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Menadione-induced Cytotoxicity in Rat Platelets: Absence of the Detoxifying Enzyme, Quinone Reductase

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The elevation of intracellular Ca^{2+} in various tissue through oxidative stress induced by menadione has been well documented. Increase of Ca^{2+} level in platelets results in aggregation of platelets. To test the hypothesis that menadione-induced Ca^{2+} elevations can play a role in platelet aggregation, we have studied the effect of menadione on aggregation of platelets isolated from female rats. Treatment with menadione to platelet rich plasma (PRP), which proved to be an adequate system, appeared to induce dose-dependent turbidity changes of platelets up to 60%, as determined by aggregometry. However, exposure of PRP to menadione leads to a loss of cell viability, as measured by lactate dehydrogenase (LDH) leakage, suggesting that menadione might induce cell lysis rather than aggregation of platelets. Turbidity changes induced by menadione were unaffected by addition of dicoumarol, which is a quinone reductase (QR) inhibitor. Consistent with these findings, no activity of QR was detected in any subcellular fractions of platelets. These data, which indicate an absence of the QR detoxifying pathway, suggest that platelets may be more susceptible to menadione-induced cytotoxicity than certain other cell, such as hepatocytes.

Key words : Menadione, Platelets, Quinone reductase, Cytotoxicity

INTRODUCTION

The involvement of platelets in hemostasis and their production of thrombus implies an important pathophysiological role for platelets in mammalian circulatory systems (Mustard and Packman, 1979, Frojmovic and Milton, 1982). Injury to a vessel disrupts the endothelium and exposes the underlying connective tissue collagen molecules. Platelets adhere to collagen and trigger the release of platelet granules which contain potent endogenous biochemical agents that affect blood vessels and cause platelet aggregation (Stormorken, 1984). Normal endothelium continuously produces nitric oxide and prostaglandins (PGE_2, PGI_2, etc.) to control aggregation of platelets (Katzenschlager *et al.*, 1991; Radomski *et al.*, 1987). This homeostatic balance is broken by damage to the vessels or pathological factors. Furthermore, excess aggregation of platelets can be induced, which results in vessel diseases (Thompson and Harker, 1987).

In all mammalian cells, the cytosolic calcium level is approximately 10,000-fold less than the extracellular calcium concentration in plasma (Nicotera *et al.*, 1992). This low cytosolic Ca^{2+} concentration is maintained mainly by ATP-dependent calcium ATPase (present in the plasma membrane and endoplasmic reticulum) and by the calcium transport system of the mitochondria (Tsien *et al.*, 1982; Kim *et al.*, 1983). Normally, unstimulated platelets maintain a low cytosolic calcium concentration and a steep plasma membrane calcium gradient (Brass, 1984). However, when activated by agonists (thrombin, collagen, etc.), a significant increase in cytosolic calcium is observed (Johnson *et al.*, 1985; Kovacs *et al.*, 1989). This increase is due to an infiux of calcium through the plasma membrane and the release of stored calcium from dense tubular systems. An increase in the level of cytoplasmic calcium is considered the primary event leading to platelet shape change due to cytoskeletal rearrangement, the secretion of granule contents, and finally aggregation of platelets (Rink *et al.*, 1982; Rink and Sage, 1990).

Menadione (2-methyl-1,4-naphthoquinone), as a component of multivitamin drugs, has been used as a therapeutic agent for hypothrombinemia, and as an anti-inflammatory agent. Menadione (quinone) can undergo either two-electron reduction to the hydroquinone or one-electron reduction to the semi-

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quinone radicals. Two-electron reduction occurs by quinone reductase which is mainly found in cytoplasm of various tissues. On the other hand, one-electron reduction occurs primarily by flavoprotein reductase (NADPH cytochrome P-450 reductase, NADH cytochrome b_5 reductase, etc.), which is bound to microsomal membrane (Lind *et al.*, 1982; Monks *et al.*, 1992). Two-electron reduction is generally known as the detoxification step by reducing quinone into the stable hydroquinone, which is readily excreted after conjugation. However, one-electron reduction produces semiquinone, which in the presence of molecular oxygen is the converted back to quinone via oxidation. In this redox sequence, molecular oxygen is converted to a superoxide anion radical. The superoxide anion radical is metabolized further to hydrogen peroxide and other reactive oxygen species. Numerous studies have shown that treatment with menadione leads to cytotoxicity through oxidative stress induced by menadione's redox cycle (Monks *et al.*, 1992). At cytotoxic concentrations of menadione, rapid changes in intracellular thiol and Ca^{2+} homeostasis were also observed (Thor *et al.*, 1982; Di Monte *et al.*, 1984a and 1984b; Smith *et al.*, 1987). Menadione increases the platelet intracellular Ca^{2+} level, which causes alterations in the cytoskeleton of the platelets (Mirabelli *et al.*, 1988; Mirabelli *et al.*, 1989). These phenomena are consistent with observed changes in platelet shape and the activation of platelet aggregation.

