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Important Role of Glutathione in Protecting against Menadione-induced Cytotoxicity in Rat Platelets

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Our previous studies demonstrate that menadione (MEN) is cytotoxic to platelets of rats by depleting glutathione (GSH). In order to clarify whether GSH has a role in protecting against menadione-induced cytotoxicity, the effect of GSH depletors as well as GSH precusors on menadione-induced cytotoxicity was investigated. Cysteine and dithiothreitol (DTT) prevent MEN-induced cytotoxicity in a dose-dependent manner, as determined by LDH leakage and change in turbidity. When platelets were treated with 1-chloro-2,4-dinitrobenzene (CDNB) and diethylmaleate (DEM), both of which deplete intracellular GSH, MEN-induced cytotoxicity was potentiated in the CDNB-treated platelets, but not in the DEM-treated platelets. These data suggest that the GSH in platelets plays an important role in protecting against cytotoxicity induced by menadione.

Key words : Menadione, Platelets, Glutathione, Cytotoxicity

INTRODUCTION

Quinones are widely distributed in nature and are often used clinically as antitumor drugs. Cytotoxicity of quinones, such as menadione (2-methyl-1,4-naphthoquinone), has been related to the "oxidative stress" caused by the redox cycling of quinones in their target cells (Monks *et al.*, 1992). The metabolism of menadione has been studied in isolated rat hepatocytes (Thor *et al.*, 1982) and it is known that quinones undergo either one- or two- electron reduction. Two-electron reduction of a quinone to its corresponding hydroquinone, mediated by quinone reductase, may represent a detoxifying pathway which protects the cell from the formation of intermediate reactive oxygen species. But the cytotoxic effects of many quinones are thought to be mediated by NADPH-cytochrome P-450 reductase through their one-electron reduction to semiquinone radicals, which subsquently enter redox cycles with molecular oxygen to produce active oxygen species and oxidative stress (Straat *et al.*, 1987).

In most cells, the toxic effects of oxidative stress, such as enzyme inactivation, DNA damage, cellular membrane damage, etc., are protected by glutathione (GSH). Incubation of isolated rat hepatocytes (Di Monte *et al.*, 1984a and 1984b) and blood platelets (Mirabelli *et al.*, 1989) with cytotoxic concentrations of menadione resulted in a marked decrease of intracellular GSH level and the amount of protein thiols. These free sulfhydryl group(s) are critical for the activity of hepatic plasma membrane Ca^{2+} translocase and the inhibition of this activity by menadione contributes to perturbation of Ca^{2+} homeostasis which precedes cell death (Nicotera *et al.*, 1985). However, the role of GSH and other sulfhydryl substances in protecting against oxidative stress damage in blood platelets has not been examined.

Our laboratory's previous research has shown that menadione causes oxidative stress to platelets. Glutathione (GSH) was depleted during incubation of platelets with menadione and reactive oxygen species were generated (Mirabelli *et al.*, 1989: Kim *et al.*, 1995a and 1995b). These reactive oxygen species caused cell lysis. The cell lysis would lead to a release of platelet granules into the blood. The release of these platelet granules could then result in excessive platelet aggregation and subsequent thrombosis and cardiovascular disease.

In the present investigation, we studied the role of intracellular GSH in menadione-induced cytotoxicity in platelets by modulating the intracellular GSH level. We examined the role of GSH in preventing LDH leakage as well as changes in platelet turbidity because our previous research demonstrated that the latter could be another indicator of plasma membrane damage in platelets (Kim *et al.*, 1995a).

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MATERIALS AND METHODS

Materials

The following chemicals and purified enzymes were purchased from Sigma (St. Louis, USA); NADH, NADPH, DTNB, diethylmaleate (DEM), cysteine, methionine, dithiothreitol (DTT), and menadione (2-methyl-1,4-naphthoquinone). All other chemicals were obtained from standard commercial sources.

