Association of Quinone-Induced Platelet Anti-Aggregation with Cytotoxicity

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Received January 9, 2001; accepted April 3, 2001

Various anti-platelet drugs, including quinones, are being investigated as potential treatments for cardiovascular disease because of their ability to prevent excessive platelet aggregation. In the present investigation 3 naphthoquinones (2,3-dimethoxy-1,4-naphthoquinone [DMNQ], menadione, and 1,4-naphthoquinone [4-NQ]) were compared for their abilities to inhibit platelet aggregation, deplete glutathione (GSH) and protein thiols, and cause cytotoxicity. Platelet-rich plasma, isolated from Sprague-Dawley rats, was used for all experiments. The relative potency of the 3 quinones to inhibit platelet aggregation, deplete intracellular GSH and protein thiols, and cause cytotoxicity was 1,4-NQ > menadione >> DMNQ. Experiments using 2 thiol-modifying agents, dithiothreitol (DTT) and 1-chloro-2,4-dintrobenzene (CDNB), confirmed the key roles for GSH in quinone-induced platelet anti-aggregation and for protein thiols in quinone-induced cytotoxicity. Furthermore, the anti-aggregative effects of a group of 12 additional quinone derivatives were positively correlated with their ability to cause platelet cytotoxicity. Quinones that had a weak anti-aggregative effect did not induce cytotoxicity (measured as LDH leakage), whereas quinones that had a potent anti-aggregative effect resulted in significant LDH leakage (84–96%). In one instance, however, p-chloranil demonstrated a potent anti-aggregative effect, but did not induce significant LDH leakage. This can be explained by the inability of p-chloranil to deplete protein thiols, even though intracellular GSH levels decreased rapidly. These results suggest that quinones that deplete GSH in platelets demonstrate a marked anti-aggregative effect. If this anti-aggregative effect is subsequently followed by depletion of protein thiols, cytotoxicity results.

Key Words: quinones; platelet anti-aggregation; cytotoxicity; protein thiols; glutathione.

Blood platelets play an important role in hemostasis, thrombosis, and initiation of various cardiovascular diseases. After exposure to the vessel wall by physical and chemical stress, platelets become rapidly activated and platelet aggregation and adhesion occur (Mustard and Packman, 1979; Stormorken, 1984). The aggregation of platelets is normally controlled by endogenous anti-aggregating factors such as nitric oxide (NO) and prostacyclin (PGI2). If these controlling factors are disrupted by certain diseases or chemical exposures, excessive platelet aggregation can occur, leading ultimately to cardiovascular disease.

To date, several potential mechanisms for inhibition of platelet aggregation have been suggested. These include inhibition of adenosine receptors and thromboxane synthase (Martinez et al., 1992; Vittori et al., 1996), increased cGMP (Bassenge and Stewart, 1988), increased NO-releasing compounds (Civelli et al., 1994), and synthesis of prostacyclin analogues (Katsube et al., 1993). Depletion of glutathione (GSH) in platelets has been correlated with inhibition of platelet aggregation by several compounds. These include helaniline (a derivative of sesquiterpene lactone) and 1-chloro-2,4-dinitrobenzene (CDNB), which deplete GSH (Bosia et al., 1985; Schroder et al., 1990), diamide, which oxidizes GSH (Matsuda et al., 1979; Patscheke and Worner, 1978), and N-ethylmaleimide (NEM), which alkylates GSH (Hill et al., 1989).

Quinones are widely distributed in nature and are used clinically as antitumor drugs, components of multivitamin formulations, and anti-allergic agents (Mandel and Cohn, 1996; Monks et al., 1992; Smith, 1985). Recently, quinones have been shown to be platelet anti-aggregative agents by several different mechanisms. Platelet aggregation was reduced by 2-chloro-3-methyl-1,4-naphthoquinone via inhibition of phosphoinositide breakdown (Ko et al., 1990). Alternatively, menadione and vitamin K analogues were suggested to interfere with the mobilization and/or utilization of intracellular Ca2+ (Blackwell et al., 1985). In addition, 2-[(4-cyanophenyl)amino]-3-chloro-1,4-naphthalenedione inhibited thromboxane A2 (TXA2) synthase (Chang et al., 1997), and 2-methoxy-5-methyl-1,4-benzoquinone inhibited TXA2 receptor binding (Lauer and Anke, 1991). Other quinone substances, such as derivatives of benzoquinone (Suzuki et al., 1997) and naphthoquinone (Lien et al., 1996), also prevent platelet aggregation, but the mechanism(s) of action have not been clarified.

