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Lipid Peroxide Overcomes the Inability of Platelet Secretory Phospholipase A_2 to Hydrolyze Membrane Phospholipids in Rabbit Platelets

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The present study investigated the effect of lipid peroxide on the ability of group IIA secretory phospholipase A_2 (IIAsPLA$_2$) to hydrolyze platelet membrane phospholipids. The treatment of rabbit platelets with *tert*-butyl hydroperoxide (BHP) and $FeSO_4$ generated malondialdehyde, an index of lipid peroxidation, and slightly induced arachidonic acid liberation and lysophosphatidylcholine formation. Further addition of IIAsPLA$_2$ purified from rabbit platelets synergistically enhanced the liberation and the formation induced by the oxidizing reagents, although the enzyme alone did not. When the IIAsPLA$_2$ was pretreated with heparin, the enhancement was not observed. The combination of IIAsPLA$_2$ with linoleic acid hydroperoxide and $FeSO_4$ also caused synergistic arachidonic acid liberation. Furthermore, IIAsPLA$_2$ enhanced thromboxane B_2 generation and platelet aggregation induced by BHP and $FeSO_4$. The synergistic aggregation was sensitive to indomethacin. With a membrane fraction as a substrate, IIAsPLA$_2$ caused arachidonic acid liberation, which was enhanced in the presence of BHP and $FeSO_4$. These results suggest that modification of membrane phospholipids by oxidizing reagents increases the accessibility of the membrane to platelet IIAsPLA$_2$, and sequential enhancement of arachidonic acid liberation may contribute to the propagation of oxidative stress-induced cellular injury.

Key words: arachidonic acid, lipid peroxidation, platelet, secretory phospholipase A_2.

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of membrane phospholipids to generate arachidonic acid and lysophospholipids, which are precursors for eicosanoids and platelet-activating factor, and thus contributes to inflammatory processes (*1, 2*). Most Ca^{2+}-dependent PLA_2s characterized and purified so far have been classified into several groups based on their primary structures (*3*). The group IV cytosolic PLA_2 is involved in stimulus-induced arachidonic acid liberation, because the cytosolic PLA_2 preferentially hydrolyzes phospholipids bearing an arachidonoyl residue at the *sn*-2 position in the presence of the physiological Ca^{2+} concentration attained within stimulated cells (*2*). The group IIA secretory PLA_2 (IIAsPLA$_2$) has been detected in synovial fluid (*4, 5*) and inflammatory cells (*6, 7*) and has been suggested to be involved in eicosanoid generation in a variety of cells (*8-11*).

Previous studies demonstrated that exogenous IIAsPLA$_2$ enhances stimulus-induced eicosanoid generation (*9-11*), although the enzyme alone has no effect. This finding suggests that priming of cells upon stimulation increases the accessibility of membrane phospholipids to IIAsPLA$_2$, probably through modification of membrane properties. Platelets have been shown to possess IIAsPLA$_2$ (*12, 13*) in addition to cytosolic PLA_2 (*14*). However, the addition of IIAsPLA$_2$ to rabbit platelets elicited no response even when the cells were preactivated (*15*). Furthermore, the enzyme enhances the generation of prostaglandin I_2, which has an inhibitory effect on platelet activation, in primed endothelial cells (*11*). This suggests that the enzyme released from activated platelets might exert negative feedback regulation on platelet activation. Therefore, the involvement of IIAsPLA$_2$ in inflammatory processes *via* platelet activation is not clear.

Oxidative stress plays an important role in the propagation of inflammation through, at least in part, induction of peroxidation of membrane lipids. It has been suggested that lipid peroxidation stimulates PLA_2 activity in pulmonary arterial endothelial cells (*16*). Previously, we also reported that the treatment of rabbit platelets with an oxidant, *tert*-butyl hydroperoxide (BHP), and $FeSO_4$ resulted in enhancement of arachidonic acid liberation in response to agonists (*17*). However, the type of PLA_2 contributing to the enhanced liberation is unclear. Recent studies have suggested that hydrogen peroxide-stimulated arachidonic acid liberation may be mediated by cytosolic PLA_2 activation in smooth muscle cells (*18*) and kidney epithelial cells (*19*). With regard to IIAsPLA$_2$ derived from snake venom, enhancement of the hydrolytic action of the enzyme by lipid peroxidation has also been reported in liposomes or kidney epithelial cells treated with BHP or hydrogen peroxide (*20, 21*). However, since the venom IIAsPLA$_2$ alone can hydrolyze membrane phospholipids in intact rabbit platelets (*15*), venom and mammalian IIAsPLA$_2$s may differ in their detailed characteristics. In

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Abbreviations: PLA_2, phospholipase A_2; IIAsPLA$_2$, group IIA secretory PLA_2; BHP, *tert*-butyl hydroperoxide.

this study, to investigate the possible involvement of platelet IIAsPLA$_2$ in the propagation of oxidative stress-mediated inflammation, we examined whether treatment of rabbit platelets with oxidizing reagents affects the hydrolytic action of the enzyme purified from the cells.