Our laboratory originally hypothesized that menadione-induced elevation of intracellular calcium in platelets leads to excessive aggregation of platelets and thence to an increased risk of cardiovascular disease. However, our investigations determined that menadione induces platelet lysis instead of platelet aggregation. As a consequence, we have studied a role of the detoxifying enzyme, quinone reductase, in menadione-induced platelet lysis.

MATERIALS AND METHODS

Materials

The following chemicals were purchased from Sigma (St. Louis, USA): dicoumarol, menadione (2-methyl-1,4-naphthoquinone), menadione sodium bisulfite, and sodium citrate.

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 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. Washed platelets (WP) were prepared by washing PRP with a washing buffer (163.3 mM NaCl, 2.8 mM KCl, 1.1 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 11.2 mM glucose, 2 mM EDTA, and 0.35% bovine serum albumin) according to a procedure described previously (Mustard *et al.*, 1972). The platelet pellets were finally suspended in a buffer solution similar to the washing buffer, but without the EDTA and bovine serum albumin. $CaCl_2$ (1 mM) was added or omitted from the buffer solutions in which the washed platelet preparations were suspended. Throughout all experiments, the platelet number was adjusted to 5×10^8 platelets/ml by diluting PRP with PPP or by diluting WP with suspension buffer (Puri *et al.*, 1989).

Measurement of platelet lysis

Platelet aggregation was induced by addition of 1.2 units of thrombin to the platelet preparations. Aggregation was measured by platelet turbidity, with 0% aggegration calibrated as the absorbance of PRP or washed platelet (WP) suspension and 100% aggregation calibrated as the absorbance of PPP or suspension buffer. PRP or WP suspension in a silicon-coated aggregation cuvette was stirred at 1,200 rpm for 1 min prior to addition of menadione. 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 of either platelet aggregation induced by thrombin or platelet lysis induced by menadione. Changes in turbidity were detected by a Lumi-aggregometer (Chrono-log Corp., USA).

Quinone reductase assay

Activity of the cytosolic enzyme, quinone reductase (QR), was determined by the spectrophotometric method of Benson *et al.* (1980). A final 3.0 ml volume of reaction mixture contained 25 mM Tris-HCl (pH 7.4), 0.07% bovine serum albumin, 0.01%

Tween 20, 5 uM FAD, 0.2 mM NADH, 0 or 10 uM dicoumarol, and an appropriate amount of cytosolic fractions. The electron acceptor, 40 uM 2,6-dichlorophenolindophenol (DCPIP) in 10 ul of water, was added to this mixture to intiate the reaction. Assays were performed at 25°C with and without 10 uM dicoumarol. The dicoumarol-sensitive part of the activity was taken as a measure of the quinone reductase activity. The initial velocity of the reduction of 2, 6-dichlorophenolindophenol was measured spectrophotometrically at 600 nm and an extinction coefficient of 21/mM/cm was used to determine specific activity.

RESULTS AND DISCUSSION

To select an adequate experimental system, attempts were made to measure menadione-induced aggregation using a turbidimetric aggregometer on three different preparations of platelets-platelet rich plasma (PRP), washed platelets containing Ca^{2+}, and washed platelets without Ca^{2+}. PRP preparations incubated with 1 mM menadione (Fig. 1) showed the most sensitive shape change (as indicated by a turbidity increase) at 20 mins and the most significant aggregation (as indicated by a turbidity decrease) thereafter. Washed platelets (WP) in the presence or absence of calcium demonstrated less response. As a consequence, PRP was selected as the experimental model for further menadione investigations.

PRP was treated with various concentrations of menadione (Fig. 2). No change of turbidity was observed at 0.1 mM. At higher concentrations, a dose-dependent change in the shape of the curve followed by decreases in turbidity were observed. Reaction times were considerably shortened as well. However, the maximum decrease in turbidity induced by the menadione was greater than 50-60%. This was unexpected, because it was known that most agonists, such as thrombin, collagen, etc. can induce 100% aggregation of platelets. It was postulated that higher levels of menadione were required to induce 100% platelet aggregation. Since menadione is relatively low in solubility in DMSO (maximum of 4 mM), menadione's effect at higher concentrations was investigated by preparing aqueous solutions of menadione sodium bisulfite, which was known to be rapidly converted into menadione in blood plasma. However, even concentrations as high as 100 mM menadione sodium disulfite (Fig. 3) failed to demonstrate decreases in turbidity beyond 60%. Further, no platelet aggregates were visible in the solution, even when examined under light microscopy.

