Biochemical Pharmacology, Vol. 33, No. 3, pp. 401–406, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

CYTOTOXIC EFFECTS OF *N*-ACETYL-*p*-BENZOQUINONE IMINE, A COMMON ARYLATING INTERMEDIATE OF PARACETAMOL AND *N*-HYDROXYPARACETAMOL

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(*Received* 8 *July* 1983; *accepted* 2 *September* 1983)

Abstract—The cytotoxic effects of *N*-acetyl-*p*-benzoquinone imine (NAPQI), a postulated ultimate reactive metabolite of paracetamol (pHAA), was studied in suspensions of isolated rat hepatocytes. Incubation of cells for 10–300 min with 0.1–0.5 mM NAPQI led to concentration dependent cell damage, as determined by increased trypan blue exclusion, lactate dehydrogenase release and glutathione (GSH) depletion. NAPQI and *N*-hydroxyparacetamol (N-OH-pHAA), a postulated proximate metabolite of pHAA, caused cytotoxic effects in the same concentration range. In contrast, no toxic effects of pHAA (≤ 20 mM) could be demonstrated. With the short half-life of NAPQI, less than 0.5% of the NAPQI added is expected to be left in the incubation medium after a 2 min incubated period. Nevertheless, 10–120 min (depending on the concentration of NAPQI) elapsed before the cells responded with increased membrane permeability. Clearly, the initial damage caused by NAPQI must be followed by subsequent cellular steps before toxicity becomes apparent. The addition of N-acetylcysteine, GSH or ascorbate during the NAPQI exposure period fully protected the hepatocytes from NAPQI damage. Lesser effects were demonstrated when these agents were added after the 5 min NAPQI exposure period. The results presented in this study further support the hypothesis that NAPQI is the ultimate reactive formed from pHAA.

Large doses of paracetamol (pHAA)‡, a commonly used analgesic and antipyretic drug, causes hepatic necrosis both in man and laboratory animals [1–4]. pHAA is chemically stable *per se*, but can be enzymatically converted in the organism to intermediate(s) that react with cellular constituents [5]. However, the initial reactions with cellular molecules must be followed by subsequent cellular responses before toxicity becomes manifest [6, 7].

The main route of elimination of pHAA involves conjugation with glucuronic and sulfuric acids [8] and subsequent urinary excretion. The formation of the reactive metabolite, most probably *N*-acetyl-*p*-benzoquinone imine (NAPQI), represents a lesser fraction of the total metabolism [8, 9]. Following therapeutic doses of pHAA, the nucleophilic tripeptide glutathione (GSH) forms a non-toxic conjugate with the electrophilic pHAA metabolite, thus protecting the hepatocytes from the cytotoxic effects seen at higher doses of pHAA, where GSH becomes depleted [5].

A cytochrome P-450 dependent *N*-hydroxylation reaction resulting in the formation of *N*-hydroxyparacetamol (N-OH-pHAA), followed by spontaneous dehydration to NAPQI, was initially proposed as a mechanism for the activation of pHAA [4]. N-OH-pHAA has since been synthesized [10] and shown to have many chemical and toxicological properties consistent with a role as a proximate toxic metabolite of pHAA [6, 7, 10–12], and Corcoran *et al.* [13] have provided evidence for NAPQI as a common arylating species in both the metabolism of pHAA and decomposition of N-OH-pHAA. However, the rate with which N-OH-pHAA dehydrates to NAPQI seems to be too slow to support the view that N-OH-pHAA is the proximate toxic metabolite of pHAA [9]. Furthermore, recent investigations indicate that NAPQI may be formed via a radical mechanism that does not involve N-O bond formation and such a reaction is mediated by cytochrome P-450 in the liver, and cytochrome P-450 or peroxidases in the kidney [14, 15]. Recently, Dahlin and Nelson [16] were able to synthesize NAPQI in the cystalline form. Preliminary decomposition kinetic and toxicity studies did not exclude NAPQI as the ultimate toxic metabolite of pHAA.