It is well known that quinones rapidly deplete intracellular GSH in various tissues including hepatocytes, kidney cells, and platelets (Brown et al., 1991; Seung et al., 1998; Stone et al., 1996). Likewise, several quinones inhibit platelet aggregation...
within a short period of time as described above. However, the relationship between inhibition of platelet aggregation and depletion of GSH by quinones has not been investigated. Our laboratory is interested in examining the effects of quinones on GSH depletion as a mechanism of platelet anti-aggregation.

Following rapid depletion of GSH, quinones can subsequently deplete protein thiols. We previously demonstrated that protein thiol depletion was closely related with cytotoxicity (Cho et al., 1997). Because both the anti-aggregative effect and the depletion of GSH occur within a few minutes following quinone exposure, whereas depletion of protein thiols and overt cytotoxicity occur at later time points, we postulated that anti-aggregation was correlated to depletion of GSH and that this event precedes protein thiol depletion and cytotoxicity. To further investigate this hypothesis, we examined the relationships among platelet anti-aggregation, depletion of GSH, protein thiol depletion, and cytotoxicity using a large number of quinone substances in platelets.

MATERIALS AND METHODS

Drugs and chemicals. The following chemicals and purified enzymes were purchased from Sigma Chemical Co. (St. Louis, MO): menadione, 1-chloro-2,4-dinitro-benzene (CDNB), dithiothreitol (DTT), duroquinone, aloe-emodin, glutathione reduced form (GSH), thrombin, ADP, oxidized glutathione (GSSG), triton X-100, trisodium citrate, pyruvic acid, DMSO, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), N-ethylmaleimide (NEM), NADPH, and NADH. The following chemicals were purchased from Aldrich Chemical Co. (Gillingham, Dorset, U.K.): 1,4-benzoquinone, 1,4-naphthoquinone, and 2,3-dichloro-1,4-naphthoquinone, 2- or 6- (1-Oxopentyl)-5,8-dimethoxy-1,4-naphthoquinone was provided by Dr. Byung-Zun Ahn (Chungnam National University, Korea) and quinoneinolinedione derivatives were provided by Dr. Chung-Kyu Ryu (Ewha Womans University, Korea). All other chemicals were obtained from standard commercial sources.

Animals. Female Sprague-Dawley rats (Laboratory Animal Center of Seoul National University, Korea) weighing 200–250 g were used throughout all experiments. Prior to experiments, animals were acclimated for 1 week in the laboratory animal facility maintained at constant temperature and humidity with ad libitum access to food and water.

Preparations of 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-anesthetized rats using a syringe and the use of glass containers and pipettes was avoided. Blood was collected from the abdominal aorta of ether-anesthetized rats using a syringe and then each agonist was added to activate platelets. The concentrations of agonists were 1.6–1.8 U/ml, 6.4–8.0 μM, or 6.0–8.0 μg/ml for thrombin, collagen, or ADP, respectively. The values of IC₅₀ and IC₁₀₀ were determined based upon the concentration-response curve using a computer program (PHARM/PCS). Changes in light transmission were detected by a Lumi-aggregometer. Data represent means ± SEM from 3 experiments.

Platelet-rich plasma was preincubated with 2,3-dimethoxy-1,4-naphthoquinone (DMNQ; 100–500 μM), menadione (5–300 μM), or 1,4-naphthoquinone (1,4-NQ; 1–100 μM) for 3 min at 37°C and then each agonist was added to activate platelets. The concentrations of agonists were 1.6–1.8 U/ml, 6.4–8.0 μM, or 6.0–8.0 μg/ml for thrombin, collagen, or ADP, respectively. The values of IC₅₀ and IC₁₀₀ were determined based upon the concentration-response curve using a computer program (PHARM/PCS). Changes in light transmission were detected by a Lumi-aggregometer. Data represent means ± SEM from 3 experiments.

