# Beneficial effect of oleoylated lipids on paraoxonase 1: protection against oxidative inactivation and stabilization

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The effect of lipids on PON1 (paraoxonase 1), one of antioxidant proteins in high-density lipoprotein, was investigated in respect to inhibition, protection against oxidative inactivation, and stabilization. When the effect of lipids on the PON1 activity was examined, a remarkable inhibition was expressed by polyenoic fatty acids ($C_{18:2}$–$C_{20:5}$). Linoleic acid, the most potent ($K_i$, 3.8 $\mu$M), showed competitive inhibition. Next, various lipids were examined for prevention against the inactivation of PON1 by ascorbate/$Cu^{2+}$, which caused a remarkable ($\geqslant$ 90 %) inactivation of PON1. Compared with saturated fatty acids ($C_6$–$C_{18}$), exhibiting a modest protection (9–40 %), monoenoic acids ($C_{16:1}$–$C_{20:1}$) showed a greater maximal protective effect ($E_{max}$, 70–82 %), with oleic acid being the most effective ($EC_{50}$, 2.7 $\mu$M). In contrast, polyenoic acids showed no protection. Noteworthy, linoleic acid prohibited the protective action of oleic acid non-competitively. In the structure–activity relationship, a negatively charged group seems to be required for the protective action. Consistent with this, dioleoylphosphatidylglycerol, negatively charged, was more protective than dioleoylphosphatidylcholine. These data, together with requirement of $Ca^{2+}$ ($EC_{50}$, 0.6 $\mu$M) for the protective action, may support the existence of a specific site responsible for the protective action. A similar protective action of lipids was also observed in the inactivation of PON1 by ascorbate/$Fe^{2+}$, peroxides or *p*-hydroxymercuribenzoate. Separately, PON1 was stabilized by oleic acid or oleoylated phospholipids, in combination with $Ca^{2+}$, but not linoleic acid. These results suggest that in contrast to an adverse action of linoleic acid, monoenoic acids or their phospholipid derivatives play a beneficial role in protecting PON1 from oxidative inactivation as well as in stabilizing PON1.

Key words: inhibition, oleic acid, oxidative inactivation, paraoxonase 1 (PON1), protection, stabilization.

## INTRODUCTION

PON1 (paraoxonase 1; aryldialkylphosphatase; EC 3.1.8.1), associated with HDL (high-density lipoprotein) [1–3], was initially identified for its ability to hydrolyse organophosphate compounds and aromatic carboxylic acid esters [1,4–6]. Its potential interest comes from the detoxification of organophosphate insecticide or neurotoxins such as soman [4,7]. The lack of this enzyme renders animals sensitive to organophosphate toxicity [8]. PON1 was also observed to hydrolyse homocysteine thiolactone [6], which could pose potential harm by homocysteinylation of some proteins [9]. Recent interest in the enzyme has arisen from the idea that PON1 protects LDL (low-density lipoprotein) and HDL from oxidation [1,3,10–15]. A support for this contention is provided by an animal model, in which a lower serum level of PON1 is associated with a greater susceptibility of LDL to oxidation and an increased risk of atherosclerosis [16]. In studies with PON1-knockout mice, PON1 has been shown to be both necessary and sufficient for *in vitro* protective effects of HDL on LDL oxidation [8]. Recent clinical investigations indicate that PON1 activity was lower in subjects with coronary heart diseases than control subjects [17–19]. Additionally, there are polymorphisms that affect the PON1 level in blood, which may alter the propensity to develop coronary vascular disease [20].

Despite the association of PON1 activity with the reduced oxidation of LDL [3,10,12–14], the mechanism by which PON1 inhibits the oxidation of LDL phospholipids is not clear. According to previous observations [12,14], one function of PON1 in reducing the level of oxidized LDL was due to the hydrolysis of oxidized PCs (phosphatidylcholines) containing core aldehydes or isoprostanes. However, such a hydrolysis function of PON1 is disproved by a recent report [21] that PON1 has no ability to hydrolyse oxidized phospholipids. Meanwhile, the role of PON1 in preventing against LDL oxidation has been proposed to be due to its antioxidant action [11–13]. In support of this, the protection by PON1 against LDL oxidation was accompanied by a partial inactivation of the enzyme [13], and the degradation of PON1 paraoxonase activity was significantly correlated with the decrease in the antioxidant action of HDL towards LDL oxidation [22]. However, the arylesterase activity of PON1 is not related to its antioxidant action against LDL oxidation [15]. Rather, the cysteine residue at position 283, which is not necessary for PON1 activity, is required for the ability of PON1 to protect against LDL oxidation [15]; Cys-283 PON1 mutants failed to prevent LDL oxidation. Nevertheless, it was supposed that some amino acid residues in the active site of PON1 might participate in the prevention of LDL oxidation, since there was a reverse relationship between oxidative inactivation of PON1 and its antioxidant capacity; the lipid peroxide-inactivated PON1, compared with native PON1, showed less protection against $Cu^{2+}$-catalysed LDL oxidation [11,15], as had been observed with PHMB (*p*-hydroxymercuribenzoate)-inactivated PON1 [15]. This might be in line with the notion that some amino acid residues in the active site might be important for the antioxidant action of cysteine residues in PON1. In this regard, the oxidative inactivation of PON1, which could lead to the reduction in the reactivity of cysteine residues via the oxidative modification of adjacent amino acid residues, might be one of causes to reduce antioxidant action of PON1. Besides, the oxidative inactivation of PON1 results in the loss of its ability to hydrolyse homocysteine thiolactones, a potential atherogenic factor [23]. Concerning the oxidative inactivation of PON1 by

Abbreviations used: LDL, low-density lipoprotein; HDL, high-density lipoprotein; PON1, paraoxonase 1; ROS, reactive oxygen species; PHMB, *p*-hydroxymercuribenzoate; PC, phosphatidylcholine; PG, phosphatidylglycerol; c.m.c., critical micellar concentration.

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ROS (reactive oxygen species), $H_2O_2$ at excess concentrations (>1 mM) was observed to inactivate PON1 [13]. Additionally, it was suggested that free-radical-induced inactivation of PON1 was responsible for the accelerated inactivation of HDL-bound PON1 in glycoxidative condition [24]. Furthermore, in the macrophage of PON1-knockout mice, the deficiency of PON1 activity was associated with the increase of oxidative stress [25].

