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ROLE OF GLUTATHIONE REDUCTASE DURING MENADIONE-INDUCED NADPH OXIDATION IN ISOLATED RAT HEPATOCYTES

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Abstract—Metabolism of menadione (2-methyl-1,4-naphthoquinone) results in the rapid oxidation of NADPH within isolated rat hepatocytes. The glutathione redox cycle is thought to play a major role in the consumption of NADPH during menadione metabolism, chiefly through glutathione reductase (GSSG-reductase). This enzyme reduces oxidized glutathione (GSSG), formed via the glutathione-peroxidase reaction, with the concomitant oxidation of NADPH. To explore the relationship between GSSG-reductase and the consumption of NADPH during menadione metabolism, isolated rat hepatocyte suspensions were exposed to non-lethal and lethal menadione concentrations (100 and 300 μM respectively) following the inhibition of GSSG-reductase with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Menadione produced a concentration-related depletion of GSH (measured as non-protein sulfhydryl content) which was potentiated markedly by BCNU. Menadione toxicity was potentiated at either concentration by BCNU based on lactate dehydrogenase leakage at 2 hr. In addition, the NADPH content of isolated hepatocytes rapidly declined following exposure to either concentration of menadione. However, at the lower menadione concentration (100 μM), the NADPH content returned to control values or above by 60 min, whereas the NADPH content of cells exposed to 300 μM menadione with or without BCNU remained depressed for the duration of the incubation. These data suggest that, although NADPH is required by GSSG-reductase for the reduction of GSSG to GSH during quinone-induced oxidative stress, this pathway does not appear to be the major route by which NADPH is consumed during the metabolism of menadione in isolated hepatocytes.

Glutathione reductase (GSSG-reductase) catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) [1] formed by the selenoprotein glutathione peroxidase during the metabolism of H_2O_2 and a variety of organic hydroperoxides [2]. The role of GSSG-reductase in protecting cells from drug-induced oxidative challenge is relatively well established and the inhibition of this enzyme by 1,3-bis(2-chloroethyl)-l-nitrosourea (BCNU) has been shown to potentiate the *in vitro* cytotoxicity of a number of agents that produce oxidative cell injury [3–7].

NADPH oxidation occurs rapidly in isolated hepatocytes exposed to menadione (2-methyl-1,4-naphthoquinone). Processes that consume NADPH during the metabolism of this quinone include activation of menadione to menasemiquinone by NADPH–cytochrome P-450 reductase [8, 9], the two electron reduction to menadiol mediated by DT-diaphorase (NAD(P)H:(quinone acceptor)oxidoreductase) [8] and reduction of GSSG by GSSG-reductase. Since these enzymes all have affinities for NADPH in the micromolar range, each may be capable of depleting hepatocytes of NADPH.

Menadione metabolism is coupled to the glutathione redox cycle via H_2O_2 generated by the dismutation of the superoxide anion radical ($O_2^{\cdot-}$) [7]. Superoxide is produced during the oxidation of menasemiquinone to menadione by molecular oxygen [8, 10]. At toxic menadione concentrations, GSSG accumulates following pyridine nucleotide oxidation [10]. The maximum rate of GSSG reduction by GSSG-reductase is approximately 8–10 $\mu mol \cdot min^{-1} \cdot g^{-1}$ [11], whereas the maximal rate of glutathione (GSH) oxidation by glutathione peroxidase is 40 $\mu mol \cdot min^{-1} \cdot g^{-1}$ [12]. Since fatty acid synthesis and mixed function oxidation normally consume only 1–2 μmol NADPH$\cdot min^{-1} \cdot g^{-1}$ [13, 14], GSSG-reductase may play a major role in the oxidation of NADPH that occurs during conditions of oxidative stress [10, 15, 16].