MATERIALS AND METHODS

Materials—Linoleic acid hydroperoxide, BHP, and indomethacin were obtained from Sigma (St. Louis, MO, USA). Heparin was from Novo Nordisk (Denmark). Sulfate-Cellulofine-m was from Seikagaku Kogyo (Tokyo). Butyl-Toyopearl 650M was from Tosoh (Tokyo). [^{3}H]Arachidonic acid (100 Ci/mmol), [^{3}H]glycerol (17.4 Ci/mmol), and 1-palmitoyl-2-[^{14}C]linoleoyl-glycerophosphoethanolamine (55 mCi/mmol) were from New England Nuclear (Boston, MA, USA). Other reagents were obtained from Wako Pure Chemical Industries (Osaka) or Nacalai Tesque (Kyoto).

Preparation of Washed Rabbit Platelets—Platelet-rich plasma was obtained from rabbit blood anticoagulated with a one-tenth volume of 1% EDTA by centrifugation at 230$\times$ *g* for 10 min. Platelets were sedimented by centrifugation of the platelet-rich plasma at 800$\times g$ for 15 min, then washed twice with HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, and 3.8 mM HEPES, pH 6.5) containing 0.35% bovine serum albumin and 0.4 mM EGTA. The washed platelets were resuspended in HEPES buffer (pH 7.4).

Purification of IIAsPLA$_2$ from Rabbit Platelets—Washed platelets (1×10^9 cells/ml) were stimulated with 10 nM platelet-activating factor in the presence of 1 mM $CaCl_2$ at 37°C for 5 min. The mixture was centrifuged at 1,000$\times g$ for 10 min at 4°C in the presence of 3 mM EGTA, and the supernatant was further centrifuged at 10,000$\times g$ for 10 min. The supernatant obtained, as an enzyme source, was loaded onto a Sulfate-Cellulofine-m column (1 cm$\times$6 cm) that had been equilibrated with 10 mM Tris-HCl (pH 7.4) containing 100 mM NaCl and 1 mM EDTA. The column was washed with 500 mM NaCl, then PLA_2 was eluted with 1 M NaCl (10 ml/h). The pooled fractions containing PLA_2 were applied to a column of Butyl-Toyopearl 650M (1 cm$\times$ 4 cm) preequilibrated with 10 mM Tris-HCl (pH 7.4) containing 1 M NaCl and 1 mM EDTA, then the column was washed with 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl. PLA_2 was eluted with glycine-HCl (pH 2.7) containing 150 mM NaCl (10 ml/h). The fractions containing PLA_2 were pooled and neutralized with 1 M Tris. The activity of PLA_2 in each fraction was determined by incubation of an aliquot with a mixture of 1-palmitoyl-2-[^{14}C]linoleoyl-glycerophosphoethanolamine and unlabeled 1-palmitoyl-2-linoleoyl-glycerophosphoethanolamine (2.6 mCi/mmol, 20 μM) as substrates. The incubation was carried out at 37°C for 5 min in the presence of 5 mM $CaCl_2$ and 100 mM Tris-HCl (pH 8.5), and [^{14}C]linoleic acid liberated was determined according to the method of Sundaram *et al.* (*22*). The protein concentration was determined with a commercial assay kit (Pierce, Rockford, IL, USA). The purity of the final enzyme preparation was determined by SDS-PAGE (15% gels), followed by staining.

Lipid Metabolism—Platelet-rich plasma was incubated with [^{3}H]arachidonic acid (1 μCi/ml) or [^{3}H]glycerol (80 μCi/ml) at 37°C for 1.5 h, then washed as above. The labeled platelets (5×10^8 cells/ml) were treated with or without 100 μM BW755C (3-amino-1-[*m*-(trifluoromethyl)phenyl]-2-pyrazoline, a cyclooxygenase and lipoxygenase inhibitor) at 37°C for 2 min in the presence of 1 mM $CaCl_2$. When thromboxane B_2 generation was analyzed, BW755C was omitted. The platelets were preincubated with BHP and $FeSO_4$ for 1 min, then further incubated with the purified IIAsPLA$_2$. Lipids were extracted and separated by TLC on silica gel G plates (Merck, German) with the following development systems: for the analysis of arachidonic acid, petroleum ether/diethyl ether/acetic acid (40 : 40 : 1, v/v/v); for the analysis of lysophosphatidylcholine, the combinations of chloroform/methanol/7 M NH_4OH (65 : 35 : 7.3, v/v/v) for the first dimension and chloroform/methanol/acetic acid/water (60 : 30 : 8: 4, v/v/v) for the second dimension; and for the analysis of thromboxane B_2, an upper phase of ethyl acetate/isooctane/acetic acid/H_2O (9 : 5 : 2 : 10, v/v/v). The area corresponding to each lipid was scraped off and the radioactivity was determined by liquid scintillation counting.

