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OXIDANT-INDUCED CHANGES IN THE CELLULAR ENERGY HOMEOSTASIS

A STUDY WITH 3,5-DIMETHYL *N*-ACETYL-*p*-BENZOQUINONE IMINE AND ISOLATED HEPATOCYTES

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Abstract—Exposure of isolated hepatocytes to 400 μM 3,5-dimethyl *N*-acetyl-*p*-benzoquinone imine (3,5-diMe NAPQI), rapidly induced the formation of plasma membrane blebs. More than 50% of the viable cells were affected after 1 min incubation with 3,5-diMe NAPQI. Rapid loss of mitochondrial ATP, and sequential increases in ADP and AMP accompanied hepatocyte blebbing. 3,5-diMe NAPQI also induced a pronounced elevation of mitochondrial NADP level, whereas the NAD concentration was unaffected. Similar alterations in the adenine and pyridine nucleotide pools were found to occur in the cytosol, although at slower rates. During the initial phase of ATP loss and NADP production, there was also a concomitant decrease in the oxygen uptake of the hepatocytes. The decreases in energy substrates occurred in parallel to an increased uptake of trypan blue into the cells. Treatment of the hepatocytes with dithiothreitol, following 4 min exposure of the cells to 3,5-diMe NAPQI, reversed the quinone imine-induced changes in nucelotide levels and reduced the cytotoxicity. It is concluded that alteration of mitochondrial function, which results in changes in the cellular energy homeostasis, is an important event in the development of cytotoxicity caused by 3,5-diMe NAPQI.

In a recent study we have shown that cell death caused by *N*-acetyl-*p*-benzoquinone imine (NAPQI‡), a reactive metabolite of acetaminophen [1], is associated with marked alterations in the energy metabolism of isolated hepatocytes [2]. These changes were characterized by an imbalance in the production and utilization of ATP. Furthermore, a rapid depletion of the mitochondrial content of ATP occurred in parallel to an increase in the formation of surface blebs, which preceded cell death. However, the precise molecular mechanisms underlying the hepatotoxic effect of NAPQI are not fully understood. This highly reactive metabolite has been demonstrated to deplete hepatocyte GSH [3], modify cellular proteins [4] and alter Ca^{2+} homeostasis in hepatocytes [5]. Since NAPQI possesses both electrophilic and oxidant characteristics [6], it is not known if the cytotoxicity induced by the compound is due primarily to arylation or oxidation.

In the present investigation, 3,5-dimethyl *N*-acetyl-*p*-benzoquinone imine (3,5-diMe NAPQI), an analogue of NAPQI that causes primarily oxidation of thiols [7], was used in order to evaluate the relationship between oxidant-induced changes in hepatocyte energy metabolism and cell death.

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‡ Abbreviations used: NAPQI, *N*-acetyl-*p*-benzoquinone imine; ATP, adenosine-5′-triphosphate; 3,5-diMe NAPQI, 3,5-dimethyl *N*-acetyl-*p*-benzoquinone imine; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione.

MATERIALS AND METHODS

Materials. Collagenase (grade II) and DTT were obtained from Boehringer (Mannheim, F.R.G.). Hepes, digitonin and DMSO were purchased from the Sigma Chemical Co. (St Louis, MO). Silicone oil, density 1.05 kg/L, from the Aldrich Chemical Co. (Milwaukee, WI), and paraffin oil, density 0.88 kg/L, from Merck (Darmstadt, F.R.G.), were mixed in a ratio 6:1 (v/v). 3,5-diMe NAPQI was synthesized and purified as previously described [8]. The quinone imine was dissolved immediately prior to use in distilled water and dried (Linde molecular sieves, type 4A) DMSO. All other reagents were obtained from local commercial sources.

Methods. Hepatocytes from male Sprague–Dawley rats (200–250 g) were used in all experiments. The animals were allowed free access to food and water up to the time of hepatocyte preparation. Hepatocytes were isolated by collagenase perfusion of rat livers as previously described [9], and cell incubations were performed in rotating, round-bottom flasks (10^6 cells/mL) at 37° under an atmosphere of 95% O_2 and 5% CO_2 in Krebs–Henseleit buffer supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4.

