The biological significance of non-enzymatic reaction of menadione with plasma thiols: enhancement of menadione-induced cytotoxicity to platelets by the presence of blood plasma

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Received 9 March 1999

Abstract To test the hypothesis that the non-enzymatic reaction of quinones with thiols in plasma can generate reactive oxygens (ROS), thereby leading to potentiated cellular toxicity, we have studied the effect of a representative quinone compound, menadione, on plasma isolated from rats. The experimental results are as follows: (1) menadione generated ROS via non-enzymatic reaction with protein thiols in plasma; (2) the presence of plasma increased menadione-induced cytotoxicity to platelets; (3) pretreatment of plasma with a thiol-depleting agent significantly suppressed menadione-induced ROS and cytotoxicity. These results suggest that the non-enzymatic reaction of menadione with plasma thiols could be an important process in quinone-induced cellular toxicity. © 1999 Federation of European Biochemical Societies.

Key words: Plasma; Menadione; Cytotoxicity; Thiol; Non-enzymatic reaction; Reactive oxygen; Platelet

1. Introduction

Oxidative stress is defined as a disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential damage [1]. It has been reported that oxidative stress is related to such chronic diseases as atherosclerosis, cancer, and diabetes mellitus. Oxidants causing oxidative stress can be generated endogenously, by immune cells such as neutrophils, and exogenously, by exposure to drugs [2,3]. Menadione (2-methyl-1,4-naphthoquinone: vitamin K\textsubscript{3}), a multivitamin component and a widely used therapeutic agent for hypothyroidemia and cancer, is cytotoxic in isolated hepatocytes and its cytotoxic mechanism has been associated with the excessive generation of reactive oxygens such as superoxide, singlet oxygen, and hydrogen peroxide. Menadione is metabolized to a semiquinone by various flavoenzymes, such as NADPH cytochrome P-450 reductase, NADH cytochrome b\textsubscript{5} reductase, and NADH-ubiquinone reductase. This semiquinone is converted to the parent quinone in the presence of oxygen, thereby generating reactive superoxides which play a critical role in cellular oxidative damage [4–6].

It has also been reported that menadione is converted to the semiquinone by non-enzymatic reaction with various thiols. In the presence of oxygen, reactive oxygens are generated by this non-enzymatic reaction in the test tube [7–10]. This menadione-thiol reaction has also been observed both in hepatic microsomal systems and within intact cells (hepatocytes). In liver microsomes, superoxide was generated in the absence of NAD(P)H, a cofactor required for enzymatic reduction of menadione, and the generation of superoxide was blocked by N-ethylmaleimide (NEM), a thiol-depleting agent [11]. In isolated hepatocytes, formation of GSSG, which is indicative of oxidative stress, is increased in the absence of NAD(P)H [12]. Weers and Sies [13] also reported that, in menadione-treated hepatocytes, less singlet oxygen could be detected in GSH-depleted hepatocytes than in control (non-GSH-depleted) hepatocytes, suggesting that non-enzymatic reaction of menadione with soluble thiol (GSH) has biological relevance in the cell. However, they also observed that the reaction of menadione and GSH catalyzed by a detoxification enzyme, glutathione \textsubscript{S}-transferase (GST), was not accompanied by the generation of reactive oxygens. Therefore, though menadione can be reacted with thiols non-enzymatically in the cell following the generation of reactive oxygens, the biological implication of this observation is currently unclear, given the relatively high intracellular activity of GST [14].

Blood plasma provides important transport functions, so plasma is readily exposed to various drugs or chemicals. Plasma is rich in protein thiols, but has only a limited amount of soluble thiol (including GSH) [15]. In addition, the activity of GST is negligible in plasma [14]. Thus we can assume that the non-enzymatic reaction of menadione with protein thiols following the generation of reactive oxygens is a major process of menadione in plasma, which could lead to cellular toxicity. This paper addresses that issue by showing that menadione reacts with plasma thiols, thereby generating reactive oxygens. In addition, we observed that the presence of plasma affected toxicity of menadione to adjacent cells such as platelets, the cells directly exposed to plasma.

