N-OH-AAF; (□) 1.0 mM N-OH-AAF; (■) 2.5 mM N-OH-AAF. Values represent mean ± SE of nine experiments.

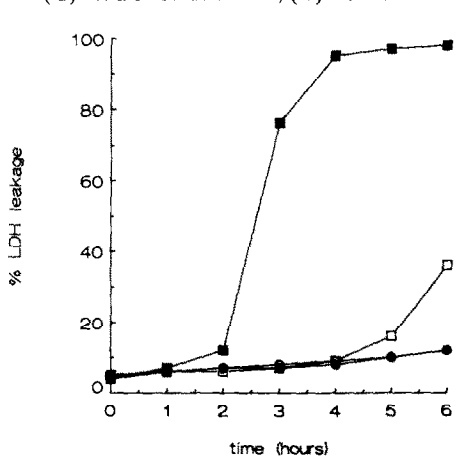

Fig. 2. N-OH-AAF-induced cell death in hepatocytes isolated from female rats. Hepatocytes were incubated and cell viability was determined as described in Materials and Methods. (○) Control; (●) 0.2 mM N-OH-AAF; (□) 1.0 mM N-OH-AAF; (■) 2.5 mM N-OH-AAF. Values represent mean ± SE of six experiments.

resulted in an earlier onset of N-OH-AAF hepatotoxicity (Fig. 5). Vit.E alone delayed the occurrence of cytotoxicity of 1.0 mM N-OH-AAF (but did not delay N-OH-AAF-induced GSH reduction) (Fig. 5).

Effect of N-OH-AAF on lipid peroxidation

Lipid peroxidation and subsequent cellular damage is regarded as an important mechanism underlying the toxicity of several xenobiotics [41, 42]. However, it does not play a role in N-OH-AAF-induced cytotoxicity: no lipid peroxidation was observed even at the highest concentration of 2.5 mM N-OH-AAF during the 6 hr exposure (Fig. 6A). On the contrary, N-OH-AAF proved to be an inhibitor of the peroxidation process, since it reduced control levels of lipid peroxidation (Fig. 6A) and prevented lipid peroxidation due to DEM (Fig. 6B).

N-OH-AAF-induced changes in protein thiols

N-OH-AAF, at the cytotoxic concentration of 1.0 mM, did not deplete protein thiols during a 5 hr

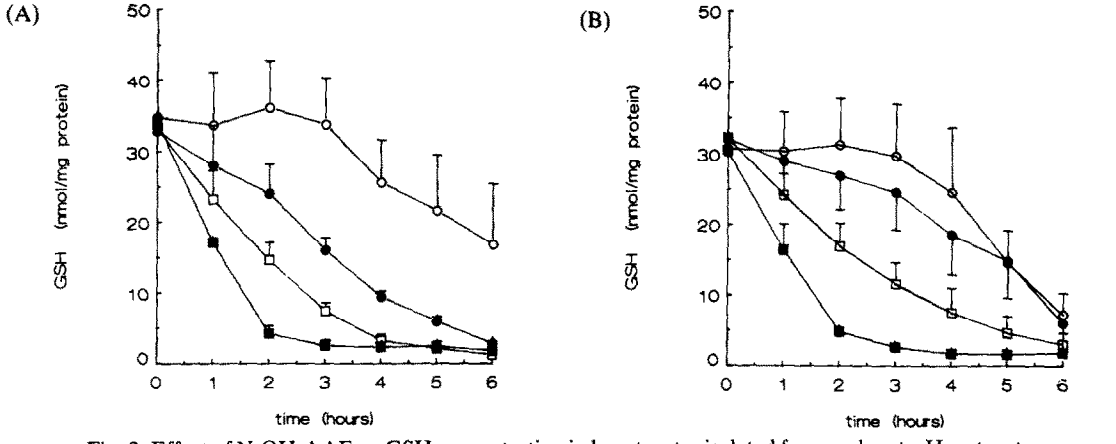

Fig. 3. Effect of N-OH-AAF on GSH concentration in hepatocytes isolated from male rats. Hepatocytes were incubated and GSH concentrations were determined as described in Materials and Methods. (A) Normal (sulfate supplemented) incubation. (B) Sulfate-deficient incubation. (○) Control; (●) 0.2 mM N-OH-AAF; (□) 1.0 mM N-OH-AAF; (■) 2.5 mM N-OH-AAF. Values represent the mean of two experiments.