3,5-diMe NAPQI and DTT were added to the incubations in dry dimethylsulfoxide (DMSO <0.5%) at a final concentration of 400 μM and 10 mM, respectively. An equal amount of DMSO was added to the control incubation. DTT was included in the incubation mixture following 4 min exposure to 3,5-diMe NAPQI. In some experiments, 5 mM DTT was added after 2 min. The results obtained with this protocol, however, did not differ

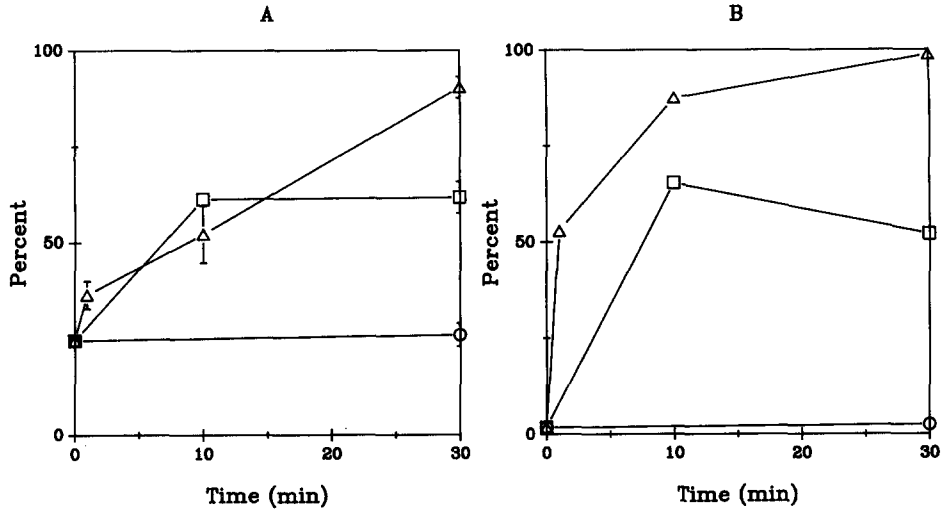

Fig. 1. Cytotoxicity (A) and surface blebs (B) caused by 3,5-diMe NAPQI in isolated hepatocytes. Hepatocytes were incubated with 400 μM 3,5-diMe NAPQI and then selected incubations were treated with DTT after 4 min as described in Materials and Methods. The number of viable cells and cells containing blebs were evaluated as described in Materials and Methods. Symbols represent: control (○); 3,5-diMe NAPQI (Δ); or 3,5-diMe NAPQI plus DTT (□). Data represent means from three to five different hepatocyte preparations.

from the standard condition, which also agrees with our previous observation [3].

Assays were performed as follows: cell viability and formation of surface blebs were determined during the course of the experiments by counting cells in a hemocytometer in the presence of 0.2% trypan blue. In order to analyse the mitochondrial and cytosolic pools of adenine and pyridine nucleotides, a 0.5-mL aliquot of the hepatocyte incubation mixture was treated with digitonin according to the method of Andersson and Jones [10], and nucleotides were determined by high-performance liquid chromatography as described in [2]. Hepatocyte oxygen uptake was analysed according to [2].

RESULTS

The time course of cell damage during exposure of isolated hepatocytes to 3,5-diMe NAPQI is shown in Fig. 1. Panel 1A demonstrates a gradual increase in the uptake of trypan blue. Following 30 min incubation with the quinone imine, more than 90% of the hepatocytes were stained by trypan blue. Treating the cells with DTT after addition of 3,5-diMe NAPQI decreased the cytotoxicity induced by the quinone imine (Fig. 1A). Preceding the trypan blue uptake was a rapid formation of blebs on the surface of the hepatocytes (Fig. 1B). After 1 min of incubation, greater than 50% of the cells were found to contain blebs, and by 30 min virtually all viable cells were blebbed. Addition of DTT reduced the number of cells containing blebs by nearly 50%.