It is important to preserve the activity of PON1 under oxidative stress, since the prevention against oxidative inactivation of PON1 could lead to the maintenance of the antioxidant function of PON1 *in vivo*. An approach to protect PON1 under oxidative stress has been achieved partly by the introduction of antioxidants such as flavonoids [11,26] or ascorbate [27]. The introduction of hypolipidaemic drugs or their metabolites significantly enhanced the ability of PON1 to reduce the level of cholesterylester hydroperoxides in oxidized LDL [28]. Another strategy to protect enzymes from the inactivation by ROS or lipid peroxidation products would be the employment of substrates, inhibitors or regulators [29,30]. However, an earlier study indicated that the inhibitors of PON1 might be inappropriate for this purpose as demonstrated from the negative role of PON1 inhibitors, such as PD65950, in preventing LDL oxidation [31]. Although phospholipids were observed to stimulate the activity of PON1 [32,33], there is no study on the effect of lipids on the PON1 activity under oxidative stress. In animal experiments [34–36], the intake of oleate was found to be beneficial in preventing atherogenesis; the oleate intake contributed to an elevation of serum PON1 activity by enhancing the synthesis and secretion of very-high-density lipoprotein [34]. The beneficial effect of the oleate intake on PON1 activity was more prominent in subjects carrying the R allele of the PON1–192, which is more susceptible to oxidation [15,37]. Thus some lipids such as oleate may interact directly with PON1 under physiological oxidative conditions. Nevertheless, little interest was given to the investigations concerning the protective effects of lipids on the activity of PON1 under oxidative stress.

In the present study, we attempted to examine the effect of fatty acids or phospholipids on the catalytic activity, oxidative inactivation and stability of PON1, and provide a strategy to preserve the activity of PON1 under oxidative stress.

## EXPERIMENTAL

### Materials

All materials, including fatty acids, were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise noted. $H_2O_2$ (30 %) was from Junsei Chemical Co. (Tokyo, Japan). Metal ions, including cupric sulphate and ferrous sulphate, were of analytical grade. Oleoyl sulphate was kindly supplied by Dr T. R. Holman (Department of Chemistry and Biochemistry, University of California, Santa Cruz, CA, U.S.A.). In these experiments, Hepes buffer was passed through Chelex 100 resin before use.

### Purification of PON1

PON1 was purified from human plasma by (pseudo) affinity chromatography using Cibacron Blue 3GA, DEAE-Sephacel chromatography, Sephacryl S-200-HR gel chromatography and finally affinity chromatography using concanavalin A–Sepharose according to a slight modification of the published procedures [5,7, 33,38]; the identification of PON1 from human plasma has been reported previously [38]. The purified enzyme, homogeneous, with a molecular mass of $\approx$45 kDa in SDS/PAGE analysis and a mass (*m*/*z*) of 45161.88 in matrix-assisted laser-desorption ionization–time-of-flight MS (Voyager DE-RP mass spectrometer; Framingham, MA, U.S.A.), possessed a specific activity of approx. 1018 and 0.552 $\mu$mol/min per mg of protein in the hydrolysis of phenylacetate and paraoxon, respectively. Separately, when the phenotype of PON1 was determined by the dual-substrate method [39], the purified PON1 was found to belong to phenotype group AB.

### Preparation of HDL

Serum HDL was isolated from the blood of fasted normolipidaemic human volunteers by ultracentrifugation as described before [11,31], and dialysed extensively overnight against 10 mM phosphate buffer (pH 7.4) with 150 mM NaCl at 4 °C. Prior to the use, HDL (6.2 mg of protein/ml) was dialysed against the same buffer containing 10 $\mu$M $Ca^{2+}$ at 4 °C.

### Assay of PON1

PON1 activity towards phenylacetate was measured by adding enzyme solution to 0.5 ml of 50 mM Tris buffer, pH 7.4, containing 1 mM $CaCl_2$ and 10 mM phenylacetate, and the rate of phenol generation was determined as described before [5,7]. Unless otherwise noted, the PON1 activity was determined using phenylacetate as a substrate. PON1 activity towards paraoxon was quantified spectrophotometrically in 50 mM Tris buffer (pH 8.0) containing 1 mM $CaCl_2$ and 1 mM paraoxon as described before [5]. One unit of enzyme activity is expressed as 1 $\mu$mol of product/min.

### Prevention by fatty acids or phospholipids against oxidative inactivation of PON1 by various oxidants

PON1 (0.5 units) was incubated with 0.5 mM ascorbate and 1 $\mu$M $Cu^{2+}$ at 38 °C in the presence of each fatty acid or phospholipid in 0.1 ml of 50 mM Hepes buffer (pH 7.4) containing 50 $\mu$M $Ca^{2+}$ and 10 min later the aliquot (20 $\mu$l) was taken for the assay of remaining activity using phenylacetate as a substrate. To see the effect of derivatization of carboxyl group on ascorbate/$Cu^{2+}$-induced inactivation, PON1 (0.5 units) was incubated with 0.5 mM ascorbic acid and 1 $\mu$M $Cu^{2+}$ in the presence of each oleic acid derivative. Similarly, the effect of each fatty acid on ascorbate/$Fe^{2+}$-induced inactivation of PON1 was examined by incubating PON1 (0.5 units) with 0.5 mM ascorbate/2 $\mu$M $Fe^{2+}$ in the presence of each lipid. Also, the protective action of fatty acid against $H_2O_2$-induced inactivation was investigated by incubating PON1 (0.5 units) with 2 mM $H_2O_2$ in the presence of each fatty acid as described above. Fatty acids and phospholipids were dissolved in ethanol, and then included in the reaction mixture (final concentration of ethanol, 1%).

### Prevention by saturated fatty acids or oleic acid against the inactivation of PON1 by PHMB

PON1 (0.5 units) was incubated with 50 $\mu$M PHMB in the presence of each saturated fatty acid in 0.1 ml of 50 mM Hepes buffer (pH 7.4) containing 50 $\mu$M $Ca^{2+}$ at 38 °C, and 30 min later an aliquot (20 $\mu$l) was taken for the assay of remaining activity. In addition, PON1 (0.5 units) was incubated with 50 $\mu$M PHMB in the presence of oleic acid at various concentrations (0–100 $\mu$M) at 38 °C.

### Inhibitory effect of detergents or polyunsaturated fatty acids on the protective action of oleic acid against the inactivation of PON1 by ascorbate/$Cu^{2+}$

PON1 (0.5 units) was incubated with 0.5 mM ascorbate and 1 $\mu$M $Cu^{2+}$ in the presence of each detergent or polyunsaturated fatty acid of various concentrations in 0.1 ml of 50 mM Hepes buffer (pH 7.4) containing 10 $\mu$M oleic acid and 50 $\mu$M $Ca^{2+}$ at 38 °C, and 10 min later the aliquot (20 $\mu$l) was taken for the assay of remaining activity. Separately, the inactivation of PON1 by 0.5 mM ascorbate and 1 $\mu$M $Cu^{2+}$ was performed by varying the concentration (0–100 $\mu$M) of oleic acid while that of linoleic acid was fixed at 5 or 10 $\mu$M.

### $Ca^{2+}$-dependent protective action of oleic acid against the inactivation of purified PON1 or HDL-PON1 by ascorbate/$Cu^{2+}$

To see the requirement of $Ca^{2+}$ for the protective effect of oleic acid, PON1 (0.5 units) was incubated with 0.5 mM ascorbic acid and 1 $\mu$M $Cu^{2+}$ for 10 min in the presence of oleic acid (10 $\mu$M) in 0.1 ml of 50 mM Hepes buffer (pH 7.4) containing $Ca^{2+}$ of various concentrations (0.2–1000 $\mu$M) at 38 °C. Separately, HDL (0.52 mg of protein/ml) was incubated with 0.5 mM ascorbate and 2 $\mu$M $Cu^{2+}$ in the presence of oleic acid in the buffer containing various concentrations of $Ca^{2+}$.