2. Materials and methods

2.1. Materials

The following reagents were obtained from Sigma (St. Louis, MO, USA): menadione, NEM, dimethyl sulfoxide (DMSO), superoxide dismutase (SOD), catalase, and 5,5’-dithiobis (2-nitrobenzoic acid) (DTNB). All other reagents were commercial products of the highest available grade of purity.

2.2. Animals

Female Sprague-Dawley rats (180–220 g) were used for all experiments. The animals were maintained at 22 ± 2°C and 45–55% humid-
ity under a 12 h light-dark cycle. The animals were allowed food and water ad libitum.

2.3. Preparations of plasma, platelet-rich plasma, or washed platelets
All procedures were conducted at room temperature, and the use of glass containers and pipettes was avoided. Blood was collected from the abdominal aorta of ether-anaesthetized rats. For plasma preparation, 3.8% sodium citrate (1:9) was used as an anticoagulant. Plasma was obtained by centrifugation at 1500 × g for 20 min. ‘Filtered plasma’ was prepared by filtering plasma through Centriprep 50 (Amicon, USA), thereby eliminating proteins higher than 50,000 Da from the plasma. ‘Boiled plasma’ was prepared by heating plasma in a test tube in boiling water for 30 s. For the preparation of platelet-rich plasma (PRP) or washed platelets (WP), acid-citrate-dextrose (ACD) (1:6; 85 mM trisodium citrate, 71 mM citric acid, 111 mM dextrose) was used as an anticoagulant. ACD blood was centrifuged at 1500 × g for 15 min to obtain condensed PRP. The platelets were pelleted by centrifugation at 500 × g for 10 min. Platelets were washed in washing buffer (138 mM NaCl, 2.8 mM KCl, 0.8 mM KH2PO4, 0.8 mM MgCl2, 10 mM HEPES, 5.6 mM dextrose, 2 mM EDTA, pH 7.4) and pelleted by centrifugation at 500 × g for 10 min. Final platelet pellets were resuspended in plasma for PRP or in suspension buffer for WP. The composition of WP suspension buffer was 138 mM NaCl, 2.8 mM KCl, 0.8 mM KH2PO4, 0.8 mM MgCl2, 10 mM HEPES, 5.6 mM dextrose, pH 7.4. For all experiments, the platelet concentration was adjusted to 5 × 108 cells/ml using light microscopically. Whole plasma, PRP or WP were incubated at 37°C in plastic flasks. Menadione was added as a 0.5% solution in DMSO; controls received DMSO, no increase in oxygen consumption was observed. When plasma was treated with the control vehicle, DMSO, no increase in oxygen consumption was observed. Addition of superoxide dismutase (SOD) and catalase at 3 min before menadione addition resulted in a significant reduction of menadione-induced oxygen consumption in plasma. These results suggest that menadione causes increased oxygen consumption in plasma, which is due to the generation of reactive oxygens.

2.4. Biochemical assays
Oxygen consumption was measured polarographically with a Clark oxygen electrode (Yellow Springs Instrument Co., USA) in magnetically stirred sample chambers at 37°C. Whole plasma, PRP or WP were preincubated under the same conditions for at least 5 min to saturate samples with air. The initial rate of oxygen consumption was determined from the time of addition of menadione or DMSO alone and allowed to equilibrate sample chambers at 37°C. Whole plasma, PRP or WP were incubated at 37°C in plastic flasks. Menadione was added as a 0.5% solution in DMSO; controls received vehicle alone. Aliquots were withdrawn for analysis after various time intervals.