Suspension- and monolayer cultures of isolated rat hepatocytes, combining many advantages of an *in vitro* system with many properties of an *in vivo* system, have proven useful in studies of drug metabolism [17–19], cytotoxicity [6, 7, 20] and genotoxicity [21, 22]. The purpose of the present investigation was to characterize toxic properties of synthetic NAPQI in suspensions of isolated rat hepatocytes in order to study whether the observed effects would be in accordance with NAPQI's postulated role as an ultimate toxic metabolite of pHAA.

‡ Abbreviations: The following abbreviations are used: pHAA, paracetamol (*N*-acetyl-*p*-aminophenol); N-OH-pHAA, *N*-hydroxyparacetamol; NAPQI, *N*-acetyl-*p*-benzoquinone imine; HEPES, *N*-2-hydroxyethyl-piperazine-*N*-2-ethane sulfonic acid; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase; GSH, glutathione; BSA, bovine serum albumin; cytochromes P-450, a collective term for all forms of the cytochrome P-450.

MATERIALS AND METHODS

Chemicals

N-OH-pHAA sulfate was a generous gift from Dr. S. S. Thorgeirsson, National Cancer Institute, Bethesda, MD (U.S.A.). N-OH-pHAA was extracted after hydrolysis with sulfatase (Sigma Type H-1), as previously described [6]. NAPQI was synthesized as previously described [16]. Other chemicals were obtained from the following sources: collagenase (type IV), HEPES, bovine serum albumin (BSA), ascorbate, menadione, tocopherole and DMSO from Sigma Chemical Company, St. Louis, MO, U.S.A.; *N*-acetylcysteine from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England; pHAA and phenacetin from the Norwegian Medicinal Depot, Oslo, Norway.

Preparation and incubation of hepatocytes

Adult male Wistar rats, weighing 180–280 g were obtained from Møllegaard Breeding Centre, Ejby, Denmark, and given a pelleted diet and water *ad libitum*. Suspensions of rat hepatocytes were prepared by the collagenase perfusion technique [23], as described elsewhere [6]. After preincubation for 30 min at 37°, the hepatocytes were washed and purified by low speed centrifugation (50 *g*, 30 sec). The viability of the cells was always greater than 90%, as judged by trypan blue exclusion. Hepatocytes (1.5×10^6 cells/ml) were incubated in glass vials in a total volume of 2 ml Hank's–HEPES medium containing 1% BSA [6]. The chemical additions were either dissolved in DMSO (final concentration not exceeding 0.5% DMSO) or directly in the incubation medium. NAPQI was dissolved in DMSO immediately before the start of the incubations. The reactions were initiated by the addition of 10 μl test substance in DMSO or DMSO only and terminated in an ice bath. In some experiments cells were separated from the medium after a 5 min incubation by centrifugation at 55 *g* for 60 sec, suspended and further incubated in medium without NAPQI.

Assays

Cell viability was determined by trypan blue exclusion [24] and by measurement of lactate dehydrogenase (LDH) released into the medium from injured cells [25].

Intracellular levels of GSH were estimated by the method of Tietze [26], as described [6]. Lipid peroxidation was determined by the colorimetric measurement of thiobarbituric acid reactive substances at 535 nm, essentially as described by Stacey *et al.* [27].

RESULTS

The cytotoxic effect of NAPQI for hepatocytes in suspension was determined both by trypan blue exclusion and by measurement of LDH release into the medium, and compared with the effects of pHAA and N-OH-pHAA (Fig. 1).

The data in Fig. 1 shows that similar indications of cell damage were obtained using both methods. Concentrations of 0.1 mM NAPQI and higher led to increased cytotoxicity measured after 5 hr of incubation, with a half maximal effect at about 0.25 mM. The apparent decrease in LDH leakage at 1.0 mM NAPQI is likely due to an inactivation of the enzyme. N-OH-pHAA was nearly as toxic as NAPQI, while no hepatocellular toxicity could be observed with concentrations up to 20 mM of pHAA.