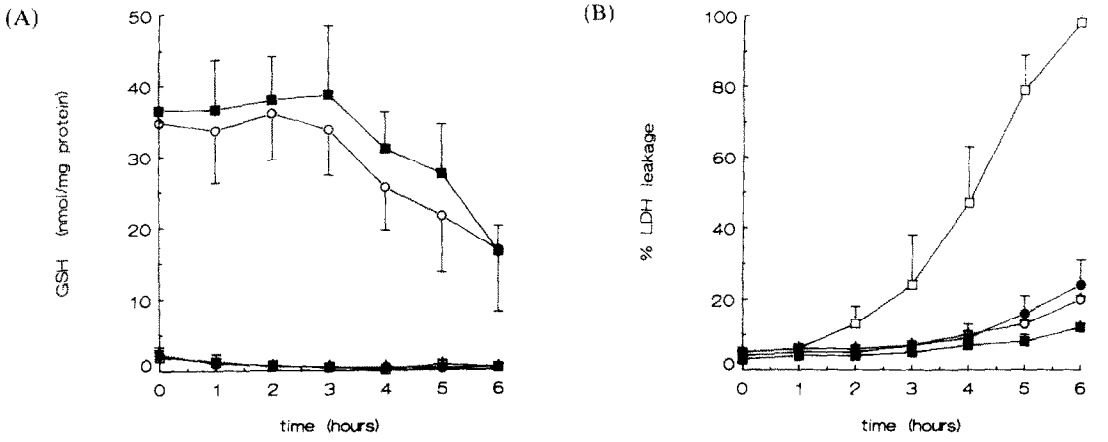

Fig. 4. Effect of DEM and vit.E on GSH concentration (A) and cell viability (B) of hepatocytes isolated from male rats. Hepatocytes were incubated, and GSH concentrations and cell viabilities were determined as described in Materials and Methods. (○) Control; (□) 5.0 mM DEM; (■) 75 mM vit.E; (●) 5.0 mM DEM plus 75 mM vit.E. Values represent mean ± SE of three experiments.