Note. Platelet-rich plasma was preincubated with 2,3-dimethoxy-1,4-naphthoquinone (DMNQ; 100–500 μM), menadione (5–300 μM), or 1,4-naphthoquinone (1,4-NQ; 1–100 μM) for 3 min at 37°C and then each agonist was added to activate platelets. The concentrations of agonists were 1.6–1.8 U/ml, 6.4–8.0 μM, or 6.0–8.0 μg/ml for thrombin, collagen, or ADP, respectively. The values of IC₅₀ and IC₁₀₀ were determined based upon the concentration-response curve using a computer program (PHARM/PCS). Changes in light transmission were detected by a Lumi-aggregometer. Data represent means ± SEM from 3 experiments.

Measurement of platelet aggregation. Platelet aggregation was measured by light transmission, with 100% calibrated as the absorbance of PPP and 0% calibrated as the absorbance of PRP. PRP suspension in a silicon-coated cuvette was stirred at 1200 rpm for 1 min before addition of quinones. Dimethylsulfoxide (DMSO) was used as the vehicle for quinones, such that the final concentration of DMSO in the incubation medium was 0.5%. This concentration was shown to have no effect on either platelet aggregation induced by agonists or platelet lysis. Changes in light transmission were detected by a Lumi-aggregometer (Chrono-log Corp., Havertown, PA).

Intracellular glutathione levels. Sample preparation was accomplished by incubating PRP with 100 μM quinones. One ml- aliquots of quinone-treated PRP (1 × 10⁷ platelets/ml) were centrifuged at 10,000 × g for 20 s at room temperature to obtain a platelet pellet. The pellets were resuspended with 0.6 ml of 0.125 M perchloric acid containing 0.4 mM EDTA and centrifuged at 10,000 × g for 2 min to obtain the acid soluble fraction (supernatant). The supernatant was mixed with 2 M KOH containing 0.3 M MOPS to remove excess perchloric acid and to adjust the pH to 7.0. A 0.2 ml aliquot of this preparation was then assayed for glutathione. Total glutathione levels were determined by the enzymatic recycling method described by Griffith (1980), with the following modification—a higher activity of glutathione reductase (1.5 kU/ml in assay buffer) was used in order to optimize conditions for assaying platelets.

Lactate dehydrogenase leakage. Leakage of lactate dehydrogenase (LDH) from platelets was measured as described by Bergmeyer et al. (1965). LDH activity was measured in both the incubation medium and platelets (lysed with 0.3% Triton X-100). LDH leakage was expressed as % of total enzyme activity.

Protein thiol levels. Protein thiol concentrations were measured using a modification of the colorimetric method of Di Monte et al. (1984). One ml of quinone-treated PRP was spun at 10,000 × g for 20 s, and the supernatant was discarded. The pellet was washed once with 5% perchloric acid and then resuspended in 2.5 ml of Tris-EDTA buffer (0.5 M Tris, 5 mM EDTA, pH 7.6). DTNB (250 μM final concentration) was added and, after 20 min, the absorbance was measured at 412 nm. Protein thiol levels were calculated on the basis of a glutathione calibration curve.

Statistical analysis. The means and standard errors of means were calculated for all treatment groups. The data were subjected to one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test to determine which means were significantly different from each other or control. In all cases, a p-value of < 0.05 was used to determine significance.

RESULTS

The capacity for 3 naphthoquinone compounds, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), menadione, and 1,4-naphthoquinone (1,4-NQ), to prevent platelet aggregation was investigated. PRP was pretreated with 1 of 3 quinones for 3 min and then thrombin, ADP, or collagen was added to activate platelets. Table 1 summarizes the relative inhibitory capacity of the 3 quinones. Quinone concentrations that resulted in 50%
inhibition of platelet aggregation (IC_{50}) or 100% inhibition of aggregation (IC_{100}) were determined. Concentrations of DMNQ as great as 500 \mu M failed to inhibit aggregation induced by all 3 agonists. On the other hand, menadione and 1,4-NQ consistently inhibited platelet aggregation, with IC_{100}s being 2 times greater than the IC_{50}s, irrespective of the specific agonist. The relative potency of quinones to inhibit platelet aggregation was 1,4-NQ > menadione > DMNQ.