The time-course of the cytotoxic effect of NAPQI

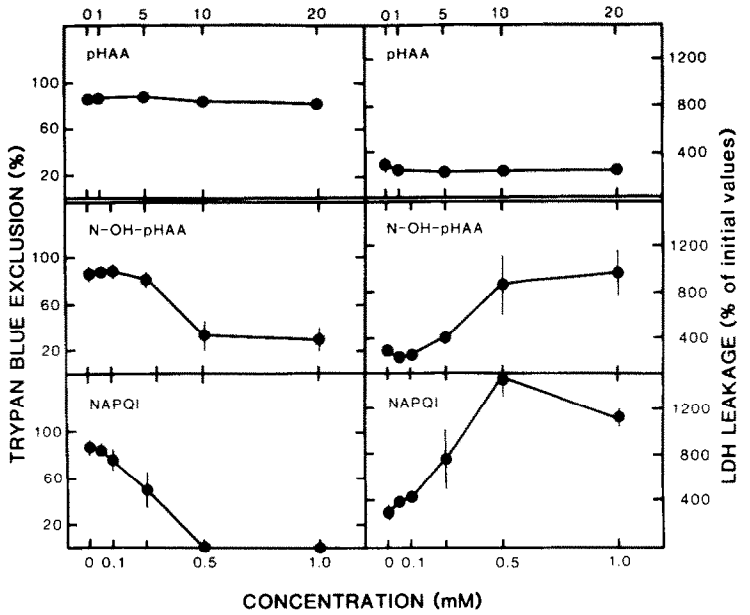

Fig. 1. Concentration dependent cytotoxicity of pHAA, N-OH-pHAA and NAPQI in suspensions of isolated rat hepatocytes, determined by trypan blue exclusion and LDH release after 5 hr incubation. Values are means ± S.D. of 3 different experiments.

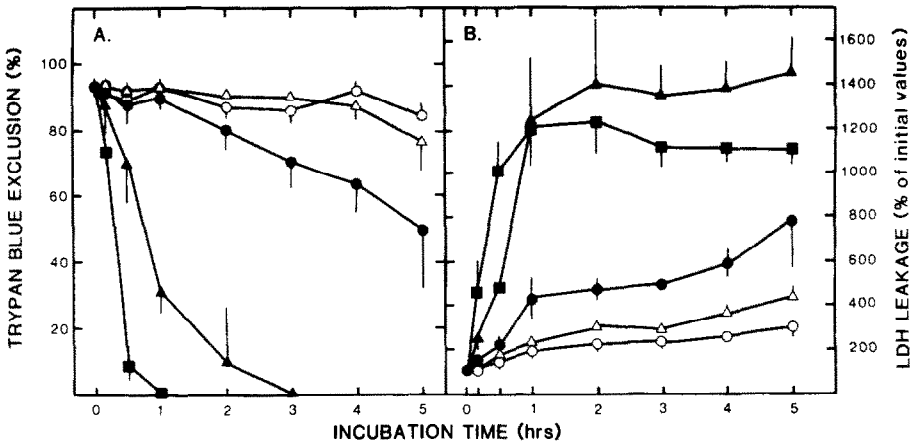

Fig. 2. Time course of cytotoxicity of different NAPQI concentrations in isolated rat hepatocytes, determined by trypan blue exclusion (A) and LDH (B) release. Values are means ± S.D. of 3 different experiments. Control (○—○), NAPQI: 0.1 mM (△—△), 0.25 mM (●—●), 0.5 mM (▲—▲), 1.0 mM (■—■).

was examined in some detail (Fig. 2). At a concentration of 1.0 mM NAPQI, a significant decrease in hepatocyte viability was seen within 10 min, with a total loss in viability after an incubation time of 60 min. A decrease of approx. 50% in the cell population excluding trypan blue was observed after 10–30 min, 30–60 min and 5 hr, respectively, with concentrations of 1.0, 0.5 and 0.25 mM NAPQI.