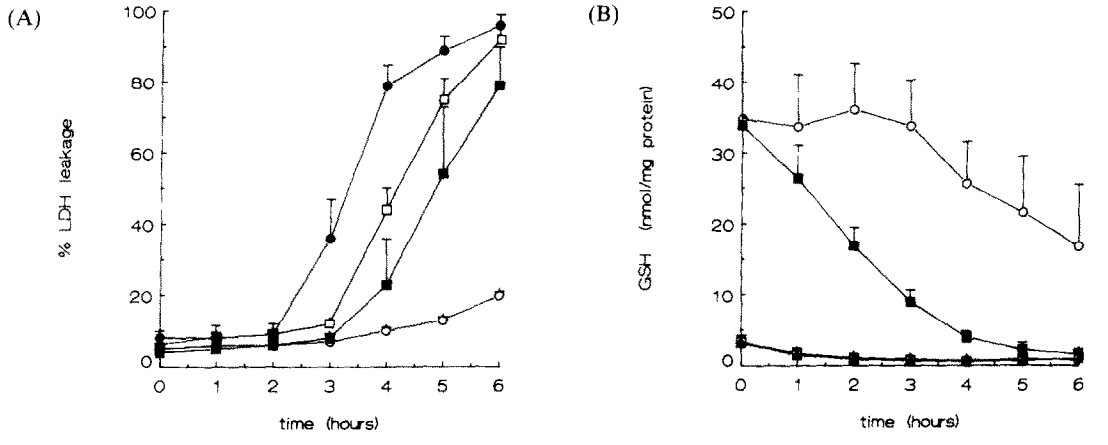

Fig. 5. Effect of DEM plus vit.E pretreatment on N-OH-AAF-induced changes in cell viability (A) and GSH status (B) of hepatocytes isolated from male rats. Hepatocytes were incubated, and GSH concentrations and cell viabilities were determined as described in Materials and Methods. (○) Control; (□) 1.0 mM N-OH-AAF (A) or 1.0 mM N-OH-AAF plus 5.0 mM DEM (B); (■) 1.0 mM N-OH-AAF plus 75 mM vit.E; (▲) and (●), 1.0 mM N-OH-AAF plus 5.0 mM DEM plus 75 mM vit.E. Values represent mean ± SE of three experiments.

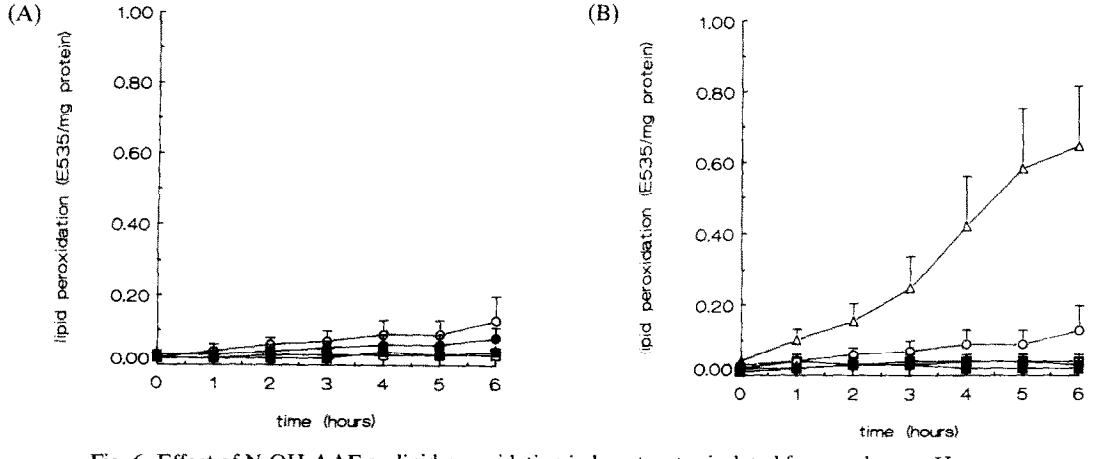

Fig. 6. Effect of N-OH-AAF on lipid peroxidation in hepatocytes isolated from male rats. Hepatocytes were incubated and lipid peroxidation was determined as described in Materials and Methods. (A): (○) Control; (●) 0.2 mM N-OH-AAF; (□) 1.0 mM N-OH-AAF; (■) 2.5 mM N-OH-AAF. (B): (○) Control; (□) 1.0 mM N-OH-AAF plus 5.0 mM DEM; (■) 1.0 mM N-OH-AAF plus 75 mM vit.E; (●) 1.0 mM N-OH-AAF plus 5.0 mM DEM plus 75 mM vit.E; (△) 5.0 mM DEM. Values represent mean ± SE of three experiments.