3,5-diMe NAPQI-induced cell injury was also found to be associated with dramatic changes in the levels of adenine and pyridine nucleotides within the hepatocytes. As shown in Fig. 2A and B, 3,5-diMe NAPQI caused depletion of ATP from both the cytosol and mitochondria. Mitochondrial ATP content had decreased by 70% after only 1 min exposure to the quinone imine (Fig. 2B), whereas the concentration of ATP in the cytosol decreased at a slower rate (Fig. 2A). Addition of DTT prevented the 3,5-diMe NAPQI-induced depletion of ATP in the cells (Table 1). After 30 min incubation with 3,5-diMe NAPQI in the presence of DTT, mitochondria and cytosol still contained 87% and 75%, respectively, of the ATP concentrations measured in control (DMSO exposed) hepatocytes (data not shown). These results are in contrast to what has been reported for NAPQI [2], where DTT treatment only partially protected hepatocytes from ATP loss.

The disappearance of ATP from hepatocytes during the first 10 min of the incubation with 3,5-diMe NAPQI was accompanied by marked increases in cellular concentrations of ADP and AMP (Fig. 2A and B). In mitochondria, ADP reached maximal level (greater than two-fold the initial concentration) after 1 min, whereas the AMP content was found to be approximately 2.5-fold the 0-time value following 10 min exposure to 3,5-diMe NAPQI. Similarly, the cytosolic concentrations of ADP and AMP increased to maximal levels after 1 and 10 min, respectively. However, when DTT was included in the incubations, the rise in AMP concentrations was totally abolished (Table 1). In contrast, the increase in the mitochondrial content of ADP was not affected by addition of DTT, whereas ADP in the cytosol remained at a constant lower value (Table 1).

Taken together, these results indicate that exposure of isolated hepatocyte to 3,5-diMe NAPQI results in marked changes in the cellular energy metabolism.

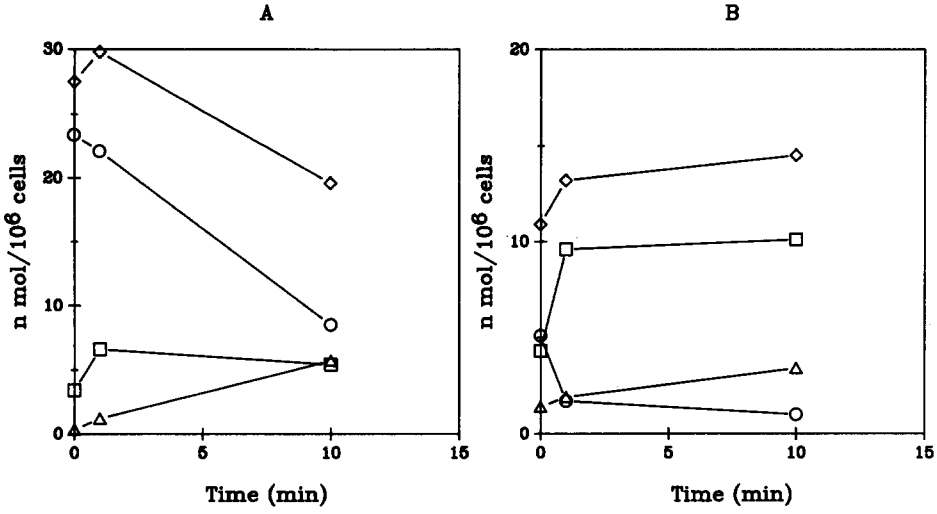

Fig. 2. Effect of 3,5-diMe NAPQI on hepatocyte levels of adenine nucleotides in cytosol (A) and mitochondria (B). Incubations were carried out with 400 μM 3,5-diMe NAPQI and then treated as described in Materials and Methods. Symbols represent: ATP (○); ADP (□), AMP (△) and Σ ATP + ADP + AMP (◇). Data represent the means of experiment utilizing three to four hepatocyte preparations.

Table 1. Effect of DTT on 3,5-diMe NAPQI-induced changes of nucleotide levels in isolated hepatocytes

	nmol/10⁶ cells					
	Cytosol			Mitochondria		
	0-time	−DTT	+DTT	0-time	−DTT	+DTT
ATP	23.4 ± 1.3	8.5 ± 2.2	24.5 ± 3.3	5.1 ± 0.36	1.0 ± 0.25	4.2 ± 0.23
ADP	3.4 ± 0.28	5.4 ± 0.40	2.2 ± 0.23	4.3 ± 0.35	10.1 ± 1.2	9.5 ± 0.75
AMP	0.34 ± 0.05	5.7 ± 1.9	0.33 ± 0.07	1.4 ± 0.20	3.4 ± 0.48	1.5 ± 0.15
NADP	0.67 ± 0.04	4.0 ± 0.32	1.7 ± 0.12	0.65 ± 0.04	4.4 ± 0.50	0.70 ± 0
NAD	7.3 ± 0.78	4.6 ± 0.31	4.9 ± 0.81	3.3 ± 0.20	3.0 ± 0.29	2.7 ± 0.38