Animals

Female Sprague Dawley rats (Yuhan Pharmaceutical Co., Korea) weighing 200 to 250 gm, were used. Prior to experiments, animals were housed for at least 3 or 4 days in the laboratory animal facility in polypropylene cages. The lighting in the animal room was regulated by an automatic control switch such that lights were on from 7 am to 7 pm and off from 7 pm to 7 am. Water was provided *ad libitum* throughout the experiments.

Preparations of platelets

Animals were sacrificed under light ether anesthesia. Blood collected from the abdominal aorta and anticoagulated with sodium citrate (3.8%, 1 : 9), was centrifuged for 15 min at 150 g at room temperature. Platelet rich plasma (PRP) was obtained from the supernatant resulting from this relatively low g-force centrifugation. Platelet poor plasma (PPP) was obtained from the supernatant of a 20 minute, 1,500 g centrifugation of the blood cell residue resulting from the first spin. Throughout all experiments, the platelet number was adjusted to 5×10^8 platelets/ml by diluting PRP with PPP.

Measurement of platelet lysis by aggregometer

Platelet lysis was measured by platelet turbidity, with 0% turbidity calibrated as the absorbance of PPP and 100% turbidity calibrated as the absorbance of PRP. PRP suspension in a silicon-coated cuvette was stirred at 1,200 rpm for 1 min prior to addition of menadione. Dimethyl sulfoxide (DMSO) was used as the vehicle for menadione, such that the final concentration of DMSO in the cuvette's incubation medium was 0.5%. This concentration was shown to have no effect on platelet lysis induced by menadione. Changes in turbidity were detected by a Lumi-aggregometer (Chrono-log Corp., USA).

Lactate dehydrogenase leakage

Leakage of lactate dehydrogenase (LDH) from platelets was measured by spectrophotometry (Bergmeyer *et al.*, 1965). After various times of incubation of the PRP with menadione, the incubation medium was centrifuged. A 0.05 ml aliquot of resulting supernatant was added to 2 ml of Tris-EDTA-NADH buffer (pH 7.4) and then incubated for 10 min at 37°C. After incubation, 0.2 ml of 14 mM pyruvate solution which had been preincubated at 37°C was added to the incubation vessel. The decrease of absorbance at 340 nm as NADH was converted to NAD^+ was measured. The extent of LDH leakage was expressed as the % of total enzyme activity lysed with 0.1% Triton X-100.

Statistical analysis

The results for LDH assay were expressed as means ±SEM of three experiments and were analyzed for statistical significance using Student t-test. Each experiment was done on cells obtained from a different animal.

RESULTS

Preliminary experiments revealed that menadione induced LDH leakage in a dose- and time-dependent manner, with 0.25 mM menadione being the effective dose tested. When platelets were exposed to 1 mM menadione, LDH leakage was 100% at 60 min (data not shown). To examine the possible role of GSH in protecting against menadione-induced cytotoxicity, the effect of a known precursor of GSH,

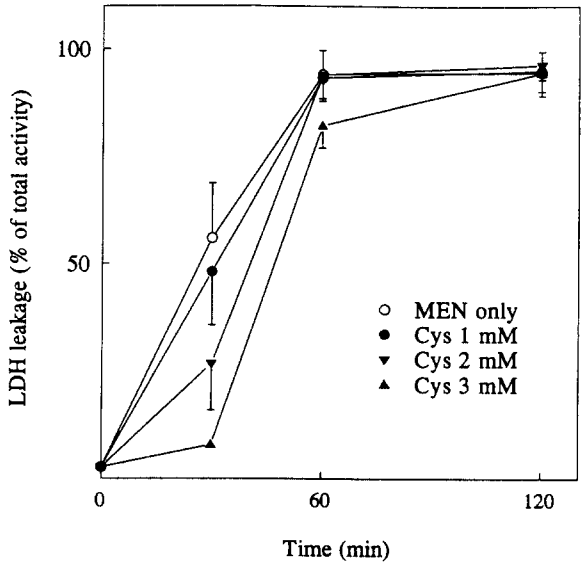

Fig. 1. Effect of cysteine (Cys) on menadione (MEN)-induced cytotoxicity in platelets. The preparation of platelet rich plasma (PRP) from rats was described in the methods section. Each platelet suspension was pretreated with various concentrations of cysteine 5 minutes prior to incubation with 1 mM menadione and then release of LDH was determined. Values are means±SEM of three experiments from three animals.