To determine if inhibition of platelet aggregation by quinones was related to the depletion of glutathione (GSH), total GSH levels in PRP were determined following exposure to 100 \mu M quinone for 3 or 10 min. Total GSH levels in platelets were determined as described in Methods section. Values represent mean ± SEM from 3 experiments. *Represent significant differences from control (p < 0.05).

![FIG. 1. Effect of quinones on total GSH levels in platelets. Platelet rich plasma (PRP) suspensions were incubated in the presence of 100 \mu M quinone for 3 or 10 min. Total GSH levels in platelets were determined as described in Methods section. Values represent mean ± SEM from 3 experiments. *Represent significant differences from control (p < 0.05).](image1)

Since previous studies demonstrated that quinones can decrease protein thiol levels following rapid depletion of GSH in platelets (Cho et al., 1997), total protein thiol levels were measured following addition of 250 \mu M DMNQ, menadione, or 1,4-NQ (Fig. 3). No effect on protein thiols was observed with DMNQ treatment. However, menadione and 1,4-NQ depleted protein thiols in a time-dependent manner, with 1,4-NQ pretreatment more rapidly depleting protein thiols than menadione. The order of the 3 quinones at 250 \mu M to deplete protein thiols was 1,4-NQ > menadione >> DMNQ.

To investigate the role of protein thiols in the cytotoxic effects of these quinone compounds on platelets, the ability of DMNQ, menadione, or 1,4-NQ to induce platelet cytotoxicity was assessed at various time points by measuring LDH leakage (Fig. 4). At equimolar concentrations of 250 \mu M, DMNQ did not induce LDH leakage, while menadione and 1,4-NQ induced LDH leakage in a time-dependent manner. 1,4-NQ appeared more effective than menadione, since LDH leakage was 70% of total LDH activity by 30 min and 100% of total by 60 min. The order of the 3 quinones at 250 \mu M to cause cytotoxicity was also 1,4-NQ > menadione >> DMNQ (Fig. 4), a similar observation to that describing effects on platelet aggregation, glutathione depletion, and protein thiols depletion.

In addition, the ability of DTT and CDNB to modulate quinone-induced cytotoxicity was investigated (Fig. 5). PRP was treated with menadione or 1,4-NQ following exposure to CDNB and DTT. Neither CDNB nor DTT alone resulted in LDH leakage (data not shown). Similar to the results shown above with GSH (Fig. 2), pretreatment with 1 mM DTT almost completely inhibited menadione- and 1,4-NQ-induced cytotox-
icity, while pretreatment with 0.2 mM CDNB greatly potentiated the effect of the 2 quinones (Fig. 5).

To determine if the apparent association between anti-aggregative effects and cytotoxicity observed with DMNQ, menadione, or 1,4-NQ was applicable to a broad class of quinones, the same 2 parameters (anti-aggregative effect and cytotoxicity) were measured for a number of additional quinone derivatives (Table 2). Quinones demonstrating a weak anti-aggregative effect (i.e., IC$_{50} > 250$ μM), such as duroquinone, 2-(1-oxopentyl)-5,8-dimethoxy-1,4-naphthoquinone (2-oxoDMNQ), 6-(2,3,4-trifluorobenzyl)-anilino-5,8-quinolinedione (FAQ 1), 6-(3-fluorobenzyl)-anilino-5,8-quinolinedione (FAQ 2), or anthraquinones, did not induce LDH leakage. Conversely, quinones demonstrating a potent anti-aggregative effect (i.e., IC$_{50} < 5$ μM; 7.0 μM), such as 1,4-benzoquinone, 2,3-dichloro-1,4-naphthoquinone (DCNQ), 6-(1-oxopentyl)-5,8-dimethoxy-1,4-naphthoquinone (6-oxoDMNQ), 6-(2,4-difluorobenzyl)-anilino-5,8-quinolinedionylchloride (FAQ 4), or menadione, induced significant LDH leakage (84–96%). In addition, the most potent anti-aggregative quinone, 2,3-dichloro-1,4-naphthoquinone (i.e., IC$_{50} = 7.0$ μM), also most rapidly induced LDH leakage (69.3 ± 2.5% of the total LDH level within 30 min, data not shown). These results indicate that, in general, the more potent the anti-aggregative effect of a quinone substance, the more cytotoxic that quinone substance will be.