Incubations were carried out for 10 min according to Materials and Methods. The 0-time value was obtained before the addition of 3,5-diMe NAPQI or DTT. Results represent the means ± SE of three to four different hepatocyte preparations.

The rapid loss of ATP in the mitochondria, followed by sequential increases in ADP and AMP, indicates a slower rate of ATP generation than consumption. In order to further investigate the effect of 3,5-diMe NAPQI on mitochondrial energy production, we measured the rates of hepatocyte oxygen uptake in the absence and presence of the quinone imine. In the presence of 3,5-diMe NAPQI, the rate of oxygen uptake was only 35% of the control rate (6.2 ± 0.2 and 17.5 ± 1.0 nmol/10⁶ cells/min, respectively). This result therefore suggests that inhibition of mitochondrial electron transport may be one mechanism by which 3,5-diMe NAPQI depresses ATP generation.

Cell damage induced by different oxidants has previously been shown to be associated with oxidation of NADPH and breakdown of NAD [2, 11–13]. Therefore, we quantitated the levels of NADP and NAD produced in hepatocytes during the metabolism of 3,5-diMe NAPQI. As demonstrated in Fig. 3, 1 min of exposure to the quinone imine resulted in dramatic changes in cellular NADP content. In the mitochondrial compartment, the concentration of NADP increased seven-fold relative to initial levels, and a four-fold increase in NADP was observed in the cytosol. Addition of DTT partially prevented the rapid increases of NADP in the cytosol, and totally reversed the increase in the mitochondria (Table 1).

In contrast to the changes in NADP, the levels of NAD did not appear to be affected greatly by exposure to 3,5-diMe NAPQI. After 10 min incubation with 3,5-diMe NAPQI, there was no change in the mitochondrial NAD pool size, and only 37% of the initial NAD content was lost from the cytosol (Fig. 3). Non-enzymatic reaction between 3,5-diMe NAPQI and standards of the adenine or oxidized pyridine nucleotides did not occur (not shown), as was previously reported for NAPQI [2].

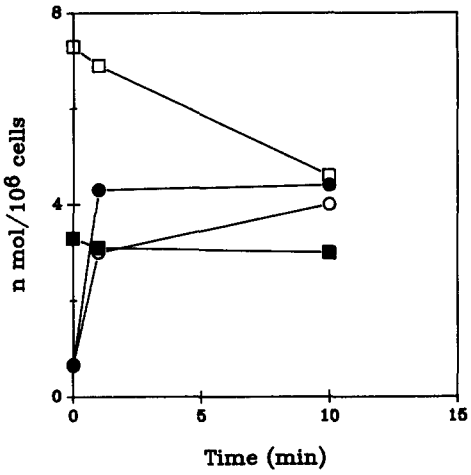

Fig. 3. Effect of 3,5-diMe NAPQI on the levels of pyridine nucleotides in hepatocytes. Incubations were carried out with 400 μM 3,5-diMe NAPQI as described in Materials and Methods. Symbols represent: NAD (□, ■), and NADP (○, ●). Open and solid symbols indicate the cytosolic and mitochondrial contents, respectively. Values are means of three to four different hepatocyte preparations.

DISCUSSION

In recent investigations we have used 3,5-diMe NAPQI and isolated hepatocytes in order to evaluate the role of oxidative stress in cytotoxicity caused by NAPQI [14, 15]. The cell damage produced by this dimethylated analogue of NAPQI was found to involve rapid oxidation of GSH and protein thiols, an elevation of the cytosolic Ca^{2+} level, and plasma membrane changes (blebbing and permeabilization). In contrast to NAPQI, covalent binding of 3,5-diMe NAPQI to hepatocyte protein only occurred to a minor extent [14]. Moreover, the thiol reducing agent, DTT, reversed both the 3,5-diMe NAPQI-induced biochemical changes and cytotoxicity. Similar results have been obtained when cytotoxicity was enhanced by treatment of hepatocytes with the glutathione reductase inhibitor 1,3-bis(2-chloroethyl)*N*-nitrosourea (BCNU) [14, 16]. Taken together, these results indicate that thiol groups, both soluble and in proteins, seem to be critical targets in the hepatotoxicity mediated by 3,5-diMe NAPQI.