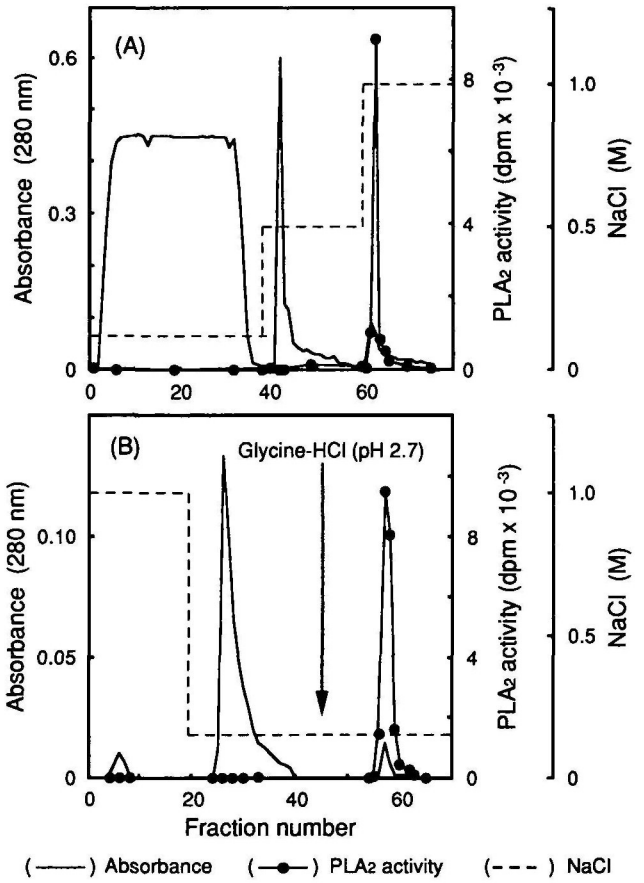
Platelet Aggregation—Platelet aggregation was determined as the increase in light transmission with an aggregometer (Hema tracer I, Niko Bioscience) as described previously (*17*).

Preparation of a Membrane Fraction—[^{3}H]Arachidonic acid-labeled platelets (1×10^9 cells/ml) suspended in 10 mM Tris-HCl (pH 7.4) containing 2 mM EGTA were sonicated, then centrifuged at 1,500$\times g$ for 10 min at 4°C. After centrifugation of the supernatant at 15,000$\times g$ for 30 min at 4°C, the pellet obtained was suspended in 10 mM Tris-HCl (pH 7.4) containing 1 M NaCl and 2 mM EGTA, then centrifuged. The pellet (membranes) was washed and resuspended in a buffer consisting of 100 mM NaCl and 10 mM Tris-HCl (pH 7.4). The labeled membranes (150 μg protein/ml) were treated with 50 μM BW755C at 37°C for 2 min, then incubated with BHP and $FeSO_4$ for 1 min in the presence of 1 mM $CaCl_2$. After addition of the purified IIAsPLA$_2$, the reaction mixture was incubated further for 2 min. [^{3}H]Arachidonic acid liberated was determined as described above.

RESULTS

Figure 1 shows the chromatographic profiles for the purification of IIAsPLA$_2$ from rabbit platelets. When the supernatant of platelet-activating factor-stimulated platelets was used as an enzyme source, sequential column chromatographies on Sulfate-Cellulofine and Butyl-Toyopearl were successful for purifying the platelet IIAsPLA$_2$. As shown in Fig. 1C, the final enzyme preparation gave a single band corresponding to a molecular mass of approximately 14 kDa. The resulting specific activity and purification were about 2,500 nmol/min/mg protein and 1,200-fold, respectively. We confirmed that maximal activity of the purified enzyme was observed in the presence of a millimolar level (1-5 mM) of calcium ions, and the enzyme activity was completely inhibited by dithiothreitol or 2-mercaptoethanol (data not shown). These enzyme properties were consistent with those of IIAsPLA$_2$ reported previously (*13*).

Using the purified IIAsPLA$_2$, we examined whether BHP and $FeSO_4$ affect the hydrolytic action of the enzyme toward membrane phospholipids. As shown in Fig. 2A, treatment of [^{3}H]arachidonic acid-labeled rabbit platelets



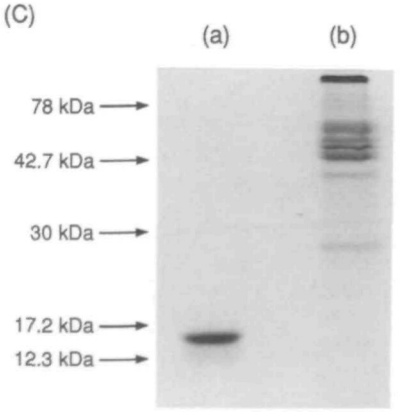


Fig. 1. **Purification of $IIAsPLA_2$ from rabbit platelets.** (A) Elution profile of $IIAsPLA_2$ on a Sulfate-Cellulofine-m column. (B) Elution profile of $IIAsPLA_2$ on a Butyl-Toyopearl 650M column. (C) $IIAsPLA_2$ obtained from the Butyl-Toyopearl 650M column (a) and the enzyme source (b) were analyzed by SDS-PAGE.

with BHP (50 μM) and $FeSO_4$ (50 μM) slightly increased arachidonic acid liberation in a time-dependent manner. Under the conditions, malondialdehyde generation, as thiobarbituric acid reactive substances, increased after 10 min from 13.8 ± 1.3 to 54.0 ± 7.4 nM ($n=3$), which was measured as an index of lipid peroxidation. When the purified $IIAsPLA_2$ (4 μg/ml) was added to platelets pretreated with BHP and $FeSO_4$, the enzyme further increased arachidonic acid liberation. The increase in arachidonic acid liberation caused by the $IIAsPLA_2$ was potentiated with increases in the concentration of the enzyme added, although the enzyme alone did not cause any liberation (Fig. 2B). Incubation with BHP and $FeSO_4$ increased arachidonic acid liberation in a BHP dose-dependent manner. However, concentrations of above 50 μM of BHP abolished the activity of exogenously added $IIAsPLA_2$ (data not shown). Figure 2C shows the effect of BHP and $FeSO_4$ on lysophosphatidylcholine formation in [^{3}H]glycerol-labeled platelets. The $IIAsPLA_2$ (4 μg/ml) synergistically increased lysophosphatidylcholine formation induced by BHP (50 μM) and $FeSO_4$ (50 μM). These results suggest that exogenous $IIAsPLA_2$ can act on phospholipids in platelet membranes exposed to oxidative stress.