An exception to this general rule, however, was p-chloranil. Among the 15 quinones tested, only p-chloranil demonstrated a potent anti-aggregative effect (IC$_{50} = 7.5$ μM) with relatively modest LDH leakage (20% of total activity). Since we observed that protein thiols depletion was associated with quinone cytotoxicity (Figs. 3 and 4), we postulated that the relatively low cytotoxicity of p-chloranil was associated with a minimal capacity to deplete protein thiols. The effects of p-chloranil on total soluble glutathione levels (Fig. 6A) and protein thiol levels (Fig. 6B) in platelets were studied. GSH
was rapidly depleted by 100 μM p-chloranil, while concentrations as high as 250 μM p-chloranil failed to induce significant depletion of protein thiols with even much longer durations of exposure.

DISCUSSION

Our current research in platelets can be summarized with the following observations: (1) quinones that deplete GSH in platelets demonstrate a marked anti-aggregative effect; the more potent the ability to deplete GSH, the more potent the anti-aggregative effect; (2) quinones that subsequently deplete protein thiols eventually lead to cytotoxicity. These observations lead us to conclude that if GSH depletion by quinones is subsequently followed by depletion of protein thiols, the anti-aggregative effect of quinones may represent an early event of cytotoxicity.

It has been previously reported that GSH plays an important role in platelet aggregation, and that GSH-depleting chemicals inhibit platelet aggregation significantly (Bosia et al., 1985; Heptinstall et al., 1987; Schroder et al., 1990). However, the association between the depletion of GSH and inhibition of aggregation in platelets has not been reported for quinones. Our data in Table 1 and Figure 1 demonstrate that the potency of GSH depletion for 3 quinone model compounds (DMNQ, menadione, and 1,4-NQ) correlates well with their potency to inhibit aggregation. In addition, among all the quinones tested, the compounds that inhibit platelet aggregation with IC_{50} < 250 μM (Table 2) also significantly deplete cellular GSH levels within 10 min (data not shown), suggesting that the anti-aggregative effect of quinones is related to GSH depletion.

To date, several mechanisms have been proposed for inhibition of platelet aggregation by GSH depletion, but the exact

FIG. 6. Depletion of GSH and protein thiols by p-chloranil. PRP suspensions were incubated in the presence of 100 μM p-chloranil (for total GSH) or 250 μM p-chloranil (for protein thiols). Total GSH and protein thiol levels were determined at the indicated time point, respectively. (A) Total GSH, (circle) Control; (triangle) 100 μM p-chloranil. (B) Protein thiols, (circle) Control; (triangle) 250 μM p-chloranil. Values represent mean ± SEM from 3 experiments. *Represent significant differences from corresponding control (p < 0.05).

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Correlation between Anti-Aggregative Effect (IC_{50}) and Cytotoxicity (LDH) by Various Quinones</th>
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<td></td>
<td>Compounds</td>
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<td>Control (DMSO)</td>
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<tr>
<td>Benzoquinones</td>
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<td>p-Chloranil</td>
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<td>1,4-Benzoxquinone</td>
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<td>Naphthoquinones</td>
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<td></td>
<td>Menadione</td>
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<td></td>
<td>2,3-Dichloro-1,4-naphthoquinone (DCNQ)</td>
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<td>(1-Oxoalkyl)-5,8-dimethoxy-1,4-naphthoquinones</td>
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<td></td>
<td>1,8-Dihydroxy-3-(hydroxymethyl)-anthraquinone (Aloe-emodine)</td>
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</table>

Note. The concentration resulting in 50% inhibition of platelet aggregation (IC_{50}) for each of 15 quinone substances was calculated to compare their relative potency. LDH leakage was measured following 120 min incubation to each quinone at 250 μM to compare relative cytotoxicity. Values represent mean ± SEM from 3 experiments.
mechanism remains unclear. Previous research (Schroder et al., 1990) has shown that GSH depletion by some substances other than quinones was associated with inhibition of platelet aggregation and suggested that this possibly caused cytoskeletal protein alterations. Our research has shown that GSH depletion by quinones also caused inhibition of platelet aggregation. It is possible that cytoskeletal alterations may be the key mechanism for our observations. This premise is supported by other studies (Bellomo et al., 1990; Thor et al., 1988) in isolated hepatocytes that showed that quinones induce cytoskeletal damage mediated by depletion of glutathione and protein thiols, leading to plasma membrane blebbing and ultimately cell death.