3. Results

3.1. The generation of reactive oxygens via non-enzymatic reaction of menadione and plasma thiols
To determine whether reactive oxygens can be generated by menadione in plasma, we measured menadione-induced oxygen consumption in plasma (Fig. 1). When 0.25 mM menadione was added to plasma, oxygen consumption increased slowly through the 10 min incubation period. Treatment with 1 mM menadione resulted in increased oxygen consumption through 5 min, at which time oxygen consumption was maximal. When plasma was treated with the control vehicle, DMSO, no increase in oxygen consumption was observed. Addition of superoxide dismutase (SOD) and catalase at 3 min before menadione addition resulted in a significant reduction of menadione-induced oxygen consumption in plasma. These results suggest that menadione causes increased oxygen consumption in plasma, which is due to the generation of reactive oxygens. It was assumed that the menadione-induced generation of reactive oxygens was due to the reaction of menadione with thiols in the plasma according to previous reports [7–10]. To test this assumption, we investigated whether thiols in plasma were depleted following the reaction of menadione with thiols (Fig. 2). Plasma treated with 1 mM menadione resulted in complete depletion of the thiols within 5 min. Treatment with 0.25 mM menadione resulted in a slower rate of thiol depletion with incomplete depletion observed even through a 30 min incubation period. These observations are consistent with the pattern of oxygen consumption in menadione-treated plasma, depicted in Fig. 1. They confirm the assumption that menadione-induced reactive oxygen generation is mediated by the reaction of menadione with thiols, as evidenced by the menadione depletion of plasma thiols.

To confirm the role of plasma thiols treated with menadione in the generation of reactive oxygens, we also pretreated plasma with NEM, a known plasma thiol-depleting agent. As seen in Fig. 3A, NEM pretreatment of plasma almost completely blocked the menadione-induced increase in oxygen consumption. To further characterize the reaction of menadione with thiols in plasma, when ‘filtered plasma’ without proteins higher than 50,000 Da was treated with menadione, a significant increase in oxygen consumption by menadione was not observed (Fig. 3B). This suggests that low molecular weight components including soluble thiols (glutathione, etc.) in plasma were not involved in the generation of reactive oxygens in the presence of menadione. However, ‘boiled plasma’ followed by menadione treatment resulted in increased oxygen consumption approximately equal to that seen in intact plasma, suggesting that the reaction of menadione with high molecular weight thiols (protein thiols) is non-enzymatic. These results, taken together with those depicted in Fig. 2, indicate that menadione can generate reactive oxygens via non-enzym...
matic reaction with protein thiols in plasma and, as a result, plasma thiols are decreased.

3.2. Potentiation of the cytotoxic effect of menadione by the presence of plasma

Menadione cytotoxicity in various tissues and blood cells, such as erythrocytes, neutrophils, and platelets, is well documented [4,18]. Since our results indicate that reactive oxygens are generated by menadione in plasma, we hypothesized that menadione-induced cytotoxicity to certain tissues might be influenced by the presence of plasma.

To test this hypothesis, we selected a platelet model for the following reasons: (1) platelets are directly exposed to plasma; (2) platelets are reported to be susceptible to menadione-induced cytotoxicity; and (3) platelet systems both with plasma (PRP) and without plasma (WP) have been well developed in extensive platelet research.

In Fig. 4, we pretreated PRP with the thiol-depleting agent NEM and cytotoxicity as assessed by LDH release was compared to that seen by menadione alone. We hypothesized that NEM pretreatment would reduce menadione-induced cytotoxicity to platelets in the PRP system because the protein thiols would no longer be available for the non-enzymatic reaction of menadione in plasma. Treatment with NEM alone does not result in release of LDH from platelets. As previous studies have shown [19], menadione (0.25 mM) induced LDH release in a time-dependent manner, such that more than 90% of LDH activity in platelets is released at 120 min. NEM pretreatment, however, significantly suppressed menadione-induced cytotoxicity, confirming our hypothesis that protein thiols present in plasma play a role in the menadione-induced cytotoxicity to platelets. We considered the possibility that NEM itself could interfere with the LDH assay by measuring known amounts of LDH activity in the presence of a 0.25 mM concentration of NEM used in our experiments. No effect was observed on LDH activity (data not shown).