Since $IIAsPLA_2$ has been suggested to hydrolyze membrane phospholipids through binding to heparan sulfate on plasma membranes in primed cells (*11*), we examined the effect of heparin on $IIAsPLA_2$-enhanced arachidonic acid liberation in platelets pretreated with BHP and $FeSO_4$ (Fig. 3). The addition of purified $IIAsPLA_2$ (4 μg/ml) further increased arachidonic acid liberation induced by BHP (50 μM) and $FeSO_4$ (50 μM), whereas no enhancement was observed by the enzyme preincubated with heparin (0.1 mg/ml). The addition of heparin alone had no effect on arachidonic acid liberation induced by BHP and $FeSO_4$. These results revealed that the enhancement of arachidonic acid liberation is actually mediated by $IIAsPLA_2$ itself.

To further examine whether the presence of lipid peroxide in membranes affects the hydrolytic action of $IIAsPLA_2$, the effect of linoleic acid hydroperoxide was examined. As shown in Fig. 4, when platelets were pretreated with linoleic acid hydroperoxide (10–50 μM) and $FeSO_4$ (50 μM), the addition of $IIAsPLA_2$ (4 μg/ml) synergistically induced arachidonic acid liberation in a hydroperoxide dose-dependent manner. At 50 μM linoleic acid hydroperoxide, no further increase was observed. This might be due to attenuation of the enzyme activity by the hydroperoxide and $FeSO_4$.

In platelets, arachidonic acid liberated is converted to thromboxane A_2, which is a potent platelet activator. We determined thromboxane B_2 generation in platelets treated with the combination of $IIAsPLA_2$ with BHP and $FeSO_4$ (Fig. 5A). The results showed that $IIAsPLA_2$ (4 μg/ml) potentiated thromboxane B_2 generation in platelets pretreated with BHP (50 μM) and $FeSO_4$ (50 μM). Figure 5B illustrates platelet aggregation in response to $IIAsPLA_2$ and the oxidizing reagents. Although the incubation of platelets with BHP (20 μM) and $FeSO_4$ (50 μM) did not cause significant aggregation, the addition of $IIAsPLA_2$ (4 μg/ml) markedly induced aggregation. The aggregation enhanced by the enzyme was completely inhibited by pretreatment with indomethacin (10 μM), suggesting that the enhancement of aggregation may be due to an increase in thromboxane A_2 generation by $IIAsPLA_2$.

Using a membrane fraction as a substrate, we further examined the effect of BHP and $FeSO_4$ on the hydrolytic action of $IIAsPLA_2$ (Fig. 6). When $IIAsPLA_2$ was added to a membrane fraction prepared from [^{3}H]arachidonic acid-labeled platelets, the enzyme increased arachidonic acid liberation in a dose-dependent manner, indicating that the enzyme could hydrolyze phospholipids in the membrane fraction. Although pretreatment of the membranes with