### Stabilizing effect of fatty acids or phospholipids on the PON1 activity during a lengthy incubation time

PON1 (0.5 units) was incubated with each fatty acid or phospholipid in 0.1 ml of 50 mM Hepes buffer (pH 7.4) containing 50 $\mu$M $Ca^{2+}$ and 50 $\mu$M butylhydroxytoluene for 12 h at 38 °C, and then the aliquot (20 $\mu$l) was taken for the assay of remaining activity. Separately, the requirement of $Ca^{2+}$ for the stabilization of PON1 by oleic acid was examined by performing the same experiment in 50 mM Hepes buffer (pH 7.4) containing $Ca^{2+}$ of various concentrations (0.5–1000 $\mu$M).

### Other analyses

pH-dependent inactivation of PON1 was performed by exposing PON1 (0.5 units) to 20 $\mu$M PHMB in 0.1 ml of 50 mM buffer (pH 6.5–9.0) containing 20 $\mu$M $Ca^{2+}$ for 10 min at 38 °C. Inhibition of PON1 by polyunsaturated fatty acids was carried out in 0.5 ml of 50 mM Hepes buffer (pH 7.4) containing phenylacetate (0.3–10 mM) and 2 mM $Ca^{2+}$ at room temperature, and the $K_i$ values of polyunsaturated fatty acids were determined by Lineweaver–Burk plot. Protection was denoted as the activity that was restored in the presence of each protective compound, expressed as a percentage of the activity lost under the inactivation conditions. $E_{max}$ was expressed as the maximal protection (%), which was achieved in the presence of each protective compound at saturating concentrations, and $EC_{50}$ was the concentration of each compound to show half-maximal protection (50 % of $E_{max}$).

## RESULTS

The oxidative inactivation of some enzymes, whose oxidative modification occurs at their active site, is known to be prevented by substrates, inhibitors or regulators [29,30,40]. However, substrates of PON1 failed to show remarkable protection against ascorbate/$Cu^{2+}$-induced inactivation of PON1 (Su, N. D. and Sok, D.-E., unpublished work). Since earlier studies indicated that lipids could affect the activity of PON1 [32,33], we examined the protective effect of lipids against oxidative inactivation of PON1. First, to define the effect of fatty acids on PON1 activity, various types of fatty acid, varying in chain length and degree of unsaturation, were examined at a low concentration (20 $\mu$M) for the effect on PON1 activity, which was determined using phenylacetate as a substrate. While polyunsaturated fatty acids showed a great inhibition (36–64 %) of PON1, saturated or monounsaturated fatty acids expressed no remarkable inhibition (< 10 %). When the inhibition by linoleic acid, the most inhibitory fatty acid, was analysed kinetically by Lineweaver–Burk plot (Figure 1), the pattern was consistent with competitive inhibition with a $K_i$ value of approx. 3.8 $\mu$M. Similar but weaker inhibition was also shown by $\alpha$-linolenic acid ($K_i$, 8.7 $\mu$M), followed by $\gamma$-linolenic acid (25.2 $\mu$M) and arachidonic acid (30.9 $\mu$M). In contrast, phospholipids such as dilauryl-PC or dioleoyl-PC (0.1 mM) showed neither inhibitory nor stimulatory effects. Also, Triton X-100 and deoxycholate at 0.1 mM failed to affect the activity of PON1.

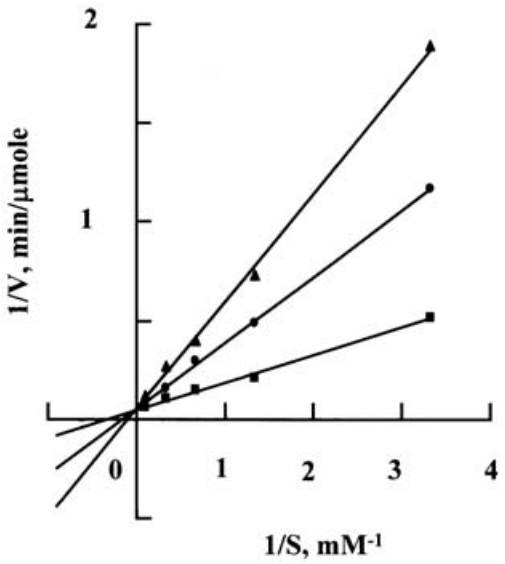


**Figure 1 Lineweaver–Burk double-reciprocal plot for the inhibition of PON1 by linoleic acid**

PON1 (4 units/ml) was incubated with phenylacetate of various concentrations (0.3–10 mM) in the absence (■) or presence of linoleic acid (5 $\mu$M, ●; 10 $\mu$M, ▲) in 50 mM Tris buffer (pH 7.4) containing 2 mM $CaCl_2$. Data are expressed means ± S.D. from triplicate determinations.

### Protective action of saturated or monounsaturated fatty acids against ascorbate/$Cu^{2+}$-induced inactivation of PON1

From the above data, it was supposed that polyunsaturated fatty acids, exhibiting prominent inhibition, would bind to the active centre of PON1. In this context, polyunsaturated fatty acids were examined for the prevention against ascorbate/$Cu^{2+}$-induced inactivation of PON1 in buffer containing 50 $\mu$M $Ca^{2+}$. However, none of polyunsaturated fatty acids at 100 $\mu$M exerted protection against ascorbate/$Cu^{2+}$-mediated inactivation of PON1, indicating that the active centre may not be a target for oxidative modification. Next, when saturated fatty acids at various concentrations (10–500 $\mu$M) were tested for protective action, all of saturated fatty acids ($C_6$–$C_{18}$) expressed a dose-dependent protection (Figure 2A). The maximal protective effect ($E_{max}$) differed greatly according to the length of acyl group (Table 1); the maximal