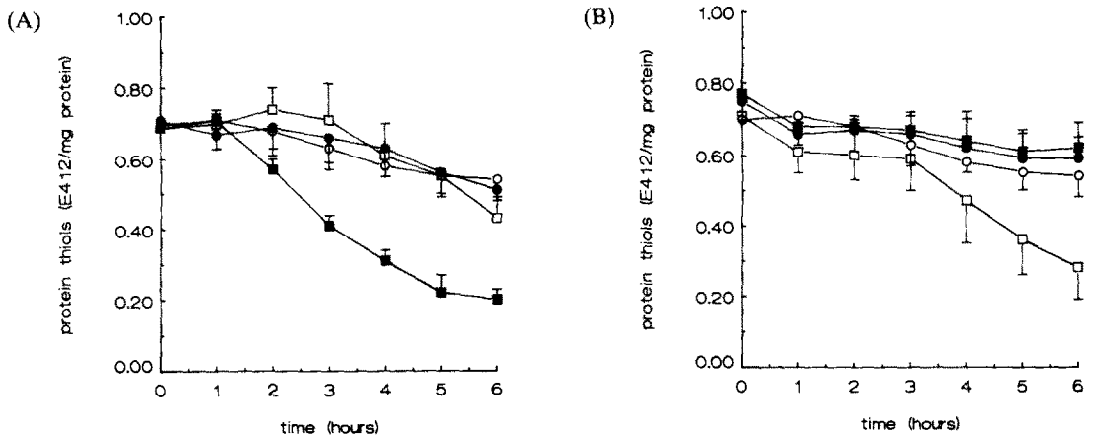

Fig. 7. Effect of N-OH-AAF, DEM and vit.E on free protein thiols in hepatocytes isolated from male rats. Hepatocytes were incubated and free protein thiols were determined as described in Materials and Methods. (A): (○) Control; (●) 0.2 mM N-OH-AAF; (□) 1.0 mM N-OH-AAF; (■) 2.5 mM N-OH-AAF. (B): (○) Control; (□) 5.0 mM DEM; (■) 75 mM vit.E; (●) 5.0 mM DEM plus 75 mM vit.E. Values represent mean ± SE of three experiments.

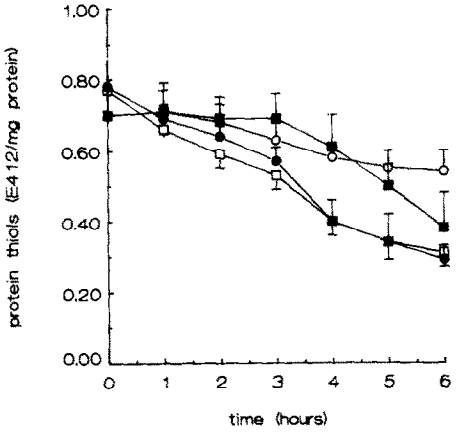

Fig. 8. Effect of N-OH-AAF, DEM and vit.E on free protein thiols in hepatocytes isolated from male rats. Hepatocytes were incubated and free protein thiols were determined as described in Materials and Methods. (○) Control; (□) 1.0 mM N-OH-AAF plus 5.0 mM DEM; (■) 1.0 mM N-OH-AAF plus 75 mM vit.E; (●) 1.0 mM N-OH-AAF plus 5.0 mM DEM plus 75 mM vit.E. Values represent mean ± SE of three experiments.

exposure (Fig. 7A). A slight decrease of protein thiols was seen thereafter. Protein thiols were severely reduced, however, by 2.5 mM N-OH-AAF.

In order to investigate the effect of N-OH-AAF on protein thiols when GSH was depleted, cells were pretreated with DEM and vit.E; this treatment with DEM and vit.E itself does not deplete protein thiols (Fig. 7B). After DEM/vit.E pretreatment, however, 1.0 mM N-OH-AAF did deplete protein thiols to about 50% at 6 hr (Fig. 8). At the same time toxicity occurred earlier (Fig. 5A).

N-OH-AAF-induced changes in intracellular ATP

Intracellular ATP levels were reduced upon exposure to N-OH-AAF (Fig. 9). At 1.0 mM N-OH-AAF this ATP depletion was dependent on the presence of sulfate in the incubation medium and preceded the occurrence of cell death. At 2.5 mM N-OH-AAF no sulfate dependence of the loss of ATP was observed (Fig. 9).