The exact molecular mechanisms whereby 3,5-diMe NAPQI causes cell death are still not fully understood. In cells, mitochondria account for greater than 90% of the ATP production under aerobic conditions [17]. Thus, chemicals that alter mitochondrial function could have a great impact on many cellular processes. The results presented in this study demonstrate the importance of the energy homeostasis in 3,5-diMe NAPQI-induced cell injury. These data are also in good agreement with the results obtained in previous studies on tert-butyl hydroperoxide-induced cytotoxicity in hepatocytes* and isolated cardiac myocytes [18], as well as H_2O_2-mediated killing of P388D_1 cells [19].

* Andersson BS and Uhlig S, submitted for publication.

During exposure of hepatocytes to 3,5-diMe NAPQI, we observed significant changes in the mitochondrial and cytosolic levels of adenine and pyridine nucleotides. Simultaneous to a rapid decrease in the hepatocyte ATP level, particularly in mitochondria, there were sequential increases in ADP and AMP levels. However, the total adenine nucleotide phosphate pool in the mitochondria did not decrease during this rapid ATP loss. These results therefore suggest that 3,5-diMe NAPQI causes a breakdown of ATP, presumably resulting from a reduced capacity of mitochondria for ADP phosphorylation. This is also indicated by 3,5-diMe NAPQI-induced inhibition of oxygen uptake. Furthermore, we found the loss of mitochondrial ATP occurred in parallel to induction of surface blebs, and preceded any substantial increase in trypan blue uptake. Because adenine nucleotides participate in many metabolic reactions, and are major factors in maintaining cellular homeostasis [20], depletion of these substances could compromise cell survival.

The relative contributions of oxidation and arylation to quinone imine-induced cell death may be evaluated by comparing the toxic effects of 3,5-diMe NAPQI to those reported for NAPQI [2]. Exposure of hepatocytes to the same concentration of the two quinone imines (400 μM), produced quite similar patterns of toxicity, characterized by an immediate formation of surface blebs, followed by uptake of trypan blue. However, a striking difference is the effect of DTT on the toxicity produced by the two quinone imine analogues. In the case of NAPQI, DTT was found only to delay cytotoxicity [2], whereas addition of DTT to hepatocyte incubations containing 3,5-diMe NAPQI markedly reduced the quinone imine-induced bleb formation and trypan blue uptake. The mechanism(s) for DTT-mediated decrease of 3,5-diMe NAPQI-induced toxicity is not clear. However, in the experiments described here, DTT was found to restore the mitochondrial and cytosolic ATP levels as well as prevent further breakdown of adenine nucleotides (Table 1). In contrast, treatment of NAPQI-exposed hepatocytes with DTT did not significantly increase mitochondrial ATP [2].

The quinone imine-induced elevation of mitochondrial NADP was totally reversed by DTT in 3,5-diMe NAPQI exposed cells, whereas there was little effect of DTT in cells treated with NAPQI [2]. These results therefore suggest that the mitochondrial capacity for NADPH generation may play an important role in oxidant-induced cytotoxicity. Taken together our data indicate that the protective effect of DTT may be related to its ability to restore the redox state of enzymes involved in energy metabolism (i.e., ATP and NADPH production). Moreover, the difference in DTT-reversibility between NAPQI and its dimethylated analogue supports further the idea that arylation of critical macromolecules is more lethal to the cell than damage caused by oxidation.

In conclusion, the results presented in this investigation, together with previous data [2], show that changes in cellular energy homeostasis resulting from mitochondrial dysfunction are early events during exposure of hepatocytes to NAPQI and its 3,5-dimethylated analogue. Furthermore, these alter-

ations appear to be critical factors in the cytotoxicity caused by these two quinone imines.

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