Experiments were then carried out to determine the effect of NAPQI on the intracellular level of GSH, the nucleophilic sulfhydryl-containing tripeptide protecting the hepatocytes from cytotoxicity elicited by electrophilic pHAA metabolites. As can be seen from Fig. 3(A), NAPQI caused a concentration dependent decrease in GSH levels, yielding approx. 10% of initial values with 0.5 mM NAPQI after a 10 min incubation period.

Under some experimental conditions, lipid peroxidation has been observed in conjunction with pHAA toxicity *in vivo* [28] and N-OH-pHAA toxicity *in vitro* [6]. With NAPQI only a small increase (with large interexperimental variation) in lipid peroxidation (Fig. 3(B)) was observed at a concentration of 0.25 mM. At 0.5 mM NAPQI no enhanced formation of thiobarbituric acid reactive substances in the cells was seen. In comparison, 6.5 mM carbon tetrachloride increased lipid peroxidation to 0.28 ± 0.1 $OD_{536}/10^6$ cells.

A number of compounds, such as *N*-acetylcysteine, ascorbate, menadione, toxopherole and phenacetin have been shown to ameliorate the toxic effects of paracetamol and/or N-OH-pHAA [7, 9]. It was therefore of interest to examine the effects of these protective agents on NAPQI toxicity in the

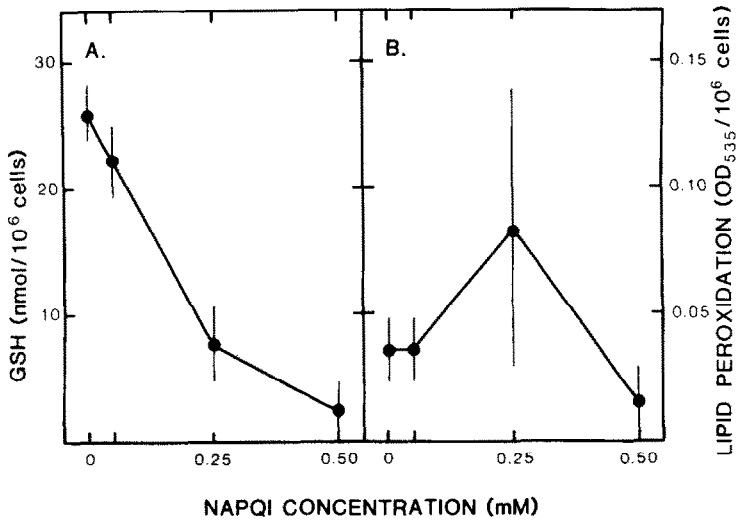

Fig. 3. Concentration dependence of NAPQI induced GSH depletion (A) and lipid peroxidation (B). Suspensions of rat hepatocytes were incubated with different concentrations of NAPQI for 10 min (A) or 120 min (B) after which cellular levels of GSH or lipid peroxidation, respectively, were determined. The values are means ± S.D. of 3 different experiments.

Table 1. Effects of various additions on the cytotoxic effect of NAPQI

Additions	Percent inhibition of induced cytotoxicity: Trypan blue exclusion	LDH release
N-Acetylcysteine (1 mM)	97 ± 3*	93 ± 6
GSH (2 mM)	91 ± 11	92 ± 2
Ascorbate (0.5 mM)	99 ± 2	96 ± 5
Menadione (0.1 mM)	48 ± 13	42 ± 19
Tocopherole (0.1 mM)	30 ± 21	24 ± 20
Phenacetin (0.5 mM)	45 ± 28	19 ± 25

The hepatocytes were incubated with 0.5 mM NAPQI and the various additions for 60 min. Trypan blue exclusion of cells exposed to NAPQI was 17 ± 9%, while 91 ± 1% of the unexposed cells excluded trypan blue. NAPQI treatment increased the LDH release to 673 ± 284% of unexposed cells.

* Mean ± S.D. of 3–4 different hepatocyte preparations.

hepatocyte suspensions. Table 1 shows the effect of these compounds on NAPQI induced cellular trypan blue exclusion and LDH release. The concentrations used were similar to those in previous experiments with N-OH-pHAA [7]. *N*-acetylcysteine, GSH and ascorbate almost totally counteracted the reduction in viability caused by NAPQI (Table 1). The addition of menadione reduced the toxic effects of NAPQI to about 50%, whereas only minor or insignificant effects were seen in experiments with tocopherole or phenacetin.