DISCUSSION

N-OH-AAF was toxic towards freshly isolated hepatocytes: more than 90% of hepatocytes isolated from male rats died during a 6 hr exposure to 1.0 mM N-OH-AAF, whereas only a slight loss of viability was observed for female cells at this concentration. Omission of inorganic sulfate from the incubation medium almost completely prevented cell death by N-OH-AAF in hepatocytes from male rats. This dependence of N-OH-AAF cytotoxicity on inorganic sulfate agrees with our previous observations *in vivo*, where N-OH-AAF hepatotoxicity could be prevented by the use of the sulfotransferase inhibitor pentachlorophenol [19]. Cytotoxicity of male hepatocytes was also observed with 2.5 mM N-OH-AAF; however, toxicity at this concentration was independent of sulfate in the incubation. Therefore, toxicity at this concentration is due to a different mechanism from that at lower N-OH-AAF concentrations. This seems to be confirmed by the results obtained with female hepatocytes: at this concentration a similar toxicity was observed as in male hepatocytes.

The sex difference with respect to N-OH-AAF hepatotoxicity observed in the rat *in vivo* was attributed to the different control of expression of sulfotransferase activity towards N-OH-AAF by male and female steroid sex hormones [17, 18, 43]. Obviously, this sex-dependent expression of N-OH-AAF sulfotransferase activity is retained in isolated hepatocytes. Because the sulfate dependency of the cytotoxicity of 1.0 mM N-OH-AAF agrees with our *in vivo* observations on the role of sulfation for N-OH-AAF toxicity, this concentration was taken for further study.

Since GSH is essential in the cellular detoxification

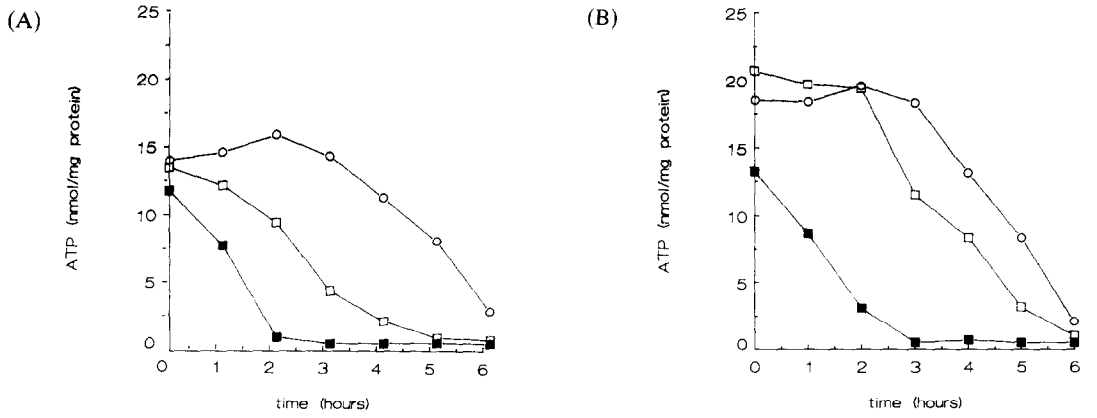

Fig. 9. Effect of N-OH-AAF on ATP concentration in hepatocytes isolated from male rats. Hepatocytes were incubated and ATP concentrations were determined as described in Materials and Methods. (A) Normal (sulfate supplemented) incubation. (B) Sulfate-deficient incubation. (○) Control; (□) 1.0 mM N-OH-AAF; (■) 2.5 mM N-OH-AAF. One experiment typical of four is shown.

of many toxic xenobiotics [11, 12], we have monitored this endogenous thiol during N-OH-AAF exposure. In the presence of N-OH-AAF, intracellular GSH levels declined and cellular GSSG efflux was reduced (unpublished data). This seems to be in agreement with our previously observed conjugation with GSH of N-OH-AAF *in vivo* [23, 24]. However, it may be questioned whether GSH is able to prevent cytotoxicity by N-OH-AAF. In the present experiments, significant LDH leakage starts to occur after GSH concentrations have declined to about 20% of the initial value. However, this value represents the mean GSH concentration in all hepatocytes; in the dying cells, GSH may have been depleted. We therefore studied two parameters known to be intimately coupled with GSH depletion: lipid peroxidation and protein thiol status [15, 16, 25–27].