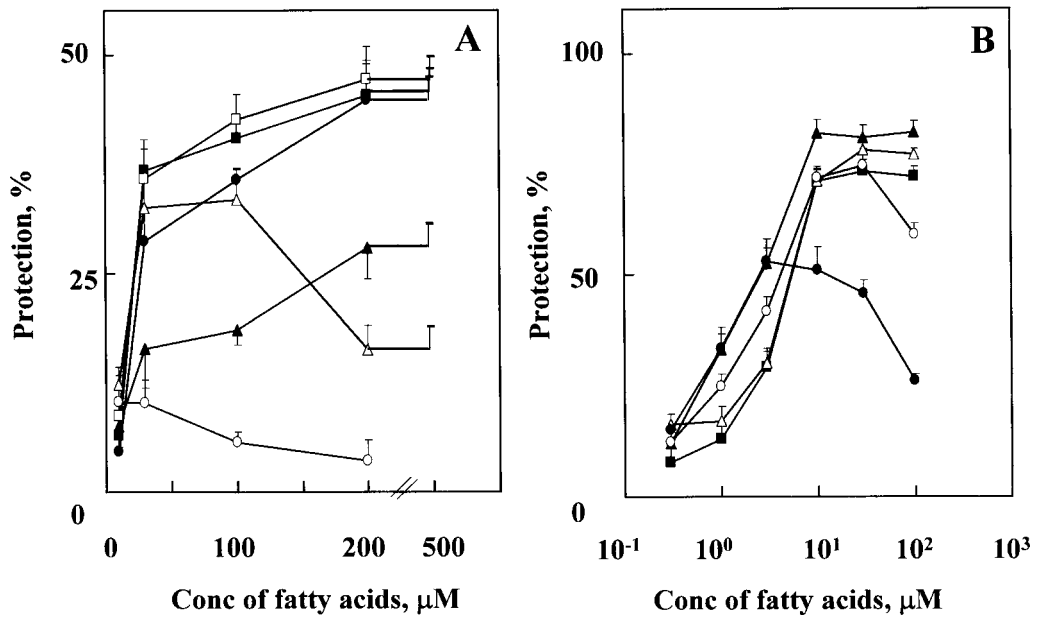


**Figure 2 Protective action of saturated or monounsaturated fatty acids against the inactivation of PON1 by ascorbate/$Cu^{2+}$**

(**A**) PON1 (0.5 units) was incubated with 0.5 mM ascorbate and 1 $\mu$M $Cu^{2+}$ in the presence of each saturated fatty acid of various concentrations (10–500 $\mu$M) at 38 °C in 0.1 ml of 50 mM Hepes buffer (pH 7.4) containing 50 $\mu$M $Ca^{2+}$ for 10 min. ▲, Caproic acid; ●, caprylic acid; ■, capric acid; □, lauric acid; △, myristic acid; ○, palmitic acid. (**B**) PON1 (0.5 units) was incubated with 0.5 mM ascorbate and 1 $\mu$M $Cu^{2+}$ in the presence of each monounsaturated fatty acid at 38 °C in 0.1 ml of 50 mM Hepes buffer (pH 7.4) containing 50 $\mu$M $Ca^{2+}$ for 10 min. The remaining activity was determined using phenylacetate as a substrate. Data are expressed as means ± S.D. from triplicate determinations. ▲, Oleic acid; △, palmitoleic acid; ■, eicosenoic acid; ○, oleoylglycine; ●, erucic acid.

**Table 1 $E_{max}$ of each saturated fatty acid in the prevention of the inactivation of PON1 by ascorbate/$Cu^{2+}$**

The protective action of each saturated fatty acid against the inactivation of PON1 by ascorbate/$Cu^{2+}$ was performed as described for Figure 2(A). $E_{max}$ is expressed as mean ± S.D. from triplicate determinations.

| Fatty acid | $E_{max}$ (%) |
|---|---|
| Butyric | 18.26 ± 3.9 |
| Caproic | 28.36 ± 2.6 |
| Caprylic | 46.25 ± 3.5 |
| Capric | 46.76 ± 2.4 |
| Lauric | 47.27 ± 3.7 |
| Myristic | 33.42 ± 3.6 |
| Palmitic | 10.26 ± 3.1 |

degree of protection was enhanced with increasing acyl-chain length up to $C_{12}$, and the extension of chain length over 12 carbons led to a corresponding decrease. Thus lauric acid was one of the most effective compounds, with an $E_{max}$ value of 47 % and an $EC_{50}$ value of approx. 20 $\mu$M, much below its c.m.c. (critical micellar concentration) [41,42].

In a related experiment, lauryl sulphate ($E_{max}$, 45 %; $EC_{50}$, 8 $\mu$M) was as effective as lauric acid, whereas laurylcholine, positively charged, had no effect, indicating that the negative charge of fatty acids is required for the protective action. Subsequently, monoenoic acids ($C_{16}$–$C_{22}$) were examined for protection against the ascorbate/$Cu^{2+}$-induced inactivation of PON1. All of the monoenoic acids, at relatively low concentrations, expressed a remarkable protection ($E_{max}$, 70–82 %), as demonstrated in Figure 2(B). The highest protection was expressed by oleic acid ($C_{18:1}$, Δ9-*cis*) with an $E_{max}$ value of approx. 82 %, followed by palmitoleic acid ($C_{16:1}$, Δ9-*cis*), eicosenoic acid ($C_{20:1}$, Δ11-*cis*) and erucic acid ($C_{22:1}$, Δ11-*cis*).

When the effect of the position of *cis*-double bond in monounsaturated $C_{18}$ fatty acids was investigated, it was found that elaidic acid (Δ9-*trans*) showed a protective action similar to that of oleic acid (Δ9-*cis*), whereas petroselinic acid (Δ6-*cis*) and vaccenic acid (Δ12-*cis*) were less effective ($E_{max}$, 70–75 %) than oleic acid. Additionally, oleoylglycine or oleoyl sulphate also showed a remarkable protective effect ($E_{max}$, 70–78 %). In contrast, ricinoleic acid, corresponding to a 2-hydroxyl derivative of oleic acid, or the derivatives such as 1-mono-oleoylglycerol, oleoylcerebroside, oleoamide or oleoyl ethylester, had no significant protective effect (< 5 %). Separately, the protective action of oleic acid was examined in the presence of detergents, which could diminish the concentration of oleic acid monomer by forming mixed micelles above their c.m.c. values [41]. As shown in Figure 3(A), Triton X-100 and deoxycholate above their c.m.c. values suppressed the protective action of oleic acid. Similarly, such inhibition was also expressed by linoleic acid or arachidonic acid at less than 0.1 mM, close to their c.m.c. values [42,43]. In particular, linoleic acid at a concentration as low as 5 $\mu$M decreased the protective action of oleic acid (10 $\mu$M) remarkably, suggestive of a selective antagonism between oleic acid and linoleic acid.

In a further study to examine the mechanism of the inhibitory action of linoleic acid, the protective action of oleic acid at various concentrations (0.3–100 $\mu$M) was examined in the presence of linoleic acid. As shown in Figure 3(B), the suppressive effect of linoleic acid (5 or 10 $\mu$M) on the protective action of oleic acid was similar irrespective of the oleic acid concentration, indicating a non-competitive antagonism between oleic acid and linoleic acid. To establish the metal-ion requirement for the protective action, the protection by oleic acid was examined in the presence of bivalent metal ions. In contrast with $Mg^{2+}$ or $Zn^{2+}$, which