In the present investigation, the role of GSSG-reductase in the consumption of NADPH during menadione metabolism was studied in isolated rat hepatocytes.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley derived, CD rats (200–250 g) were purchased from Charles River, Inc. (Wilmington, MA) and housed in individual hanging wire cages in a temperature-, humidity-, and light-controlled room. Animals were allowed food (Ralston Purina Certified Chow No. 5002) and water *ad lib*.

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Materials. Bovine serum albumin (BSA; fraction V) was purchased from Miles Scientific (Naperville, IL). *N*-2-Hydroxy-ethyl-piperazine-N^1-2-ethanesulfonic acid (HEPES), β-nicotinamide adenine dinucleotide (reduced form, disodium salt-grade III), β-nicotinamide adenine dinucleotide phosphate (reduced and oxidized forms, sodium salt), tris[aminomethyl]aminomethane hydrochloride (TRIZMA), reduced glutathione, and menadione (sodium bisulfite salt) were purchased from the Sigma Chemical Co. (St. Louis, MO). Collagenase (Type III) was purchased from Worthington Biochemicals (Freehold, NJ). MEM amino acids (50 concentrate) and L-glutamine were purchased from GIBCO Laboratories (Grand Island, NY). Glycerol-3-phosphate dehydrogenase and glutamate dehydrogenase were purchased from Boehringer Mannheim (Indianapolis, IN). 1,3-bis(2-Chloroethyl)-1-nitrosourea (BCNU) was supplied by the Developmental Therapy Program, National Cancer Institute, Bethesda, MD. All other reagents used were of the highest analytical grade commercially available.

Hepatocyte isolation. Hepatocytes were prepared by the method of Moldeus *et al.* [17] as described by Rush *et al.* [18]. Briefly, rat livers were perfused for 5 min with a modified Hanks' buffer (Ca^{2+}-free) containing 25.0 mM HEPES, 0.5 mM ethyleneglycolbis(amino - ethylether)tetra - acetate (EGTA) and 2% BSA. The perfusate was then changed to a modified Hanks' buffer containing 0.1% collagenase, 2.9 mM $CaCl_2$ and 2% BSA, and the perfusion was continued for an additional 10 min. The hepatocytes were then washed twice in Krebs–Henseleit buffer containing 25 mM HEPES and 20 mM glucose. An aliquot of cells was then combined with trypan blue, and the initial viability was determined [17]. Hepatocyte preparations with an initial viability of less than 90% were not used.

Incubation of hepatocytes with BCNU and menadione. In preliminary experiments, BCNU was found to deplete the GSH content of isolated hepatocytes. Therefore, it was necessary to reestablish the hepatocellular content of GSH in BCNU-treated hepatocytes before challenging cells with menadione. The protocol used was similar to that of Eklow *et al.* [3]. Briefly, following the isolation of hepatocytes, cells were pretreated with BCNU (60 μm) or the appropriate control vehicle by incubating them (2×10^6 cells/ml) for 30 min in a modified Krebs–Henseleit buffer containing 20 mM glucose, 20 mM HEPES, and supplemented with MEM amino acids (30 ml of 50× concentrate/l). Cells were incubated in 200-ml Erlenmeyer flasks at 37° under constant gassing with 95% O_2/5% CO_2. BCNU was prepared in dimethyl sulfoxide and aliquots were added directly to hepatocyte suspensions. The final dimethyl sulfoxide concentration was always less than 1% and did not affect cell viability. After 30 min, cells were pelleted (400 rpm, 2 min) to remove BCNU and resuspended in incubation buffer without BCNU. Incubations were then continued over a period of 2 hr to allow for the recovery of GSH to control values. During this time, GSSG-reductase activity remained inhibited by greater than 85% in BCNU-pretreated cells. Following recovery of GSH in the BCNU-pretreated cells, cells were washed to remove amino acids from the incubation buffer, resuspended in amino acid-free incubation buffer, and incubated in 50-ml Erlenmeyer flasks (2×10^6 cells/ml) for a period of 2 hr. Menadione was prepared as a stock solution in Krebs–Henseleit buffer and added to the cell suspensions at concentrations indicated in the text.