In four different experiments, the hepatocytes were incubated with 0.5 mM for 5 min whereafter they were centrifugated, resuspended in fresh medium and further incubated for 55 min before cytotoxicity was determined. Trypan blue exclusion of cells exposed to NAPQI for 5 min was not significantly different from those incubated with NAPQI without any medium change, being 21 ± 15 [4] and 36 ± 19 [4] respectively. Inclusion of *N*-acetylcysteine, GSH or ascorbate during this period of NAPQI exposure completely protected the hepatocytes from NAPQI induced damage (Table 2). On the other hand, the addition of these chemicals after an initial 5 min NAPQI exposure, resulted in less and more variable protection against NAPQI cytotoxicity. Menadione reduced the NAPQI dependent decrease in cell viability by approx. 50%, both during and after NAPQI exposure.

Table 2. Effects of various substances on NAPQI cytotoxicity together with or after NAPQI addition

Additions	Percent inhibition of induced trypan blue exclusion: With NAPQI	After NAPQI
N-Acetylcysteine (1 mM)	101 ± 7*	37 ± 20
GSH (2 mM)	101 ± 4	50 ± 17
Ascorbate (0.5 mM)	97 ± 5	30 ± 30
Menadione (0.1 mM)	47 ± 30	49 ± 22

The hepatocytes were incubated with 0.5 mM NAPQI for 5 min. The other test chemicals were either added together with or after NAPQI. After NAPQI exposure, the cells were centrifuged, resuspended in control medium with or without test chemicals, and further incubated for 55 min before cytotoxicity was determined.

* Mean ± S.D. of 4 different hepatocyte preparations.

DISCUSSION

In agreement with previous reports [29], no toxic effects of pHAA (≤ 20 mM) could be demonstrated in suspensions of isolated rat hepatocytes, as indicated by loss of cell membrane integrity. This corresponds well with the relative resistance against pHAA-induced liver damage observed in rats [30–32]. In contrast, profound cytotoxic effects were demonstrated with the postulated toxic pHAA metabolites, N-OH-pHAA and NAPQI, at fairly low concentrations (~ 0.5 mM). These findings suggest that the low hepatotoxic potential of pHAA in the rat could, at least in part, be explained by a low capacity of rat liver to activate pHAA to toxic metabolites. This is corroborated by the finding of a low V_{max} and high K_m of rat liver microsomal activation of pHAA to covalently protein bound intermediates [30, 33].

The rate with which N-OH-pHAA dehydrates to NAPQI seems to be too slow to support the view that N-OH-pHAA is the proximate toxic metabolite of pHAA [9]. On the contrary, the half-life of NAPQI, which is expected to be less than 10 sec in the presence of nucleophiles and reductants [34], is in accordance with its postulated role as an ultimate metabolite of pHAA. Nevertheless, N-OH-pHAA and NAPQI caused a concentration dependent cytoxic effect of similar magnitude. This result is, however, in agreement with the postulate that a major rate of N-OH-pHAA decomposition is a spontaneous dehydration to NAPQI in aqueous solution [12, 33]. NAPQI was dissolved in DMSO immediately before the start of the incubations and was stable during this period (data not shown). The reason why an even higher cytotoxic effect of NAPQI was not observed could be due to the reactivity and hydrophobicity of NAPQI [10], in which case decomposition occurs before reaching nucleophilic groups of vital importance in the hepatocytes.

With the short half-life of NAPQI, less than 0.5% of the NAPQI added is expected to be left in the incubation medium after a 2 min incubation period. Nevertheless, no significant changes in the viability of the hepatocytes were observed after 5 min incubation with 0.5 mM NAPQI. Clearly, the initial damage caused by NAPQI must be followed by subsequent cellular steps before toxicity becomes manifest. With N-OH-pHAA, an even longer period of time elapsed (120–180 min) before the cells showed evidence of toxicity [6, 7]. The greater stability of N-OH-pHAA [6] compared to that of NAPQI [34] accounts for the difference in the time course in cytotoxicity between NAPQI and N-OH-pHAA.