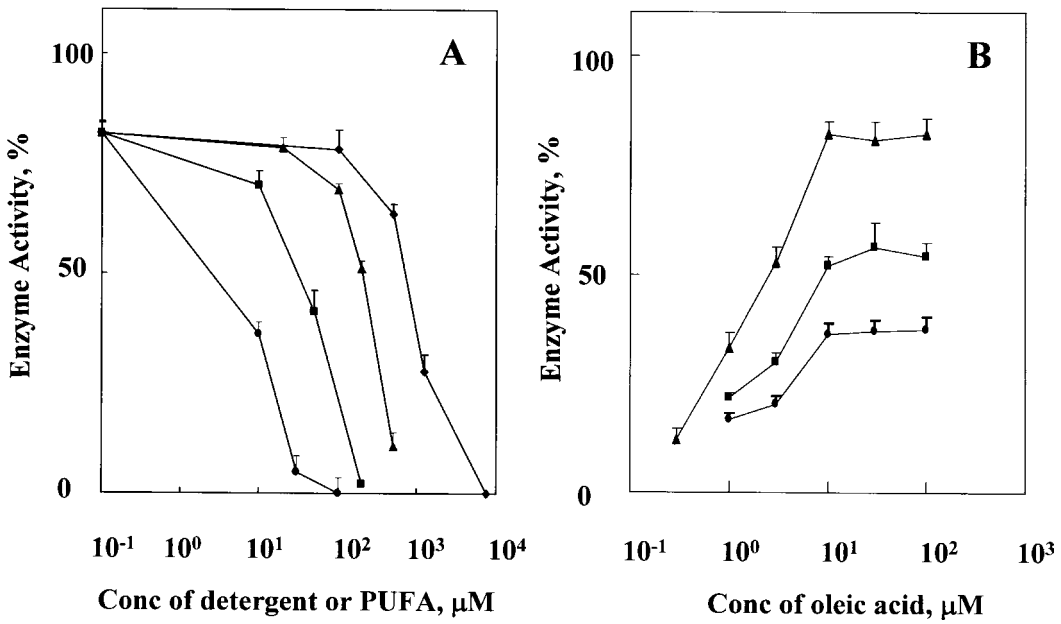


**Figure 3 Inhibitory effect of detergents or PUFA (polyunsaturated fatty acids) on the protective action of oleic acid against the inactivation of PON1 by ascorbate/$Cu^{2+}$**

(**A**) The protective action of oleic acid (10 $\mu$M) against the inactivation of PON1 by ascorbate/$Cu^{2+}$ was examined in the presence of each detergent or polyunsaturated fatty acid at various concentrations (0.1–7500 $\mu$M) as described in Figure 2(B). ◆, Deoxycholate; ▲, Triton X-100; ■, arachidonic acid; ●, linoleic acid. (**B**) Concentration-dependent protective action of oleic acid (0.3–100 $\mu$M) was investigated in the absence (▲) or presence of linoleic acid at 5 $\mu$M (■) or 10 $\mu$M (●) as described above.

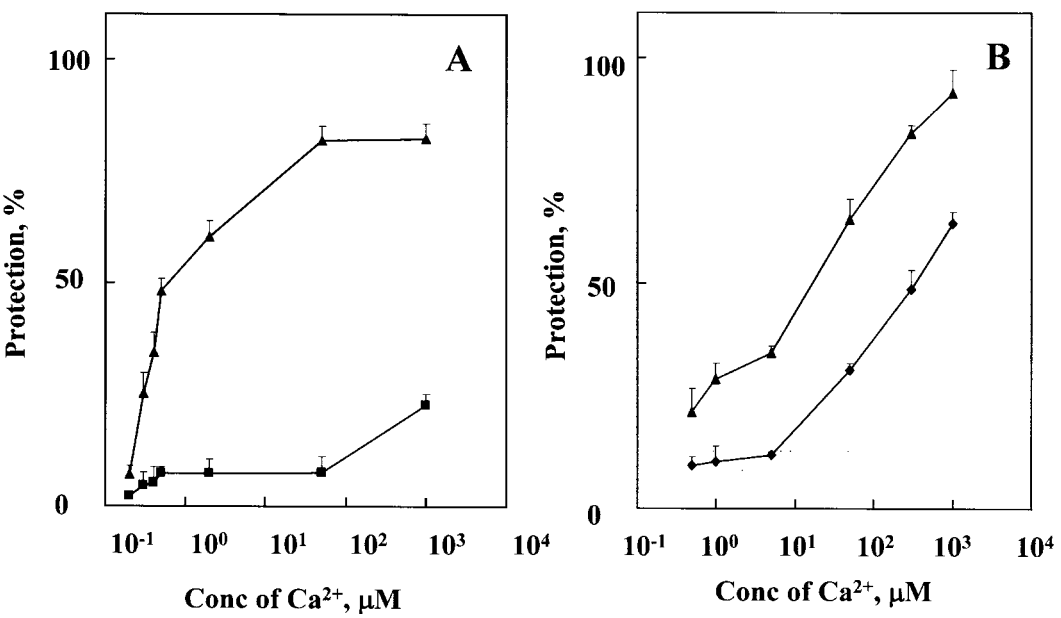


**Figure 4 $Ca^{2+}$-dependent protective action of oleic acid against ascorbate/$Cu^{2+}$-induced inactivation of PON1 or HDL-PON1**

(**A**) PON1 (0.5 units) was incubated with 0.5 mM ascorbic acid and 1 $\mu$M $Cu^{2+}$ in the presence (▲) or absence (■) of oleic acid (10 $\mu$M) in 0.1 ml of 50 mM Hepes buffer (pH 7.4) containing $Ca^{2+}$ at various concentrations (0.2–1000 $\mu$M) at 38 °C for 10 min. (**B**) HDL (0.52 mg of protein/ml) was incubated with 0.5 mM ascorbic acid and 2 $\mu$M $Cu^{2+}$ in the presence (▲) or absence (◆) of oleic acid (10 $\mu$M) at 38 °C in 0.1 ml of 50 mM Hepes buffer (pH 7.4) containing $Ca^{2+}$ of various concentrations (0.5–1000 $\mu$M) for 10 min. Data are expressed as means ± S.D. from triplicate determinations.

had no effect, $Ca^{2+}$ augmented the protective action of oleic acid in a concentration-dependent manner (Figure 4A); the $EC_{50}$ value of $Ca^{2+}$ required for the protective action of oleic acid was graphically estimated to be 0.6 $\mu$M. Separately, the requirement of $Ca^{2+}$ for the prevention against oxidative inactivation of HDL-PON1 was examined in the presence or absence of oleic acid (10 $\mu$M; Figure 4B). Although it seemed that a high concentration of $Ca^{2+}$ was required for the protection by oleic acid against ascorbate/$Cu^{2+}$-induced inactivation of HDL-PON1, the $EC_{50}$ value ($\approx$0.5 $\mu$M) of $Ca^{2+}$ for the net protective action of oleic acid was close to that (0.6 $\mu$M) of $Ca^{2+}$ for the protection of purified PON1, reflecting a similar $Ca^{2+}$ requirement between HDL-PON1