Based on these observations, it was postulated that platelet aggregation was not the cause of the observed decreases in turbidity induced by menadione. Instead, it was postulated that menadione-induced cell lysis might be the cause. In order to determine whether menadione induces platelet aggregation or lysis, the effect of menadione on platelet lactate dehydrogenase (LDH) leakage was investigated. When PRP was incubated with menadione, LDH leakage was increased in a dose- and time- dependent manner (data not shown).

The flavoprotein quinone reductase (DT-diaphorase) is commonly found in many cells, including hepatocytes, and is responsible for the reduction of quinones into corresponding hydroquinones. These hy-

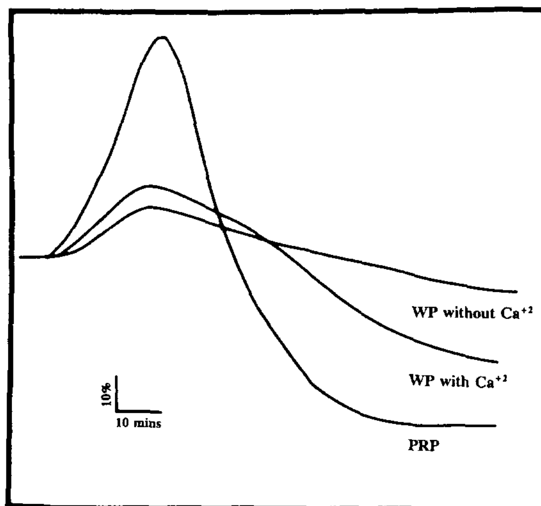

Fig. 1. Effect of menadione on trubidity changes by platelets in aggregometer under three different preparations of platelets. Preparations of platelet rich plasma (PRP) and washed platelet (WP) in the absence or presence of Ca^{+2} were described in Materials and Methods section. Each platelet suspension was incubated with 1 mM menadione. The X-axis represents the time of incubation and the Y-axis represents the percentage change in turbidity.

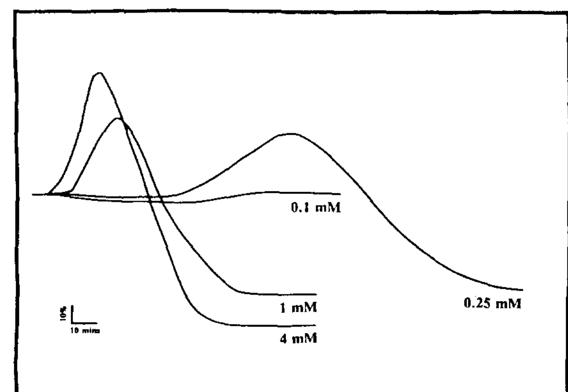

Fig. 2. Dose-dependent changes in turbidity in platelet suspension induced by menadione. PRP was incubated with various concentrations of menadione in a platelet aggregometer. The X-axis represents the time of incubation and the Y-axis represents the percentage change in turbidity.

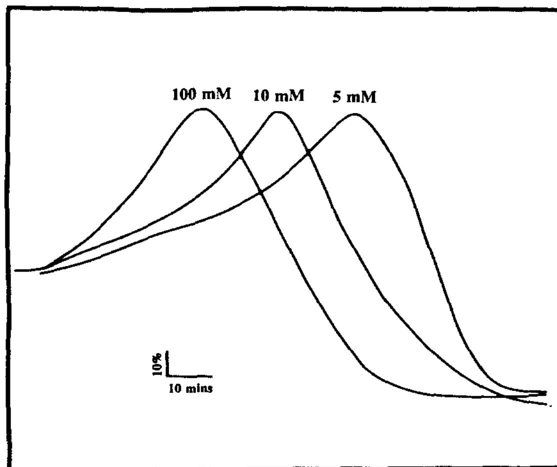

Fig. 3. Effect of water-soluble menadione sodium bisulfite on turbidity changes in platelet suspension. PRP was incubated with various concentrations of menadione sodium bisulfite in a platelet aggregometer. The X-axis represents the time of incubation and the Y-axis represents the percentage change in turbidity.