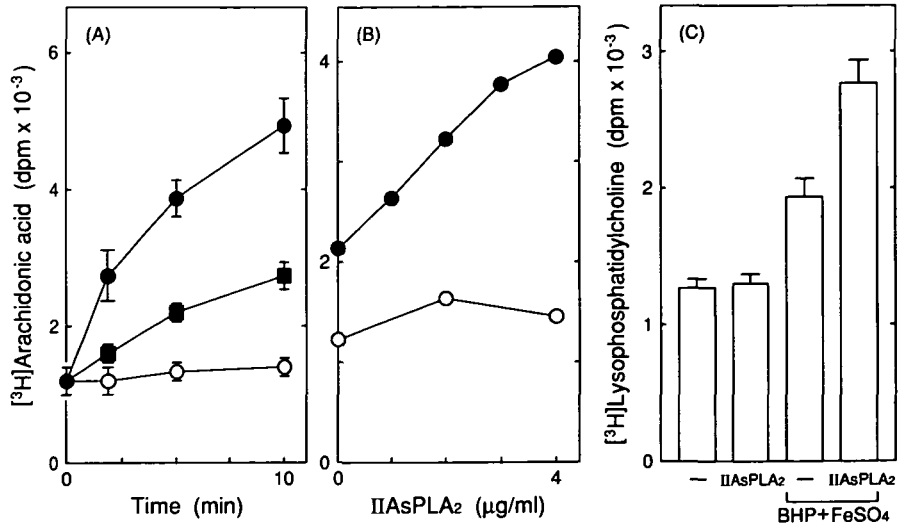


Fig. 2. **Enhancement of arachidonic acid liberation (A, B) and lysophosphatidylcholine formation (C) by IIAsPLA$_2$ in platelets pretreated with BHP and FeSO$_4$.** (A) [^{3}H]Arachidonic acid-labeled platelets were treated with (closed symbols) or without (open symbols) 50 μM BHP and 50 μM FeSO$_4$ for 1 min, then incubated with (●) or without (■,○) 4 μg/ml IIAsPLA$_2$ for the indicated times. Each point represents the mean±SE of three experiments performed in duplicate. (B) [^{3}H]Arachidonic acid-labeled platelets were treated with (closed symbols) or without (open symbols) BHP and FeSO$_4$ as in (A), then incubated with various concentrations of IIAsPLA$_2$ for 5 min. Each point represents the mean of two experiments performed in duplicate. (C) [^{3}H]Glycerol-labeled platelets were treated with or without BHP and FeSO$_4$ as in (A), then incubated with or without 4 μg/ml IIAsPLA$_2$ for 10 min. Each value represents the mean±SE of three experiments performed in duplicate.

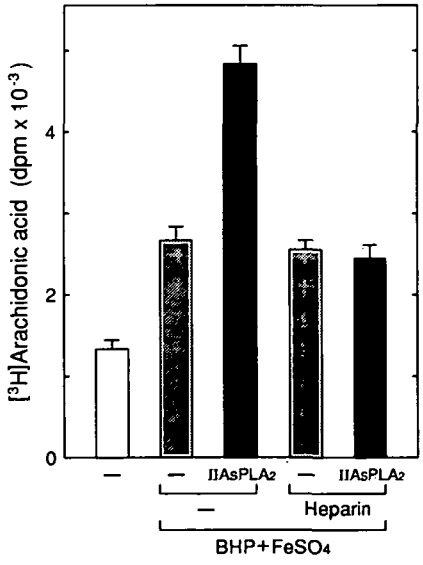


Fig. 3. **Effect of heparin on IIAsPLA$_2$-enhanced arachidonic acid liberation.** [^{3}H]Arachidonic acid-labeled platelets were treated with or without 50 μM BHP and 50 μM FeSO$_4$ as in Fig. 2A, then incubated for 10 min with or without 4 μg/ml IIAsPLA$_2$, which had been preincubated with or without 0.1 mg/ml heparin at 4°C for 10 min. Each value represents the mean±SE of three experiments.

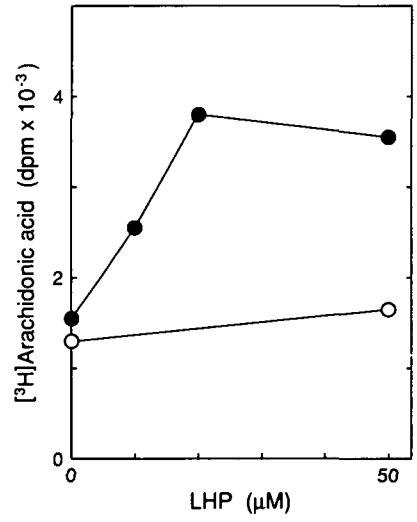


Fig. 4. **Enhancement of arachidonic acid liberation by IIAsPLA$_2$ in platelets pretreated with linoleic acid hydroperoxide and FeSO$_4$.** [^{3}H]Arachidonic acid-labeled platelets were treated with 50 μM FeSO$_4$ and various concentrations of linoleic acid hydroperoxide (LHP) for 1 min, then incubated with (●) or without (○) 4 μg/ml IIAsPLA$_2$ for 10 min. Each point represents the mean of two experiments performed in duplicate.

BHP (50 μM) and FeSO$_4$ (50 μM) did not cause significant arachidonic acid liberation, the oxidizing reagents synergistically increased IIAsPLA$_2$-catalyzed arachidonic acid liberation.

DISCUSSION

It has been shown that IIAsPLA$_2$ is released from platelets upon stimulation (*12, 13*). Although the IIAsPLA$_2$ has been suggested to be involved in eicosanoid generation in a variety of cells (*8-11*), the enzyme cannot act on membrane phospholipids in platelets even when the cells are preactivated (*15*). In the present study, however, we demonstrated that arachidonic acid liberation and lysophosphatidylcholine formation in rabbit platelets pretreated with BHP and FeSO$_4$ were enhanced on the addition of purified platelet IIAsPLA$_2$, which alone did not cause the lipid metabolism. The enhancement of arachidonic acid liberation was abolished by pretreatment of IIAsPLA$_2$ with heparin. This indicates that the enhancement is mediated by IIAsPLA$_2$ itself, because heparin binds to the enzyme, thereby interfering with its susceptibility to membrane phospholipids (*11*). Furthermore, similar synergism with regard to arachidonic acid liberation was observed with linoleic acid hydroperoxide and the enzyme. These results revealed that IIAsPLA$_2$ can hydrolyze phospholipids in platelet membranes pretreated with oxidizing reagents. The finding that BHP and FeSO$_4$ generated malondialde-