To clarify this observation, we compared the cytotoxicity of menadione in PRP and WP (Fig. 5). One complication which should be considered was the binding of menadione to plasma protein which could cause substantial decreases in the concentration of free menadione. To compensate for this effect, we utilized previously observed data [20], which indicated that the free menadione concentration in PRP incubation solution containing 1 mM menadione was equivalent to the free menadione present in WP incubation solution containing 0.25 mM menadione. We therefore compared 1 mM menadione-treated PRP with 0.25 mM menadione-treated WP, as well as 0.25 mM menadione-treated PRP with 0.25 mM menadione-treated WP.

Fig. 5A shows a comparison of oxygen consumption rates of each of these systems. In each system, the control DMSO treatment caused little or no effect on oxygen consumption rate, although slight elevations in oxygen consumption were observed due to the presence of the platelets and their basal metabolism. As shown in Fig. 1, menadione in plasma (relatively low in cells) resulted in dose-dependent, increased non-
enzymatic oxygen consumption due to menadione reaction with plasma thiols. In WP (free of plasma), statistically significantly increased oxygen consumption by 0.25 mM menadione was also observed, but 1 mM menadione was not tested because of its solubility in washing buffer. As the menadione-induced increase in oxygen consumption rate in WP (free of plasma) was compared with that in PRP, the latter is larger than the former. This indicated that the presence of plasma has an additive effect on the increased oxygen consumption rates induced by the menadione treatments in our model tissue, platelets.

Based on this result, we postulated that the presence of plasma would also affect menadione-induced cytotoxicity to platelets. We observed the cytotoxicity of menadione in each system by measuring LDH release from platelets (Fig. 5B). Menadione-treated PRP (1 mM and 0.25 mM) showed a dramatically faster and greater LDH release than menadione-treated WP (0.25 mM). The two doses of menadione-PRP together shown in Fig. 5B indicate that the thiols in the PRP system must have increased the toxicity of menadione to some degree. Increased LDH release is observed in PRP compared to that in WP, even though a nominally equivalent menadione dose (0.25 mM) is present. The amount of free menadione in the nominal 0.25 mM menadione-PRP system is very likely less than 0.25 mM, because it has been shown that some menadione is bound to the protein in the PRP system. Despite this, however, the toxic response was increased by using the thiol-containing plasma (PRP) system. A 1 mM menadione-PRP treatment was also run, because experiments have shown that the free amount of menadione in this system is roughly equivalent to that present in the 0.25 mM WP treatment. Even greater and more rapid toxicity is seen with the 1 mM PRP dose. These LDH release results are well correlated with the oxygen consumption rates observed in each system (Fig. 5A) and strongly suggest that menadione-induced cytotoxicity can be increased by the presence of blood plasma.

4. Discussion

This investigation has shown that menadione reacts non-enzymatically with protein thiols in plasma, causing generation of reactive oxygens. This reaction affects the potency of menadione toxicity to platelet cells in plasma. Plasma may serve as a critical component in the expression of menadione toxicity and affect cells (e.g. immune cells, blood cells) in other tissues (e.g. bone marrow) exposed to the plasma, as well as in the vascular system.

We demonstrated that menadione causes increased oxygen consumption (Fig. 1), which is due to the generation of reactive oxygens by reacting with thiols (Fig. 2 shows depletion of thiols). The possibility that soluble thiols (e.g. glutathione) in plasma played an important role in this reaction is very unlikely because filtered plasma (excluding 50000 Da molecular weight protein thiols) did not generate reactive oxygen (Fig. 3). We also showed that the reaction with protein thiols was non-enzymatic by demonstrating that menadione in boiled plasma was still capable of generating reactive oxygens (Fig. 3).
In order to determine whether reactive oxygens non-enzymatically generated by menadione in plasma could affect adjacent cells, a platelet system with plasma (PRP) and without plasma (WP) was used. We examined the effect of plasma by comparing toxicity to platelets using 0.25 mM menadione in both PRP and WP. In addition, we compared toxicity to platelets using 1 mM menadione in PRP with 0.25 mM menadione in WP. This comparison was made because our previous paper [20] demonstrated that the concentration of free menadione in PRP with 1 mM menadione was equivalent to that of WP with 0.25 mM menadione. We demonstrated that greater and more rapid toxicity resulted from 1 mM PRP (Fig. 5B), even though the amount of free menadione was equal to that in the WP system.