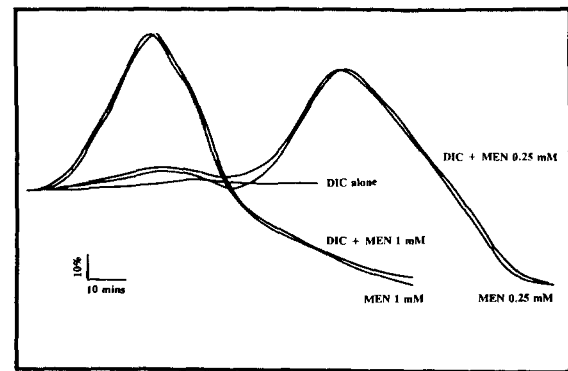

Fig. 4. Effect of dicoumarol on the menadione-induced change of turbidity in platelet suspension. PRP was pretreated with dicoumarol (DIC) 3 minutes prior to addition of menadione (MEN). The X-axis represents the time of incubation and the Y-axis represents the percentage in turbidity.

Table I. Activity of quinone reductase in rats

	Platelets	Liver (nmol/min/mg protein)
Cytosol	ND	807±126
Microsome	ND	165±24

Cytosolic and microsomal fractions from platelets and liver were prepared for quinone reductase assay. Activities of quinone reductase were determined by the spectrophotometric method. Values are means±SEM for three experiments.
ND: not detected.

droquinones may undergo a conjugation reaction, which is the detoxifying mechanism for quinone-induced toxicity (Thor *et al.*, 1982). If the quinone reductase detoxifying pathway occurs in platelets, then the menadione-induced platelet cytotoxicity should be attenuated. To investigate the possible role of quinone reductase (QR) in protecting platelets from menadione toxicity, PRP was treated with a quinone reductase inhibitor (dicumarol). When PRP was preincubated with dicoumarol for 3 mins prior to the addition of menadione, no change in turbidity was observed (Fig. 4). Furthermore, subsequent assays for quinone reductase activity in rat platelets failed to show any presence of the enzyme (Table 1).

It was originally hypothesized that an increase in Ca^{2+} concentration in platelets induced by menadione could lead to platelet aggregation, thereby resulting in excessive thrombus formation that could provoke cardiovascular diseases. However, it was determined that treatment of menadione to platelets leads to cell lysis instead of cell aggregation. Two lines of evidence support this view: 1) unlike agonists, such as thrombin, the turbidity of PRP was decreased only 60% at levels as high as 100 mM menadione sodium blsulfite: 2) intracellular lactate dehydrogenase (LDH) was released into the incubation medium in a dose-dependent manner, consistent with the increases in turbidity of PRP.

Quinone reductase catalyzes the two-electron of quinones, such as menadione, and is generally considered to play a protective role against menadione-mediated toxicity (Monks *et al.*, 1992). Using hepatic subcellular fractions and purified enzymes, it has been demonstrated that inhibition of quinone reductase with dicoumarol increases menadione-induced oxygen consumption and cytotoxicity (Wefers and Sies, 1983). Dicoumarol has also been shown to greatly potentiate the toxicity of menadione in isolated rat hepatocytes (Thor *et al.*, 1982). In our studies, the presence of dicoumarol in intact platelets did not affect turbidity changes induced by menadione (Fig. 4), suggesting that the platelets are more susceptible to quinone toxicity than other cells, such as hepatocytes. However, this interpretation could be complicated by recent *in vitro* studies (Dicker and Cederbaum, 1993) which demonstrate that quinone reductase, in the presence of iron, may not always serve a protective role against free radical damage. If this is also true *in vivo*, then the absence of quinone reductase in platelets will favor the prevention of free radical generation, thus protecting the cell from oxidative damage.

Many medical researchers have utilized platelet aggregometry for *in vitro* screening of antithrombotic activity (Lasslo and Quintana, 1984). Caution should be taken, however, before using only turbidity readings to screen for platelet anti-aggregating agents derived from synthetic chemicals and natural products, because our results indicate that turbidity alterations may be due to cell lysis instead of aggregation. This may possibly be the case with some

phenolic compounds, including quinones, which have been postulated to be platelet anti-aggregating agents due to observed alterations in platelet turbidity (Ko *et al.*, 1990). These phenolic compounds, when administered in small quantities, could possibly inhibit platelet aggregation, since they are known to have some antioxidant capacity. However, administration of larger quantities to assay systems might cause the lysis of platelets, thereby decreasing observed turbidity.

On the basis of these results, it is concluded that further investigations into the mechanism of menadione-induced platelet cytotoxicity should be undertaken to determine if platelet lysis could be a possible cause of cardiovascular disease.

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