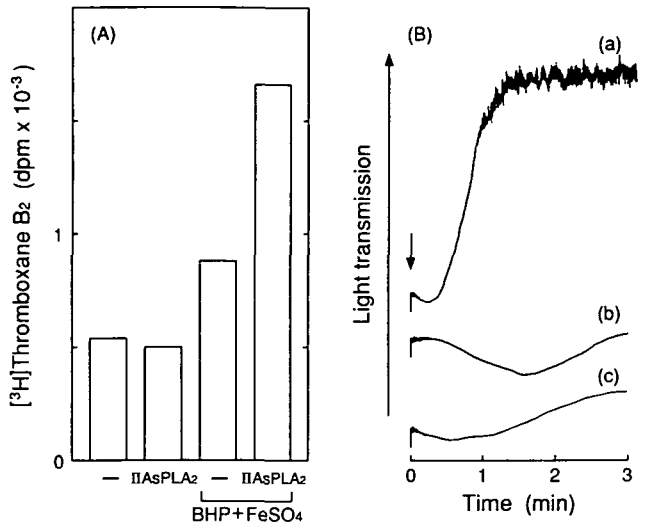


Fig. 5. **Enhancement of thromboxane B_2 generation (A) and aggregation (B) by $IIAsPLA_2$ in platelets pretreated with BHP and $FeSO_4$.** (A) [^{3}H]Arachidonic acid-labeled platelets were treated with or without 50 μM BHP and 50 μM $FeSO_4$ at 37°C for 2 min, then incubated with or without 4 μg/ml $IIAsPLA_2$ for 5 min. Data represent the means of two experiments performed in duplicate. (B) Washed platelets were treated with (b) or without (a, c) 10 μM indomethacin at 37°C for 2 min in the presence of 1 mM $CaCl_2$, then incubated with 20 μM BHP and 50 μM $FeSO_4$ for 1 min. $IIAsPLA_2$ (4 μg/ml, a, b) or buffer (c) was added at the arrow, then platelet aggregation was monitored for 3 min. The results shown are representative of three separate experiments.

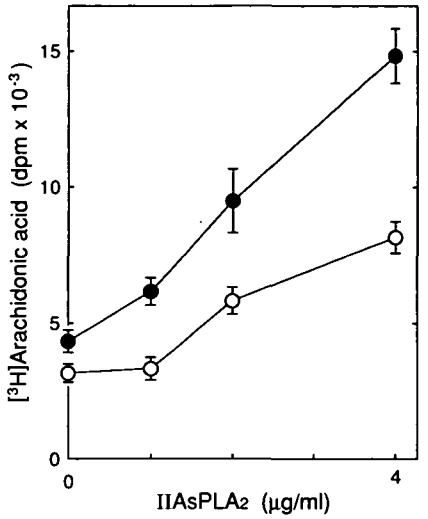


Fig. 6. **Effects of BHP and $FeSO_4$ on the hydrolysis of platelet membranes by $IIAsPLA_2$.** A membrane fraction of [^{3}H]arachidonic acid-labeled platelets was treated with (●) or without (○) 50 μM BHP and 50 μM $FeSO_4$ at 37°C for 1 min in the presence of 1 mM $CaCl_2$, then incubated with various concentrations of $IIAsPLA_2$ for 2 min. Each point represents the mean ± SE of three experiments performed in duplicate.

hyde, an indicator of lipid peroxidation, suggests that the oxidizing reagents may affect the hydrolytic action of $IIAsPLA_2$, probably through changes in membrane properties resulting from the accumulation of lipid hydroperoxides. Recently, another possibility has been proposed that the enhancement of arachidonic acid liberation by hydrogen peroxide and snake venom $IIAsPLA_2$ in kidney epithelial cells may result from hydrogen peroxide-induced degradation of proteoglycans covering the cell surface, which probably prevents the action of the venom $IIAsPLA_2$ on membrane phospholipids (*21*). We have no evidence that this mechanism is also involved in the effects of the oxidizing reagents used in this study. However, since membrane lipids are prime targets for BHP and linoleic acid hydroperoxide, which are relatively hydrophobic compounds compared with hydrogen peroxide, it is conceivable that the presence of lipid peroxide in membranes may increase the accessibility of membrane phospholipids to platelet $IIAsPLA_2$.

We previously reported that potentiation of collagen-induced platelet aggregation by BHP and $FeSO_4$ results from enhancement of thromboxane A_2 generation (*17*). The present study further showed that the combination of $IIAsPLA_2$ with BHP and $FeSO_4$ synergistically induced thromboxane B_2 generation followed by potentiation of aggregation. This suggests that the further increase in thromboxane B_2 (A_2) caused by $IIAsPLA_2$ may partially contribute to the amplification of platelet activation, although the involvement of the enzyme in thromboxane A_2 generation enhanced by collagen and oxidizing reagents is unclear. On the contrary, it has been shown that treatment of primed endothelial cells with $IIAsPLA_2$ enhances the generation of prostaglandin I_2, which inhibits platelet activation (*11*). This finding suggests that the enzyme released from activated platelets might exert negative feedback regulation on platelet activation, resulting in protection against excessive thrombogenesis. However, when reactive oxygen species are generated during inflammation or ischemia-reperfusion, oxidative stress induces vascular injury resulting in increased vascular permeability, thrombogenesis, and atherosclerosis. Under pathological conditions, therefore, platelet $IIAsPLA_2$ may be further involved in inflammatory processes, including amplification of platelet activation through enhancement of thromboxane A_2 generation.