The relative potency (IC_{50}s and IC_{100}s) for the 3 model naphthaquinones to inhibit platelet aggregation was 1,4-NQ > menadione >> DMNQ, regardless of which of 3 agonists (collagen, ADP, thrombin) was used (Table 1). Both menadione and 1,4-NQ were most effective at inhibiting platelet aggregation induced by collagen, as compared to aggregation induced by the other 2 agonists. There is currently no known explanation for why collagen’s action is most easily affected by quinones, but this observation has been reported in several studies using quinones (Chang et al., 1997; Lien et al., 1996). The effects of DTT and CDNB on the inhibition of platelet aggregation by quinones were obtained when platelet aggregation was induced by thrombin (Fig. 2). Similar effects, however, were observed using ADP and collagen agonists (data not shown), suggesting that GSH-depletion by quinones in platelets plays a key role in modulating platelet aggregation induced by any of the 3 agonists.

In general, quinones rapidly deplete GSH and subsequently deplete protein thiols in various tissues such as liver, kidney, and heart (Brown et al., 1991; Di Monte et al., 1984; Tzeng et al., 1994). In platelets, menadione also rapidly depletes GSH within 10 min and subsequently depletes protein thiols for up to 2 h, finally leading to cytotoxicity (Cho et al., 1997). In an attempt to confirm a role for GSH and protein thiols in platelet aggregation and cytotoxicity, we pre-exposed cells to CDNB and DTT, 2 thiol modifying agents, to determine their ability to modulate thrombin-induced platelet aggregation and quinone-induced cytotoxicity. DTT was used as a donar of intracellular thiols based upon the report that DTT donates sulfhydryl groups to GSH and protein thiols (Sandy et al., 1988). CDNB was used to deplete intracellular thiols. CDNB alone did not affect thrombin-induced platelet aggregation. However, pretreatment with CDNB potentiated the inhibitory effects of 1,4-NQ and menadione on platelet aggregation (Fig. 2). The major target of CDNB in platelets appears to be GSH (Bosia et al., 1985), but in our experimental system, CDNB may also induce alteration of protein thiol levels, since it subsequently depletes protein thiols by 20% throughout 2-h incubation (data not shown). We have demonstrated that pretreatment with CDNB potentiates quinone-induced cytotoxicity in platelets, whereas DTT completely protects (Fig 5), suggesting that protein thiols also play a critical role in platelet cytotoxicity as well.

*p-Chloranil, an exceptional quinone among 15 quinones tested, has a potent anti-aggregative effect but it induces a weak cytotoxicity. *p-Chloranil was effective at depleting intracellular GSH, but did not significantly deplete protein thiols (Fig. 6). This modification of intracellular GSH but not protein thiols by *p-chloranil resulted in significant anti-aggregative effect in the absence of significant cytotoxicity. These results suggest that some quinones, such as *p-chloranil, which selectively deplete GSH without affecting protein thiol levels, may be potentially effective anti-platelet drugs. However, caution is necessary in interpreting the potential use of quinone compounds as anti-platelet drugs because quinones also deplete intracellular GSH in tissues other than platelets and this depletion has itself been shown to be toxic to cells in these tissues. For example, *p-hydroxybenzoate ester-induced cytotoxicity in hepatocytes (Nakagawa and Moldeus, 1998), 2-amino-5-chlorophenol-induced toxicity in renal cortical slices (Valentovic et al., 1999), and glutamate-induced cytotoxicity in brain cells (Pereira and Oliveira, 1997) are primarily dependent upon the depletion of intracellular GSH.

In summary, the depletion of GSH by quinones results in the inhibition of platelet aggregation, and the anti-aggregative effect of the quinones may be an early event in cytotoxicity, as represented by protein thiol depletion. As a consequence, although much research has focused on the possibility that quinone substances could be developed as anti-platelet drug therapy, our results suggest that caution must be applied, since it appears that quinone inhibition of platelet aggregation precedes cytotoxicity.

ACKNOWLEDGMENTS

This work was supported by National Research Laboratory (NRL) Program of the Korean Ministry of Science and Technology and by 2000 BK21 project for Medicine, Dentistry and Pharmacy. We thank Dr. David Thompson for all his help to review this manuscript.

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