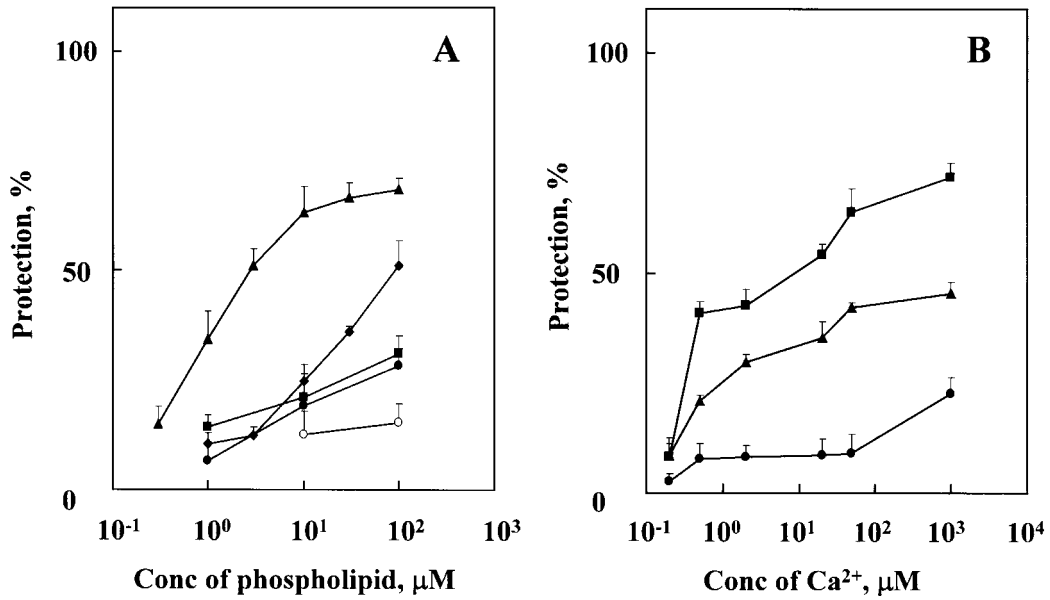


**Figure 5 Prevention by phospholipids against oxidative inactivation of PON1 by ascorbate/$Cu^{2+}$**

(**A**) PON1 (0.5 units) was preincubated with 0.5 mM ascorbate and 1 $\mu$M $Cu^{2+}$ in the presence of each phospholipid at 38 °C in 0.1 ml of 50 mM Hepes buffer (pH 7.4) containing 50 $\mu$M $Ca^{2+}$. ▲, Dioleoyl-PG; ◆, dioleoyl-PC; ■, $\beta$-oleoyl-$\gamma$-myristoyl-PC; ●, $\beta$-palmitoyl-$\gamma$-oleoyl-PC; ○, $\beta$-linoleoyl-$\gamma$-palmitoyl-PC. (**B**) The requirement of $Ca^{2+}$ for the protective action. PON1 was incubated with 0.5 mM accorbate and 1 $\mu$M $Cu^{2+}$ in the presence or absence of each phospholipid in the above buffer containing $Ca^{2+}$ of various concentrations (0.2–1000 $\mu$M). ●, None; ▲, dioleoyl-PC; ■, dioleoyl-PG.

and purified PON1. Of note, the protective action of $Ca^{2+}$ (1 mM) alone was greater for HDL-PON1 (63 %; Figure 4B) than purified PON1 (23 %; Figure 4A), indicating that HDL-PON1 could be protected from the oxidative inactivation by $Ca^{2+}$ at physiological concentration. Meanwhile, the protective action of oleic acid, in combination with $Ca^{2+}$, was smaller for HDL-PON1 than purified PON1. This led to the notion that phospholipids, a component of HDL membrane, might exert protective action in concert with $Ca^{2+}$.

### Protection by phospholipids against ascorbate/$Cu^{2+}$-induced inactivation of PON1

In this regard, various phospholipids were tested for the prevention against ascorbate/$Cu^{2+}$-induced inactivation of PON1. As shown in Figure 5(A), dioleoyl-PC and dioleoyl-PG (where PG is phosphatidylglycerol) showed concentration-dependent protection of 50 % and 68 %, respectively, at 100 $\mu$M. Similar but weaker protection was also observed with either $\beta$-oleoyl-$\gamma$-myristoyl-PC (31 % at 100 $\mu$M) or $\beta$-palmitoyl-$\gamma$-oleoyl-PC (28 %), which was more protective than $\beta$-linoleoyl-$\gamma$-palmitoyl-PC (15 %) or didecanoyl-PC (15 %). These results demonstrate that PON1 may interact preferentially with the oleoyl group in the acyl portion of phospholipids. Moreover, negatively charged dioleoyl-PG, exhibiting a saturation pattern of dose-dependent protection with an $E_{max}$ value of 68 % and an $EC_{50}$ of approx. 1 $\mu$M, was more protective than dioleoyl-PC, which is zwitterionic, at the concentrations used, underlying the importance of a negative charge in phospholipids. In contrast, lysolecithins such as mono-oleoyl lyso-PC or monopalmitoyl lyso-PC were not effective ($E_{max}$, $<10$ %). In a separate experiment, where the protective action of dioleoyl-PC (30 $\mu$M) was examined in the presence of linoleic acid, linoleic acid (10 $\mu$M) was found to diminish the protective action of dioleoyl-PC by 37 % (results not shown). In addition (Figure 5B), the protective action of dioleoyl-PC or dioleoyl-PG (10 $\mu$M) was found to require a low concentration of $Ca^{2+}$ ($EC_{50}$, $<1$ $\mu$M), as observed with the protective action of oleic acid.

### Prevention by fatty acids against inactivation of PON1 by other oxidants or PHMB

Subsequently, we turned to the protection by other oxidation systems against the inactivation of PON1. First, the protective effect of saturated fatty acids (100 $\mu$M) on ascorbate/$Fe^{2+}$ or $H_2O_2$-induced inactivation of PON1 was examined (Figure 6A). The protective effect of fatty acids in preventing against oxidative inactivation was dependent on the length of the acyl chain with 8–12 carbons being the most effective for ascorbate/$Fe^{2+}$-inactivation, and 12–14 carbons for $H_2O_2$-induced inactivation. In an additional experiment (Figure 6B), the oxidative inactivation of PON1 by $H_2O_2$ was prevented fully by oleic acid at 10 $\mu$M, whereas full protection against ascorbate/$Fe^{2+}$-induced inactivation required a higher concentration (100 $\mu$M) of oleate. Thus there seemed to be differing targets for oxidation between $H_2O_2$-induced inactivation and ascorbate/$Fe^{2+}$-induced inactivation.