Biochemical assays. The GSSG-reductase activity in cells was determined according to the method of Babson *et al.* [4]. Briefly, a 0.5-ml aliquot of cell suspension was sonicated (15 pulses, Fisher 300 Sonic Dismembrator; relative output 0.6), the resulting homogenate was pelleted (13,000 *g*, 2 min), and the supernatant fraction was placed on ice until assay. For the assay of GSSG-reductase, 0.4 ml of sample supernatant was placed in a plastic cuvette along with 1.4 ml of assay buffer which consisted of KCl (200 mM), EDTA (1 mM), and GSSG (1 mM). To initiate the reaction, 0.2 ml of a 1 mM NADPH stock solution was added. The oxidation of NADPH at 340 nm (25°) was monitored in a Beckman DU-8 UV/VIS spectrophotometer.

The viability of hepatocytes was monitored as leakage of lactate dehydrogenase (LDH) [17] following separation of cells from incubation buffer by rapid centrifugation through dibutyl phthalate as described by Fariss and Reed [19]. The GSH content of hepatocytes was measured as non-protein sulfhydryl (NPSH) content by the method of Ellman [20] as described by Rush *et al.* [21]. Hepatocyte NADPH content was quantitated by a modification of the method of Klingenberg [22] in which hepatocytes (2×10^6 cells) were suspended in 0.5 ml alcoholic KOH (0.5 N) and allowed to stand for 30 min on ice. Triethanolamine HCl (1 M) was then added to adjust the pH of the solution to 7.75–7.85, and the solution was sedimented to remove particulate material. The NADH in the clear supernatant fraction was selectively oxidized by glycerol-3-phosphate dehydrogenase. NADPH was then measured in the same cuvette by following the decrease in absorbance at 340 nm in a Beckman DU-8 UV/VIS spectrophotometer following addition of glutamate dehydrogenase.

Statistical analyses. Data are expressed as means ± SE. Where appropriate, data were analyzed by the general linear model procedure. Differences between groups were determined by the LSMEANS procedure [23]. The criteria for significance was $P < 0.05$.

RESULTS

A concentration- and time-dependent inhibition of GSSG-reductase was observed in cell sonicates prepared from BCNU-treated rat hepatocytes (Fig. 1). BCNU produced a marked inhibition of this enzyme at all concentrations tested (20–100 μM) by 30 min. At concentrations of 40 μM or greater, the inhibition of GSSG-reductase by BCNU appeared to be near-maximal (70–80%), whereas maximal inhibition of the reductase by 20 μM BCNU was not apparent until between 90 and 120 min. While BCNU did not affect viability of hepatocytes during this 2-hr incubation, as determined by LDH leakage,

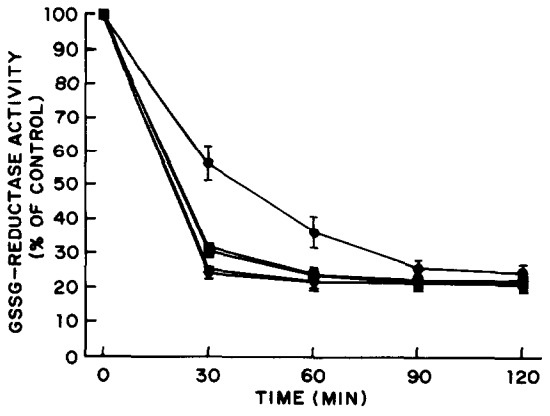

Fig. 1. Inhibition of glutathione reductase by BCNU in cell sonicates prepared from isolated rat hepatocytes. Values represent the $\bar{X} \pm$ SEM from three separate hepatocyte preparations. The absolute value for 100% glutathione reductase activity at time 0 was 0.065 ± 0.003 μmol NADPH·min^{-1}·(10^6 cells)$^{-1}$. Key: 20 μM (●—●), 40 μM (■—■), 60 μM (▲—▲), 80 μM (▼—▼), and 100 μM (◆—◆) BCNU.