cysteine, on LDH leakage was investigated (Fig. 1). When various concentrations of cysteine were added 5 minutes prior to 1 mM menadione, LDH release was inhibited in a dose-dependent manner at 30 minutes. However, inhibition of LDH release by cysteine at levels as high as 3 mM was still only partially effective at 30 minutes and was not effective at 60 minutes. The effect of cysteine on platelet rich plasma (PRP) turbidity, another parameter of menadione cytotoxicity which was recently reported by our laboratory (Kim *et al.*, 1995a), was examined (Fig. 2). As reported previously, when PRP is incubated with menadione in an aggregometer (see MEN only in Fig. 2), an increase in turbidity is initially observed. This increase is associated with a change in platelet shape. Subsequent to this event, a decrease in turbidity is observed. This decrease is associated with cell lysis. Addition of cysteine to our incubation system delays the magnitude of cell lysis and the time of occurrence in a dose-dependent manner. These results are consistent with those illustrated for LDH leakage in Fig. 1.

Methionine is also known to function as a precursor of GSH biosynthesis in isolated hepatic cells,

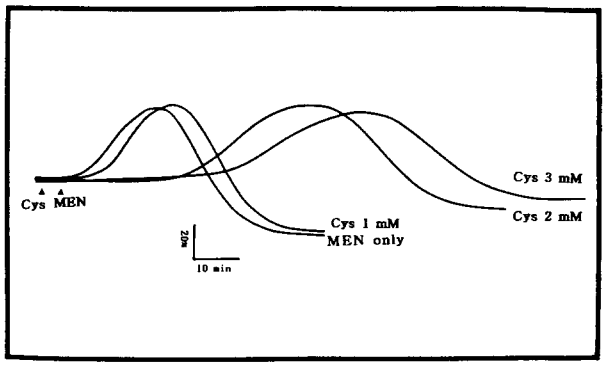

Fig. 2. Effect of cysteine (Cys) on menadione (MEN)-induced changes in turbidity. PRP was pretreated with various concentrations of cysteine 5 minutes prior to incubation with 1 mM menadione and then the turbidity changes in aggregometer were measured. The X-axis represents the time of incubation and the Y-axis represents the percentage change in turbidity.

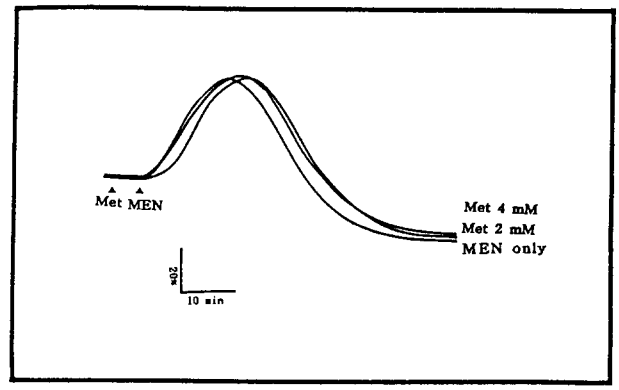

Fig. 4. Effect of methionine (Met) on menadione (MEN)-induced changes in turbidity. PRP was pretreated with various concentrations of methionine 5 minutes prior to incubation with 1 mM menadione and then the turbidity changes in aggregometer were measured. The X-axis represents the time of incubation and the Y-axis represents the percentage change in turbidity.