Many quinone compounds are both naturally occurring and often used as drugs [2,3]. Like menadione, other quinone compounds would be able to bind with thiols, thereby resulting in the generation of reactive oxygens in physiologically relevant systems, such as our laboratory’s in vitro platelet systems (shown by our data) and, of course, in vivo exposures. Several other representative quinone substances, such as benzoquinones [21] and many naphthoquinones [22], are reported to be able to form reactive oxygens via the same non-enzymatic reaction. It is possible that the presence of plasma would increase the generation of reactive oxygens by these quinones, thereby affecting their toxicity to adjacent tissues. Plasma may also enhance the toxicity of non-quinone compounds by similar or different mechanisms. It was recently reported that phenylhydrazine-induced toxicity to erythrocytes appeared to be increased in the presence of plasma [23]. The authors, however, did not speculate on the possible cause for their observations and so the exact mechanism of phenylhydrazine-induced toxicity and its enhancement by plasma remains to be identified.

Most in vitro studies for toxicological drug evaluation are performed in buffer systems. However, our studies revealed that these in vitro studies using buffer could underestimate in vivo toxicity, if plasma enhances the cytotoxicity of the drug, as it did for the quinone, menadione, in our study. Previous studies showed that therapeutic application of menadione induced hemolysis in erythrocytes [24,25], but in vitro studies required higher concentrations of menadione to induce hemolysis [26]. Our unpublished results showed that the presence of plasma potentiated menadione-induced hemolysis mediated by non-enzymatic reaction with protein thiols. It is therefore recommended that investigators of drug toxicity, especially toxicity of quinones, give consideration to the fact that their drugs may be more toxic in vivo, due to the presence of plasma. They should therefore consider also assessing in vitro cytotoxicity in the presence of plasma. Such experiments are complicated in most cell or tissue systems, but relatively simple in a platelet and plasma system such as we used in this study.

Blood plasma has various antioxidant biomolecules which can protect the vascular endothelial cells, blood cells, and circulating lipids and proteins from both endogenous and exogenous sources of oxidative damage [14]. Previously it was reported that plasma could inhibit cell damage by oxidative stress due to the antioxidant capacity of plasma [27]. This is due to the presence of soluble antioxidants such as ascorbate, α-tocopherol, and pyruvate, as well as ceruloplasmin and transferrin, which bind metal and therefore block the formation of more reactive free radicals [14]. In contrast, we have observed that menadione-induced cytotoxicity to platelets is actually potentiated in the presence of plasma. We believe that this occurs due to the generation of reactive oxygens from the non-enzymatic reaction of menadione with protein thiols in the plasma ultimately exceeding the antioxidant capacity of plasma. This means that plasma itself may be a primary target of menadione cytotoxicity.

This premise is supported by our observations that sudden and rapid decreases in plasma thiols occurred due to menadione (Fig. 2). Plasma thiols have antioxidant capacity [28] and their depletion could be related to such diseases as rheumatoid arthritis [29] and congestive heart failure [30]. In addition to decreasing plasma thiols, the reactive oxygens formed by menadione can lower soluble antioxidants such as ascorbate, so it is likely that menadione decreases the total plasma antioxidant capacity.

In summary, this study has demonstrated that protein thiols in plasma react non-enzymatically with menadione to generate reactive oxygens, which directly lead to potentiated cellular injury to a type of cell (platelets). So far, plasma has been known to decrease the pharmacological effects of many drugs. Since our study suggests that plasma may also enhance the toxicity of a model quinone (menadione), attention should be paid to this possibility for quinones and some other drugs.

Acknowledgements: This work was supported by the Korea Science and Engineering Foundation (KOSEF, 97-04-03-11-01-3).

References