the intracellular GSH (non-protein sulfhydryls) content was decreased by 50% or more in the absence of exogenous sulfur-containing amino acids (data not shown). To determine if the changes in menadione-induced oxidative cell injury in BCNU-treated hepatocytes were due to GSH depletion or to GSSG-reductase inhibition, it was necessary to allow the cells to resynthesize GSH by supplementing the incubation buffer with sulfur-containing amino acids. Following the 30-min BCNU pretreatment, cells were incubated in buffer supplemented with MEM amino acids and L-glutamine for 2 hr. In this buffer, methionine (0.15 mM) was the major sulfur-containing amino acid. When hepatocytes were exposed to BCNU (60 μM) for 30 min, the resulting GSH content was 70% of control, and the GSSG-reductase activity was approximately 30% of control (Fig. 2). Following the removal of BCNU from the incubation buffer, the resynthesis of GSH was nearly complete by 2 hr (greater than 90% of control). After amino acids were removed from the incubation buffer, the GSH content of the cells remained between 90 and 100% of control for the next 2 hr. During the entire 4-hr incubation process, which followed the 30-min BCNU treatment, the activity of GSSG-reductase remained between 10 and 20% of control (Fig. 2). Thus, this preparation allowed study of menadione-induced oxidative cell injury in GSSG-reductase-inhibited cells which contained a normal GSH content.

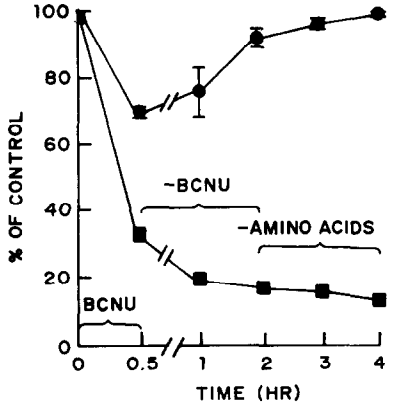

Fig. 2. Recovery of GSH content and inhibition of glutathione reductase following treatment of isolated hepatocytes with BCNU (60 μM). Values represent the $\bar{X} \pm$ SEM from three to four separate hepatocyte preparations. Following a 30-min treatment of cells with 60 μM BCNU, cells were washed and incubated for 2 hr. After 2 hr cells were washed again to remove amino acids. Key: GSH content (●—●), and glutathione reductase activity (■—■). Control GSH content (100%) at time 0 was 39.08 ± 3.76 nmol/10^6 cells. Glutathione reductase activity (100%) was 0.072 ± 0.004 μmol·min^{-1}·(10^6 cells)$^{-1}$ at time 0.

The exposure of hepatocytes to menadione resulted in a rapid, concentration-related depletion of GSH (Fig. 3, A and B). After 60 min, the GSH content of cells exposed to 100 and 300 μM menadione was 18.9 ± 3.2 and 7.9 ± 2.9 nmol GSH/10^6 cells respectively. A potentiation of the menadione-induced depletion of GSH was observed in BCNU pretreated hepatocytes. However, this response was not concentration-dependent since the time course and magnitude of GSH depletion produced by either 100 or 300 μM menadione in BCNU-pretreated cells were identical (Fig. 3, A and B).

At either 100 or 300 μM menadione, intracellular NADPH was reduced significantly by 15 min (Fig. 4, A and B). Clearly, this initial depletion of NADPH was not prevented in hepatocytes pretreated with BCNU. In hepatocytes exposed to the lower menadione concentration (100 μM), the NADPH content returned towards control values by 60 min. However, in cells exposed to either 300 μM menadione or to the combination of menadione (100 or 300 μM) plus BCNU, there was no recovery of NADPH. BCNU treatment alone did not affect the NADPH content.