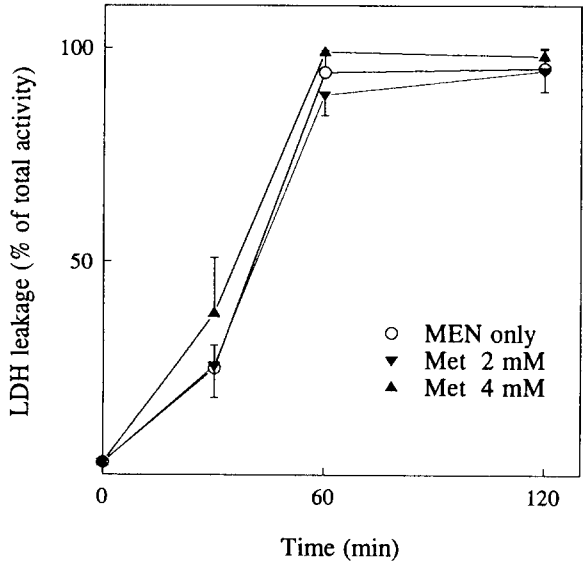

Fig. 3. Effect of methionine (Met) on menadione (MEN)-induced cytotoxicity in platelets. PRP was pretreated with various concentrations of methionine 5 minutes prior to incubation with 1 mM menadione and then release of LDH was determined. Values are means±SEM of three experiments from three animals.

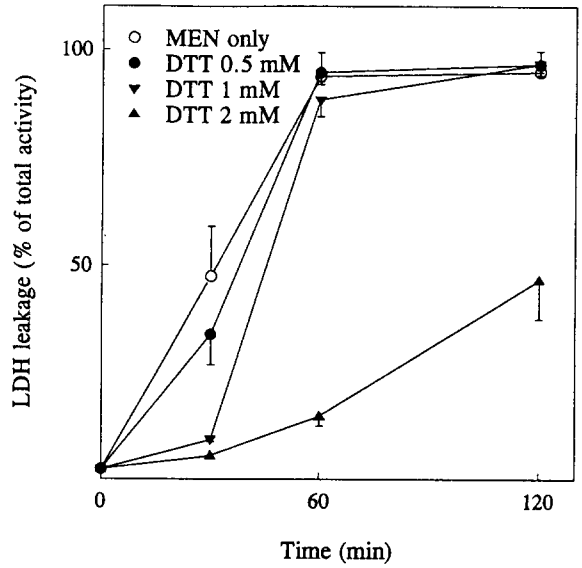

Fig. 5. Effect of dithiothreitiol (DTT) on menadione (MEN)-induced cytotoxicity in platelets. PRP was pretreated with various concentrations of cysteine 5 minutes prior to incubation with 1 mM menadione and then release of LDH was determined. Values are means±SEM of three experiments from three animals.

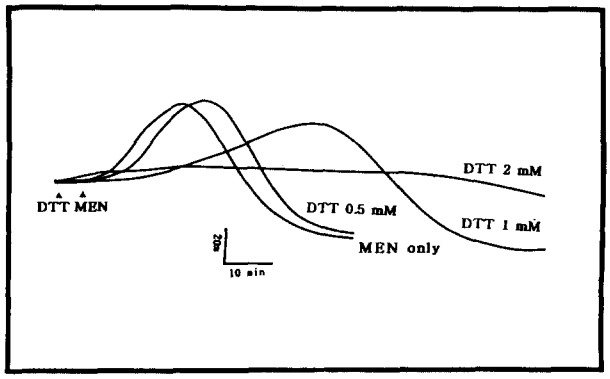

Fig. 6. Effect of dithiothreitol (DTT) on menadione (MEN)-induced changes in turbidity. PRP was pretreated with various concentrations of dithiothreitol 5 minutes prior to incubation with 1 mM menadione and then the turbidity changes in aggregometer were measured. The X-axis represents the time of incubation and the Y-axis represents the percentage change in turbidity.