As GSH is essential for the detoxification of hydrogen peroxide and various organic hydroperoxides that are continuously formed under normal aerobic conditions, depletion of GSH will result in lipid peroxidation [16, 25, 26, 44]. However, no lipid peroxidation was observed in the presence of N-OH-AAF. On the contrary, N-OH-AAF even prevented lipid peroxidation after GSH depletion by DEM and also reduced endogenous lipid peroxidation in control incubations. An explanation for these observations may be the metal ion chelating properties of hydroxamic acids [45]. Chelation of iron will result in a blockade of the Fenton reaction, thereby preventing the formation of lipid peroxidation-inducing reactive oxygen species [46, 47]. Thus, our results clearly indicate that lipid peroxidation is not involved in N-OH-AAF-induced cytotoxicity.

Another parameter related to GSH status is the amount of intracellular free protein thiols; normally, depletion of GSH results in a reduction of the amount of free protein sulfhydryl groups [15, 27]. This may be caused by two different mechanisms. Firstly, lipid peroxidation (induced by the depletion of GSH) generates reactive aldehydes [48, 49], which may react with protein thiols [45, 50]. Such a mechanism, however, can be excluded in the presence of N-OH-AAF, because no lipid peroxidation occurred. Secondly, the xenobiotic compound or its reactive metabolite(s) itself may react with protein thiols. In the presence of a cytotoxic concentration of 1.0 mM N-OH-AAF, only a small decrease in the amount of protein thiols was found during the last hour of exposure. No reduction was found during the preceding 5 hr, although over 75% of the cells had died during that period as judged by LDH leakage. Therefore, a loss of free protein thiols is most likely not the cause of the cytotoxicity of N-OH-AAF, although it cannot be excluded that a "critical" protein thiol may have been modified. Protein thiol depletion may, however, play a role in the sulfate independent toxicity of the high concentration of 2.5 mM N-OH-AAF, because in the presence of DEM and vit.E, a treatment known to result in complete GSH depletion without a concomitant protein thiol depletion (due to the antioxidant properties of vit.E), N-OH-AAF reduced the amount of free sulfhydryl groups. In addition, cell death with this latter treatment occurred earlier. Therefore, GSH probably offers protection against N-OH-AAF-induced depletion of protein thiols in control cells.

Exposure of hepatocytes to N-OH-AAF resulted in a loss of intracellular ATP. At 1.0 mM N-OH-AAF this ATP depletion was dependent on the presence of sulfate in the incubation medium and preceded the occurrence of cell death. These data suggest that cell death induced by N-OH-AAF is the consequence of the loss of cellular energy charge. The resulting impairment of cellular homeostasis may then be the trigger for loss of viability [51]. A correlation between hepatotoxicity and the loss of cellular ATP has been reported for other chemicals, such as allyl alcohol, aziridine derivatives and 1-methyl-1,2,3,6-tetrahydropyridine [28–30]. As yet, the mechanism responsible for this ATP depletion by N-OH-AAF is unclear.

In conclusion, the present data show that sulfotransferase activity probably plays a major role in N-OH-AAF-induced toxicity in freshly isolated hepatocytes from male rats. Lipid peroxidation is not involved in this cytotoxic mechanism. Also, cell death due to N-OH-AAF is not preceded or

accompanied by depletion of protein thiols. Therefore, GSH is unable to prevent N-OH-AAF-induced hepatotoxicity. It does, however, prevent depletion of free protein thiols by N-OH-AAF and ensures that no additional cytotoxicity occurs. The critical factor in N-OH-AAF-induced hepatotoxicity may be ATP loss, as this N-OH-AAF-induced effect is also sulfate-dependent and precedes cell death.

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