The existence of a receptor for group IB secretory PLA_2 has been demonstrated in Swiss 3T3 cells (*23*), rat smooth muscle cells (*24*), and rabbit skeletal muscle cells (*25*). Although it is unknown whether rabbit platelets possess a receptor for $IIAsPLA_2$, the present study and other investigators (*15*) showed that exogenous $IIAsPLA_2$ elicited no response in rabbit platelets. Furthermore, we showed in the present work that the hydrolysis of a membrane fraction by the enzyme was enhanced on pretreatment with BHP and $FeSO_4$. Therefore, these results suggest that the increase in arachidonic acid liberation caused by $IIAsPLA_2$ in whole cells treated with the oxidizing reagents may be due to the direct action of the enzyme on membrane phospholipids.

Treatment with only BHP and $FeSO_4$ induced a slight increase in arachidonic acid liberation. Under the conditions, $IIAsPLA_2$ activity was not detected in the extracellular medium (data not shown). Therefore, the liberation caused by the oxidizing reagents may be mediated by cytosolic PLA_2. A recent report has shown that hydrogen peroxide-stimulated arachidonic acid liberation is potentiated in cytosolic PLA_2-expressing epithelial cells but not $IIAsPLA_2$-expressing cells (*19*), suggesting the contribu-

tion of cytosolic PLA_2 but not $IIAsPLA_2$ to oxidant-induced cellular injury. The report noted that $IIAsPLA_2$ activity could not be detected in the extracellular medium of cells expressing the enzyme after incubation with hydrogen peroxide, in spite of the detection of $IIAsPLA_2$ proteins. Therefore, it seems likely that the lack of potentiation in the $IIAsPLA_2$-expressing cells might result from inactivation of the enzyme by hydrogen peroxide. We also observed inactivation of platelet $IIAsPLA_2$ activity in the medium after incubation with BHP at above 50 μM (data not shown).

The accumulation of peroxidized fatty acids in membranes is known to activate endogenous PLA_2 (*16, 26, 27*). It has been suggested that arachidonic acid liberation enhanced under the conditions is mediated by cytosolic PLA_2 activation (*18, 19*). Furthermore, hydrogen peroxide has been shown to affect the activation of phospholipase C (*28*) and phospholipase D (*29*). Thus, oxidative stress modulates the activation of intracellular phospholipases, resulting in excessive cell activation or cell damage. In the present study, we further demonstrated that treatment of rabbit platelets with oxidizing reagents affected the ability of platelet $IIAsPLA_2$ to hydrolyze membrane phospholipids. In addition to intracellular phospholipases, $IIAsPLA_2$ also may play an important role in the facilitation of oxidative stress-induced cellular injury, probably through an increase in the liberation of arachidonic acid, a precursor for lipid chemical mediators such as thromboxane A_2.