Hepatocyte viability, determined by leakage of LDH, was not affected significantly by 100 μM menadione over a period of 120 min. Significant LDH leakage was observed, however, after 90 min in cells exposed to 300 μM menadione with or without BCNU pretreatment and at 120 min in cells exposed to 100 μM menadione plus BCNU (Fig. 5, A and B). Over the 120-min period of incubation, the leakage of LDH from BCNU-treated cells was not different than that of control.

DISCUSSION

Inhibition (80–90%) of glutathione reductase activity was observed in isolated rat hepatocytes exposed to the selective inactivator BCNU as reported previously [4] (Figs. 1 and 2). Subsequent exposure of BCNU-treated cells to menadione resulted in a significant potentiation of GSH depletion at the lethal and non-lethal menadione concentrations (Fig. 3). While a concentration-related depletion of GSH by menadione was not observed in BCNU-pretreated cells, it is possible that the sampling interval (15 min) was too late or that residual non-protein sulfhydryl groups (e.g. methionine) masked any potential differences. Since the hepatocytes were washed prior to incubation in an amino

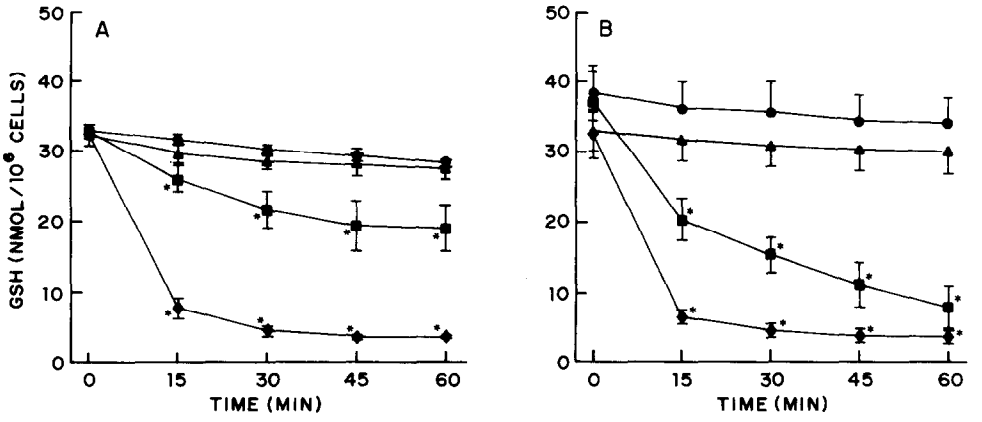

Fig. 3. Effect of menadione at 100 μM (A) and 300 μM (B) alone or in combination with BCNU (60 μM) on the content of GSH in isolated rat hepatocytes. Values represent the $\bar{X} \pm$ SEM from three to four separate hepatocyte preparations. (*) Indicates significantly different from control ($P < 0.05$). Key: control (●—●), menadione (■—■), BCNU (▲—▲), and menadione plus BCNU (◆—◆).