Next, in order to probe the binding site of protective lipids, the protective effect of fatty acids on the inactivation of PON1 by PHMB, a cysteine modifier, was evaluated. First, when the inactivation of PON1 by PHMB (20 $\mu$M) was performed at various pH values, the pH for 50 % maximal inactivation was estimated to be around 7.5 (Figure 7A, inset), indicating that the cysteine residue of PON1 may be so reactive at neutral pHs. When the protective action of saturated fatty acids against PHMB-induced inactivation was examined (Figure 7A) the optimal length of the acyl chain was 12 carbons. Thus the dependence of the maximal protection on the length of acyl chain was more critical for the prevention of PHMB-induced inactivation than ascorbate/$Cu^{2+}$-induced inactivation. Also, oleic acid expressed a dose-dependent protection against PHMB-induced inactivation of

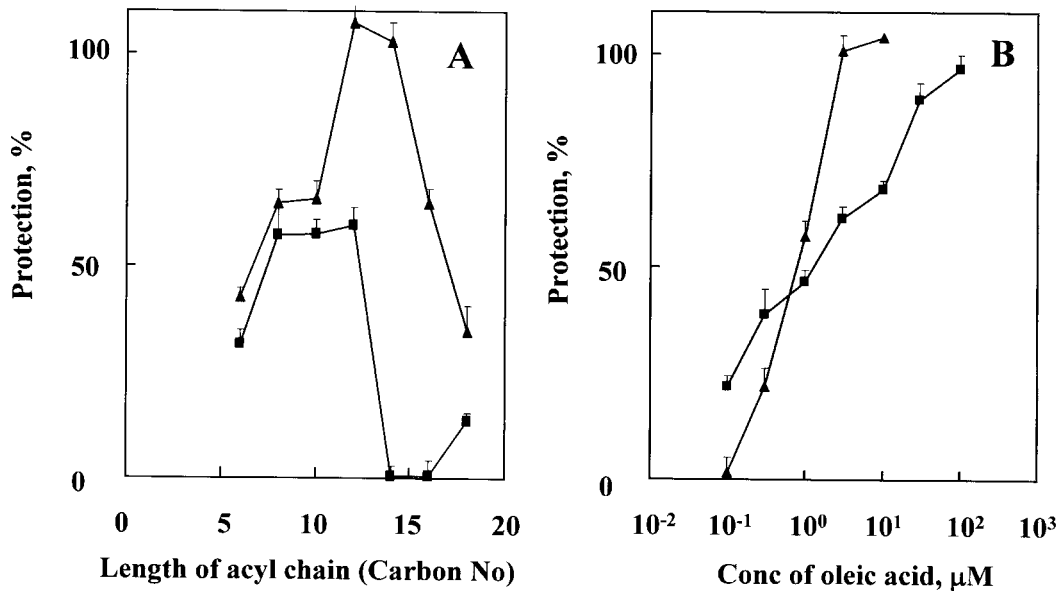


**Figure 6 Prevention by saturated fatty acids or oleic acid against ascorbate/$Fe^{2+}$- or $H_2O_2$-induced inactivation of PON1**

(**A**) PON1 (0.5 units) was incubated with 0.5 mM ascorbate and 2 $\mu$M $Fe^{2+}$ in the presence of fatty acids with different acyl-chain length in 0.1 ml of 50 mM Hepes buffer (pH 7.4) containing 50 $\mu$M $Ca^{2+}$ at 38 °C for 30 min (■). Separately, PON1 (0.5 units) was incubated with 2 mM $H_2O_2$ in the presence of each fatty acid for 30 min (▲). (**B**) PON1 (0.5 units) was incubated with 0.5 mM ascorbate and 2 $\mu$M $Fe^{2+}$ in the presence of oleic acid at various concentrations (0.1–100 $\mu$M) as described above (■). Separately, PON1 (0.5 units) was incubated with 2 mM $H_2O_2$ in the presence of oleic acid at various concentrations (▲).

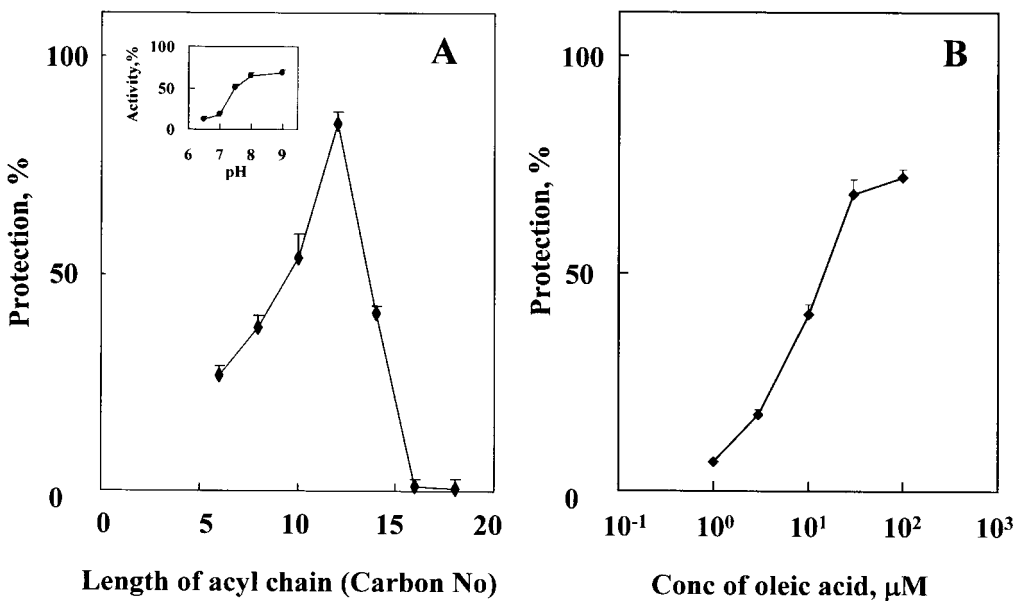


**Figure 7 Prevention by saturated fatty acids or oleic acid against PHMB-induced inactivation of PON1**

(**A**) PON1 (0.5 units) was incubated with 50 $\mu$M PHMB in the presence of saturated fatty acids (100 $\mu$M) with different acyl length in 0.1 ml of 50 mM Hepes buffer (pH 7.4) containing 50 $\mu$M $Ca^{2+}$ at 38 °C for 30 min. Inset: pH-dependent inactivation of PON1 by PHMB (50 $\mu$M). (**B**) The inactivation of PON1 by PHMB (50 $\mu$M) was performed the presence of oleic acid at various concentrations (1–100 $\mu$M).

PON1 in the presence of 50 $\mu$M $Ca^{2+}$ (Figure 7B); the maximal protective effect of oleic acid (10 $\mu$M), in combination with $Ca^{2+}$, was weaker for PHMB-induced inactivation than ascorbate/$Cu^{2+}$-induced inactivation (42 compared with 82%).