REFERENCES

1. van den Bosch, H. (1980) Intracellular phospholipases A. *Biochim. Biophys. Acta* **604**, 191-246
2. Kudo, I., Murakami, M., Hara, S., and Inoue, K. (1993) Mammalian non-pancreatic phospholipase A_2. *Biochim. Biophys. Acta* **1170**, 217-231
3. Dennis, E.A. (1997) The growing phospholipase A_2 superfamily of signal transduction enzymes. *Trens Biochem. Sci.* **22**, 1-2
4. Seilhamer, J.J., Pruzanski, W., Vadas, P., Plant, S., Miller, J.A., Kloss, J., and Johnson, L.K. (1989) Cloning and recombinant expression of phospholipase A_2 present in rheumatoid arthritic synovial fluid. *J. Biol. Chem.* **264**, 5335-5338
5. Kramer, R.M., Hession, C., Johansen, B., Hayes, G., McGray, P., Chow, E.P., Tizard, R., and Pepinsky, R.B. (1989) Structure and properties of a human non-pancreatic phospholipase A_2. *J. Biol. Chem.* **264**, 5768-5775
6. Wright, G.W., Ooi, C.E., Weiss, J., and Elsbach, P. (1990) Purification of a cellular (granulocyte) and an extracellular (serum) phospholipase A_2 that participate in the destruction of *Escherichia coli* in a rabbit inflammatory exudate. *J. Biol. Chem.* **265**, 6675-6681
7. Inada, M., Tojo, H., Kawata, S., Tarui, S., and Okamoto, M. (1991) Preferential distribution of group-II-like phospholipase A_2 in mononuclear phagocytic cells in rat spleen and liver. *Eur. J. Biochem.* **197**, 323-329
8. Pfeilschifter, J., Schalkwijk, C., Briner, V.A., and van den Bosch, H. (1993) Cyotokine-stimulated secretion of group II phospholipase A_2 by rat mesangial cells: Its contribution to arachidonic acid release and prostaglandin synthesis by cultured rat glomerular cells. *J. Clin. Invest.* **92**, 2516-2523
9. Murakami, M., Kudo, I., and Inoue, K. (1991) Eicosanoid generation from antigen-primed mast cells by extracellular mammalian 14-kDa group II phospholipase A_2. *FEBS Lett.* **294**, 247-251
10. Hara, S., Kudo, I., and Inoue, K. (1991) Augmentation of prostaglandin E_2 production by mammalian phospholipase A_2 added exogenously. *J. Biochem.* **110**, 163-165
11. Murakami, M., Kudo, I., and Inoue, K. (1993) Molecular nature of phospholipase A_2 involved in prostaglandin I_2 synthesis human umbilical vein endothelial cells: Possible participation of cytosolic and extracellular type II phospholipase A_2. *J. Biol. Chem.* **268**, 839-844
12. Horigome, K., Hayakawa, M., Inoue, K., and Nojima, S. (1987) Purification and characterization of phospholipase A_2 released from rat platelets. *J. Biochem.* **101**, 625-631
13. Mizushima, H., Kudo, I., Horigome, K., Murakami, M., Hayakawa, M., Kim, D.-K., Kondo, E., Tomita, M., and Inoue, K. (1989) Purification of rabbit platelet secretory phospholipase A_2 and its characteristics. *J. Biochem.* **105**, 520-525
14. Kim, D.-K., Kudo, I., and Inoue, K. (1991) Purification and characterization of rabbit platelet cytosolic phospholipase A_2. *Biochim. Biophys. Acta* **1083**, 80-88
15. Mounier, C., Vargaftig, B.B., Franken, P.A., Verheij, H.M., Bon, C., and Touqui, L. (1994) Platelet secretory phospholipase A_2 fails to induce rabbit platelet activation and to release arachidonic acid in contrast with venom phospholipase A_2. *Biochim. Biophys. Acta* **1214**, 88-96
16. Chakraborti, S., Gurtner, G.H., and Michael, J.R. (1989) Oxidant-mediated activation of phospholipase A_2 in pulmonary endothelium. *Am. J. Physiol.* **257**, L430-L437
17. Hashizume, T., Yamaguchi, H., Kawamoto, A., Tamura, A., Sato, T., and Fujii, T. (1991) Lipid peroxide makes rabbit platelet hyperaggregable to agonists through phospholipase A_2 activation. *Arch. Biochem. Biophys.* **289**, 47-52
18. Rao, G.N., Runge, M.S., and Alexander, R.W. (1995) Hydrogen peroxide activation of cytosolic phospholipase A_2 in vascular smooth muscle cells. *Biochim. Biophys. Acta* **1265**, 67-72
19. Sapirstein, A., Spech, R.A., Witzgall, R., and Bonventre, J.V. (1996) Cytosolic phospholipase A_2 (PLA_2), but not secretory PLA_2, potentiates hydrogen peroxide cytotoxicity in kidney epithelial cell. *J. Biol. Chem.* **271**, 21505-21513
20. Salgo, M.G., Corongiu, F.P., and Sevanian, A. (1992) Peroxidation and phospholipase A_2 hydrolytic susceptibility of liposomes consisting of mixed species of phosphatidylcholine and phosphatidylethanolamine. *Biochim. Biophys. Acta* **1127**, 131-140
21. Dan, P., Nitzan, D.W., Dangan, A., Ginsburg, I., and Yedgar, S. (1996) H_2O_2 renders cells accessible to lysis by exogenous phospholipase A_2: A novel mechanism for cell damage in inflammatory processes. *FEBS Lett.* **383**, 75-78
22. Sundaram, G.S., Mohamed Shakir, K.M., Barnes, G., and Margolis, S. (1978) Release of phospholipase A and triglyceride lipase from rat liver. *J. Biol. Chem.* **253**, 7703-7710
23. Arita, H., Hanasaki, K., Nakano, T., Oka, S., Teraoka, H., and Matsumoto, K. (1991) Novel proliferative effect of phospholipase A_2 in Swiss 3T3 cells via specific binding site. *J. Biol. Chem.* **266**, 19139-19141
24. Hanasaki, K. and Arita, H. (1992) Characterization of a high affinity binding site for pancreatic-type phospholipase A_2 in the rat: Its cellular and tissue distribution. *J. Biol. Chem.* **267**, 6414-6420
25. Lambeau, G., Ancian, P., Barhanin, J., and Lazdunski, M. (1994) Cloning and expression of a membrane receptor for secretory phospholipase A_2. *J. Biol. Chem.* **269**, 1575-1578
26. Beckman, J.K., Borowitz, S.M., and Burr, I.M. (1987) The role of phospholipase A activity in rat liver microsomal lipid peroxidation. *J. Biol. Chem.* **262**, 1479-1481
27. Borowitz, S.M. and Montogomery, C. (1989) The role of phospholipase A_2 in microsomal lipid peroxidation induced with *t*-butyl hydroperoxide. *Biochem. Biophys. Res. Commun.* **158**, 1021-1028
28. Meij, J.T.A., Suzuki, S., Panagia, V., and Dhalla, N.S. (1994) Oxidative stress modifies the activity of cardiac sarcolemmal phospholipase C. *Biochim. Biophys. Acta* **1199**, 6-12
29. Natarajan, V., Taher, M.M., Roehm, B., Parinandi, N.L., Schmid, H.H.O., Kiss, Z., and Garcia, J.G.N. (1993) Activation of endothelial cell phospholipase D by hydrogen peroxide and fatty acid hydroperoxide. *J. Biol. Chem.* **268**, 930-937