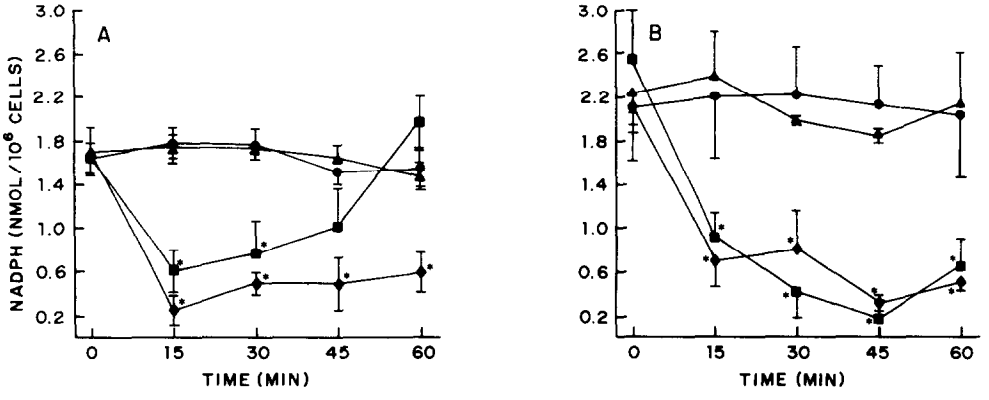

Fig. 4. Effect of menadione at 100 μM (A) and 300 μM (B) alone or in combination with BCNU (60 μM) on the content of NADPH in isolated rat hepatocytes. Values represent the $\bar{X} \pm$ SEM from three to four separate hepatocyte preparations. (*) Indicates significantly different from control ($P < 0.05$). Key: control (●—●), menadione (■—■), BCNU (▲—▲), and menadione plus BCNU (◆—◆).

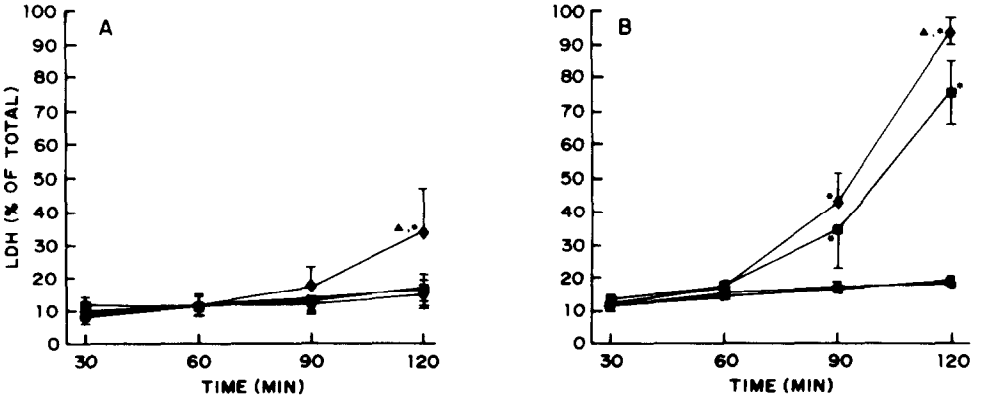

Fig. 5. Effect of menadione at 100 μM (A) and 300 μM (B) alone or in combination with BCNU (60 μM) on the leakage of lactate dehydrogenase (represented as percent of the total releasable enzyme) from isolated rat hepatocytes. Values represent the $\bar{X} \pm$ SEM from three to four separate hepatocyte preparations. (*) Indicates significantly ($P < 0.05$) different from control, (▲) indicates significant ($P < 0.05$) difference between menadione and menadione plus BCNU. Key: control (●—●), menadione (■—■), BCNU (▲—▲) and menadione plus BCNU (◆—◆).

acid deficient buffer, the latter possibility is probably not responsible for the lack of a concentration-related depletion of GSH (NPSH) by menadione. These results are consistent with the role of the glutathione redox cycle under conditions of oxidative stress [4] during which the GSSG, formed via the reaction between glutathione peroxidase and H_2O_2, is reduced to GSH by glutathione reductase with the subsequent oxidation of NADPH to $NADP^+$. Therefore, inhibition of this pathway leads to further GSH depletion in cells during menadione metabolism.

In these experiments, we observed a potentiation of menadione-induced cell lethality by BCNU (Fig. 5). While a more marked potentiation of menadione toxicity by BCNU has been demonstrated using hepatocyte cultures [6], this difference may be explained by: (1) the use of different test systems—hepatocyte suspensions versus monolayer cultures and (2) the use of a 5-fold greater concentration of BCNU in the hepatocyte culture system as compared with the cell suspensions to produce a comparable inhibition of glutathione reductase.