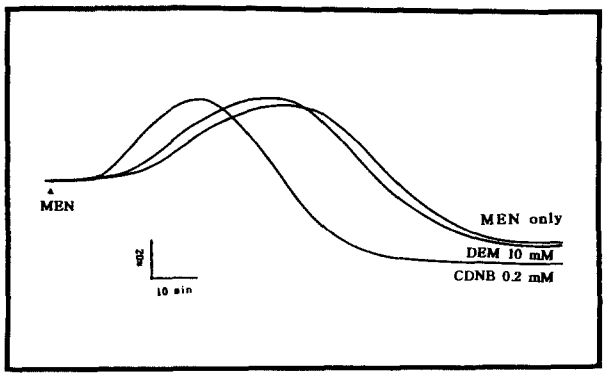

Fig. 8. Effect of glutathione depletion on menadione (MEN)-induced changes in turbidity. PRP was pretreated with 0.2 mM 1-chloro-2,4-dinitrobenzene (CDNB) or 10 mM diethylmaleate (DEM) 30 minutes prior to incubation with 0.25 mM menadione and then the turbidity changes in aggregometer were measured. The X-axis represents the time of incubation and the Y-axis represents the percentage change in turbidity.

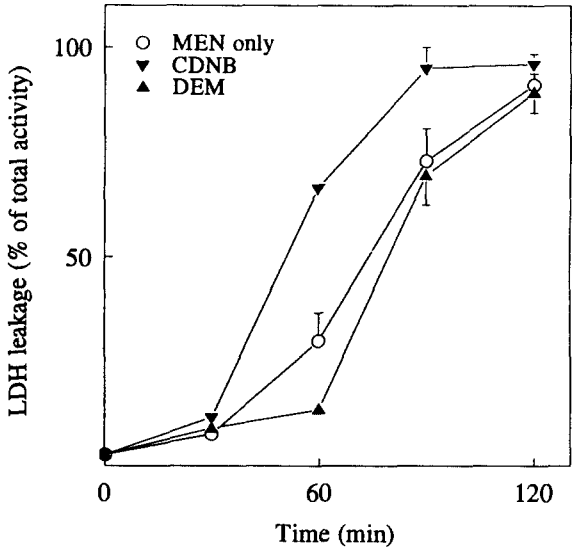

Fig. 7. Effect of glutathione depletion on menadione (MEN)-induced cytotoxicity in platelets. PRP was pretreated with 0.2 mM 1-chloro-2,4-dinitrobenzene (CDNB) or 10 mM diethylmaleate (DEM) 30 minutes prior to incubation with 0.25 mM menadione and then release of LDH was determined. Values are means±SEM of three experiments from three animals.

because it is converted to cysteine via the cystathionine pathway (Orrenious *et al.*, 1983: Beatty and Reed, 1980). To determine if methionine might also serve as a precursor for GSH in platelets, we examined the effect of methionine on menadione toxicity in platelets. When PRP was pretreated with methionine prior to treatment of menadione for time periods up to 120 minutes, the rate of LDH leakage (Fig. 3) and the curve for platelet turbidity (Fig. 4) was not different from those demonstrated by PRP treated with menadione alone.

The effect of a third sulfhydryl substance, which is known to protect against oxidative stress in hepatic cells, dithiothreitol (DTT), was also examined. DTT inhibited LDH leakage (Fig. 5) in a dose-dependent manner. A 2 mM DTT dose was almost completely effective at 60 minutes and remained partially effective through 120 minutes. The effect of DTT on PRP turbidity was also examined (Fig. 6). DTT delayed changes in turbidity in a dose-dependent manner and the 2 mM dose completely prevented all menadione-induced turbidity changes.

To confirm that glutathione (GSH) plays a role in the protection of menadione-induced cytotoxicity, we examined the effect of two well known GSH depletors, 1-chloro-2,4-dinitrobenzene (CDNB) and diethylmaleate (DEM), in our system. CDNB potentiated the cytotoxicity of 0.25 mM menadione, as indicated by LDH leakage (Fig. 7) and turbidity change (Fig. 8). However, DEM did not affect either parameter of menadione cytotoxicity, even at concentrations as high as 10 mM (Fig. 7 and 8).