Similar to N-OH-pHAA, NAPQI causes a rapid concentration dependent decrease in the cellular levels of GSH. No significant damage of the plasma membrane is seen during this time period, thus

indicating that NAPQI is sufficiently stable and lipophilic to traverse the plasma membrane and react with nucleophilic molecules in the cytosol.

In addition to protecting the hepatocytes from the toxic effect of NAPQI by serving as a donor of nucleophilic groups, GSH can also indirectly, via GSH peroxidase, protect the hepatocytes from endogenously formed active oxygen species such as oxygen radicals capable of initiating lipid peroxidation [35]. Lipid peroxidation has been proposed to be an important process in cellular damage following GSH depletion in general [36]. Lipid peroxidation has also reportedly been demonstrated in relation to pHAA hepatotoxicity, especially in situations with altered nutritional status [29]. The lipid peroxidation caused by NAPQI in the present experiments was low, variable, and seemed to be a late event in the cytotoxic process. Lipid peroxidation did not appear to be a prerequisite for cellular damage, since higher concentrations of NAPQI led to increases in cell toxicity, but decreases in lipid peroxidation. We have reported similar findings with N-OH-pHAA [6, 7].

The cytotoxic properties demonstrated with NAPQI and the effects of protective agents on NAPQI cytotoxicity, further strengthens the notion that NAPQI is the ultimate toxic metabolite of pHAA. Of the possible inhibitors of NAPQI toxicity tested, the most effective were N-acetylcysteine, GSH and ascorbate. These protective agents may modify the initial phase of cell damage simply by decreasing the concentration of the toxic metabolite or on the other hand modify the cellular response to the toxic agent [6, 7]. The addition of these compounds during the NAPQI exposure period was sufficient to fully protect the hepatocytes from NAPQI damage. A direct interaction between the protective agents and the reactive species presumably best explains the decrease in cytotoxic response observed. Ascorbate most likely produces its effects through a reduction of NAPQI back to pHAA [12]. On the other hand, *N*-acetylcysteine and GSH probably acts as both reductants and nucleophiles as previously described for the effects of cysteine on N-OH-pHAA [12]. Assuming that the protective agents used simply decrease the concentration of NAPQI, one would not expect them to decrease the NAPQI toxicity when added 5 min after the reactive metabolite. However, this is not so in the case of GSH and also to a lesser extent for the other compounds used. In similar experiments with N-OH-pHAA, GSH caused an even more pronounced inhibition of the cytotoxic response of the hepatocytes [7]. The effect did not seem to be due to a direct effect of GSH on the trypan blue staining of the cells (data not shown). The nature of the subsequent cellular steps that results in increased membrane leakage is unknown at the present time. Lipid preoxidation did not appear to be a prerequisite for the cellular damage. However, the inhibition of the hepatocytes cytotoxic response by GSH, which is believed not to cross the plasmamembrane, could indicate that endogenously formed reactive molecules (i.e. active oxygen species) are involved in the subsequent cellular steps.

When pHAA is added to suspensions of hepatocytes or administered *in vivo*, toxic metabolites will be generated inside the liver cells at a relatively low rate over a longer period of time. On the other hand, when N-OH-pHAA or NAPQI are added to cell suspensions, the cells will be exposed for a short period of time to high concentrations of the toxic metabolites, exposure initially occuring at the exterior surface of the plasma membranes. In spite of these obvious differences, this study and similar studies [6, 7] show that experiments performed by adding possible toxic metabolites to suspensions of hepatocytes, can give valuable information for the understanding of how chemical compounds exert their hepatotoxic effects.

Acknowledgements—This study was supported by grant GM-25418 from the National Institutes of Health, U.S.A. The authors wish to thank Ms. L. T. Haug and Ms. B. Sevaldson for technical assistance and Ms. I. Støren for typing the manuscript.

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