It has been suggested that NADPH regeneration is the rate-limiting step in the glutathione peroxidase-dependent reduction of *tert*-butyl hydroperoxide (TBHP) [24]. Furthermore, the increase in intracellular GSSG which occurs at toxic menadione concentrations is thought to be due to insufficient regeneration of NADPH required by GSSG-reductase [10]. Thus, NADPH consumption via glutathione reductase may contribute to the rapid depletion of NADPH which is observed during oxidant-induced cell injury. Experimental evidence is available in support of this hypothesis in hydroperoxide-induced NADPH depletion [24–26]. For example, the rapid depletion of NADPH induced by TBHP in isolated hepatocytes is reduced markedly in BCNU-treated cells, whereas GSH depletion is potentiated [25]. However, our results suggest that glutathione reductase is not a primary source of NADPH consumption during menadione metabolism by isolated rat hepatocytes. This is indicated by the failure of BCNU to prevent the rapid decline in NADPH following exposure of cells to menadione (Fig. 4). Since at least one molecule of NADPH must be oxidized to generate one molecule of H_2O_2 via the metabolic scheme described above, the NADPH-dependent reduction of GSSG by glutathione reductase can, at most, account for only half of the NADPH oxidized. Thus, it is not surprising that menadione caused some initial depletion of NADPH in the BCNU-treated cells. However, what is surprising is that BCNU did not prevent any of the observed NADPH loss. In fact, the NADPH content of isolated hepatocytes that had limited glutathione reductase activity ($< 20\%$ of control) following BCNU pretreatment was slightly below that of hepatocytes exposed to menadione alone after 15 min (Fig. 4). If a significant consumption of NADPH had occurred via glutathione reductase, the inhibition of this enzyme should have resulted in less NADPH depletion than in the non-BCNU-treated cells exposed to menadione.

Indirect support for this conclusion is provided by Ca^{2+}-release experiments performed in isolated rat liver mitochondria in which the release of mitochondrial Ca^{2+} is dependent upon the oxidation of NAD(P)H [26–28]. In these studies, the release of Ca^{2+} by Ca^{2+}-loaded mitochondria during menadione metabolism was apparently not mediated by the glutathione peroxidase-glutathione reductase system since depletion of substrate (GSH) for glutathione peroxidase did not prevent menadione-induced Ca^{2+} release [26]. Furthermore, in mitochondria lacking the selenoenzyme glutathione peroxidase, menadione caused a rapid oxidation of NAD(P)H and subsequent Ca^{2+} release [27]. Hence, the loss of NAD(P)H which occurred following menadione addition in isolated mitochondria was not related to its consumption via the glutathione redox cycle but was suggested to be related to metabolism of menadione by NADH-ubiquinone oxidoreductase [27].

At non-lethal concentrations of menadione, the NADPH content was re-established by 60 min (Fig. 4A). In BCNU-treated cells which were subsequently exposed to 100 μM menadione, however, a recovery of NADPH was not observed. While an explanation for this failure of recovery of NADPH under these conditions is currently being sought, preliminary studies have demonstrated that BCNU does not inhibit menadione-inclined stimulation of the hexose monophosphate shunt, a major source of NADPH regeneration [29]. The observation that BCNU-pretreated cells were more sensitive to menadione-induced toxicity (Fig. 4) may provide a possible explanation for the failure of these cells to regenerate NADPH. Conversely, the failure of these cells to regenerate NADPH may have been responsible for their increased sensitivity to menadione. For example, a correlation was observed recently between the failure of NADPH recovery and lethal cell injury in isolated hepatocytes exposed to menadione [29].

In conclusion, menadione caused a concentration-related depletion of GSH and release of LDH from isolated rat hepatocytes. The GSH depletion was potentiated by BCNU, a potent inhibitor of glutathione reductase. Additionally, NADPH was rapidly oxidized during metabolism of menadione. The failure of BCNU to prevent this rapid oxidation of NADPH suggests that glutathione reductase is not the primary source of NADPH consumption during menadione-induced oxidative cell injury and underlines the potential significance of other NADPH-consuming pathways.

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