DISCUSSION

These results suggest that the role of glutathione (GSH) is important in protecting against menadione-induced cytotoxicity in platelets. So far, studies on GSH in platelets mainly focus on the role of GSH in prostaglandin metabolism and as antioxidants which protect against platelet aggregation (Bryant *et al.*, 1982: Hill *et al.*, 1989: Bosia *et al.*, 1985). There are currently no studies available which investigate how platelets synthesize GSH from its precursors. Our study shows that methionine does not prevent menadione-induced cytotoxicity, but cysteine was capable

of delaying and preventing it. In hepatocytes, however, the reverse is true. Methionine is more effective in preventing menadione-induced cytotoxicity than are comparable doses of cysteine (Thor *et al.*, 1978). The reason for this is that cysteine is autooxidized in the incubation medium; this reduces the level of cysteine available to the hepatic cells (Thor *et al.*, 1979). However, methionine does not have a sulfhydryl group and therefore does not undergo autooxidation, so methionine is more readily available to the hepatocytes for synthesis to GSH. Since our study failed to demonstrate a protective effect from methionine, it appears likely that platelets may lack the pathway which converts methionine to GSH.

In hepatocytes, intracellular GSH is converted to GSSG and/or GSH-menadione conjugate in the presence of menadione. Once GSSG is formed, it can be reduced back to GSH by glutathione reductase. However, if this pathway is saturated, GSSG could be excreted extracellularly (Orrenius *et al.*, 1983). In platelets, we were unable to detect the menadione-induced formation of GSSG from GSH due to our experimental conditions and thus we can not determine the metabolic pathway of GSH. There could be two reasons why we were unable to detect GSSG. First, the GSSG could have undergone substantial recycling back to GSH, resulting in an overall minimal production of GSSG that was present at too low a concentration for our assay to detect. Second, most of the GSH may have been converted to GSH-menadione conjugate. If this is the case, it is possible that further menadione-induced damage could occur *in vivo* to the platelets and other possible target sites, such as kidney cells. This has been suggested previously (Thor *et al.*, 1982: Redegeld *et al.*, 1989) that the GSH-menadione conjugate could be excreted from hepatic cells and then taken up by kidney cells. Once there, the GSH-menadione conjugate could undergo redox cycling, leading to cell death.

Finally, when GSH was depleted by CDNB and DEM pretreatment, menadione-induced cytotoxicity was potentiated by CDNB, but not by DEM pretreatment. This was unexpected, since other *in vitro* cell systems using similar DEM pretreatments have shown that potentiation of quinone-induced toxicity occurred (Di Monte *et al.*, 1984b). Two explanations appear likely for DEM's failure to promote menadione toxicity in platelets. The first is that DEM pretreatment fails to deplete GSH in a platelet preparation. However, this possibility was eliminated after we measured total GSH levels in platelets with and without DEM pretreatment and confirmed that our pretreatment regimen did completely deplete the GSH (data not shown). The second possible explanation is that, in our platelet preparation, the DEM pretreatment regimen affected menadione metabolism or uptake, such that menadione levels in platelets were reduced to less toxic levels. If this is the case, then there must be some key difference between platelets and hepatocytes in either the method of menadione uptake or the enzyme system responsible for menadione metabolism. This explanation is plausible because our previous research (Kim *et al.*, 1995a) has shown that the menadione metabolizing enzyme system in platelets is located both in the microsomal fraction (i.e. the dense tubular system) as well as the plasma membrane. This is in contrast to hepatocytes, where menadione is primarily metabolized in the endoplasmic reticulum.

Since menadione and other quinones receive extensive therapeutic applications, it is important for further research to continue to investigate the mechanism of menadione cytotoxicity in platelets and determine how the possible risk to cardiovascular disease from menadione can be reduced.

ACKNOWLEDGEMENT

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