In an independent experiment, the requirement of $Ca^{2+}$ for the prevention against PHMB-induced inactivation of purified PON1 and HDL-PON1 was compared. While there was remarkable co-operation between $Ca^{2+}$ and oleic acid in preventing PHMB-induced inactivation of purified PON1 (Figure 8A), such co-operation was not reproduced in the PHMB-induced inactivation of HDL-PON1 (Figure 8B). Moreover, the inactivation of HDL-PON1 by PHMB was not affected by $Ca^{2+}$ (1 mM), suggesting that the cysteine residue in HDL-PON1 under physiological conditions might be almost fully exposed to the modification by PHMB. Separately, although the protective effect of oleic acid on the inactivation of PON1 by diethylpyrocarbonate, which had

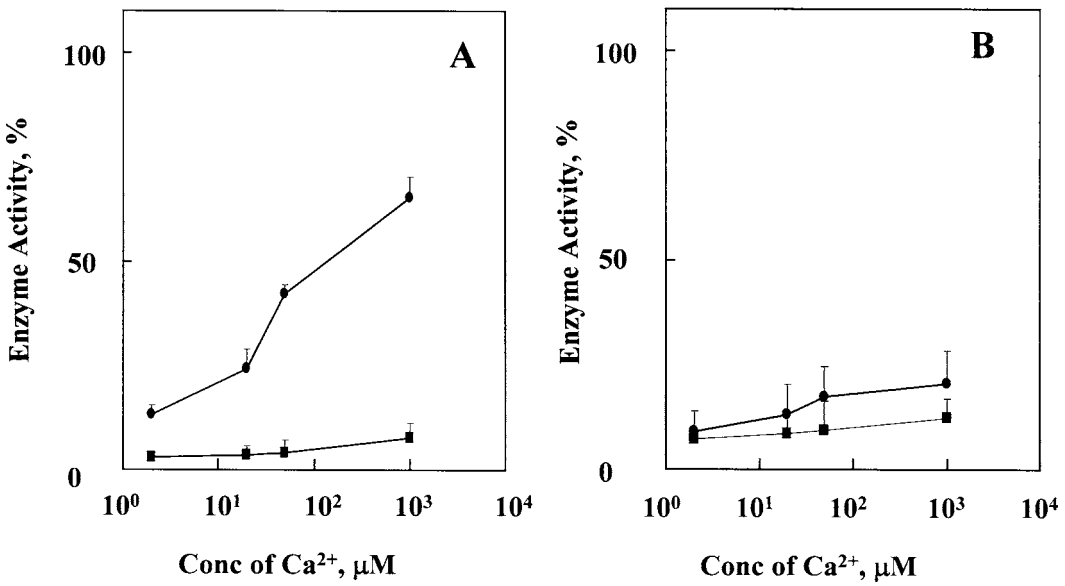


**Figure 8 $Ca^{2+}$-dependent protective action of oleic acid against PHMB-induced inactivation of PON1 or HDL-PON1**

(**A**) PON1 (0.5 units) was incubated with 50 $\mu$M PHMB in the presence (●) or absence (■) of 10 $\mu$M oleic acid in 0.1 ml of 50 mM Hepes buffer (pH 7.4) containing $Ca^{2+}$ of various concentrations (2–1000 $\mu$M) at 38 °C for 30 min. (**B**) HDL (0.52 mg of protein/ml) was incubated with 50 $\mu$M PHMB in the presence (●) or absence (■) of 10 $\mu$M oleic acid in 0.1 ml of 50 mM Hepes buffer (pH 7.4) containing $Ca^{2+}$ of various concentrations (2–1000 $\mu$M). Data are expressed as means ± S.D. from triplicate determinations.

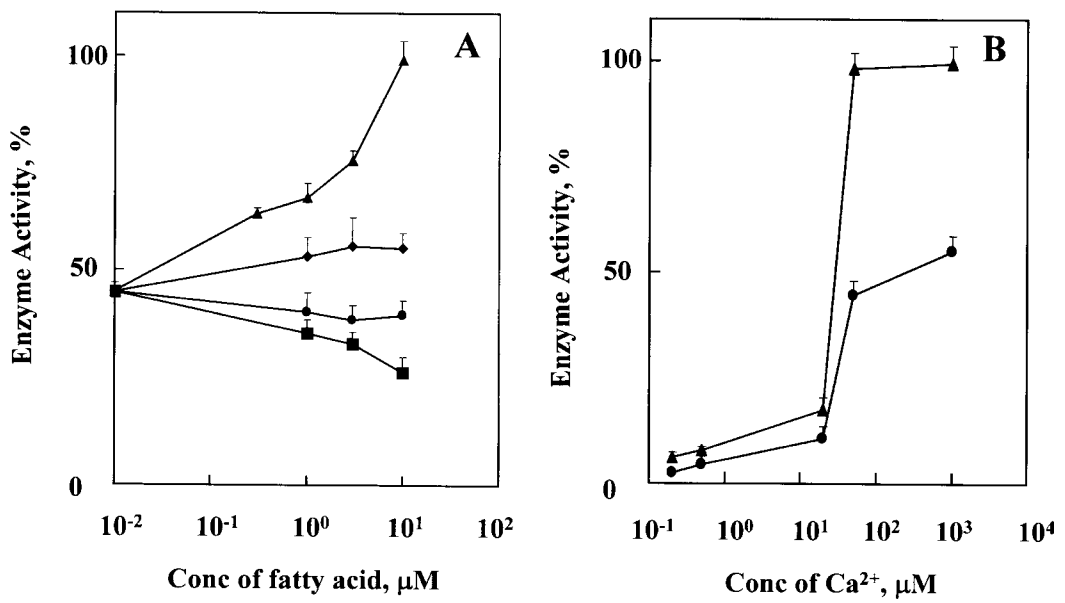


**Figure 9 Stabilizing effect of fatty acids on PON1 during long-term incubation**

(**A**) PON1 (0.5 units) was incubated with each fatty acid in 0.1 ml of 50 mM Hepes buffer (pH 7.4) containing 50 $\mu$M $Ca^{2+}$ for 12 h at 38 °C, and then an aliquot (20 $\mu$l) was taken for the assay of remaining activity. ▲, Oleic acid; ◆, lauric acid; ●, arachidonic acid; ■, linoleic acid. (**B**) Separately, the requirement of $Ca^{2+}$ for the stabilization of PON1 was examined in the absence or presence of 10 $\mu$M oleic acid by performing the same experiment in 50 mM Hepes buffer (pH 7.4) containing $Ca^{2+}$ of various concentrations (0.2–1000 $\mu$M). ●, $Ca^{2+}$ alone; ▲, $Ca^{2+}$ with oleic acid.

been observed to non-specifically modify histidine residues of PON1 [44], was examined, no remarkable protection was observed.

### Stabilizing effect of fatty acids or phospholipids on deactivation of PON1

Next, the stabilizing effect of fatty acids on PON1 activity during a lengthy incubation was examined by storing PON1 in buffer containing 50 $\mu$M $Ca^{2+}$ for 12 h at 38 °C. When PON1 was incubated in the absence of any fatty acid, it lost approx. 55% activity. Meanwhile, the inclusion of oleic acid prevented the deactivation of PON1 in a concentration-dependent manner over the range 0.3–10 $\mu$M, as shown in Figure 9(A). At 3 and 10 $\mu$M oleic acid, respectively, approx. 50% stability and an almost complete stabilization effect were achieved, whereas oleic acid at 0.01 $\mu$M failed to show any stabilization effect. Lauric acid at 3 and 10 $\mu$M showed a slight stabilization effect (5–10%), but,

**Table 2 Stabilizing effect of phospholipids on PON1 activity during long-term incubation**

PON1 (0.5 units) was incubated with dioleoyl-PC, dioleoyl-PG or $\gamma$-myristoyl-$\beta$-oleoyl-PC of different concentrations (0.3–10 $\mu$M) in 0.1 ml of 50 mM Hepes buffer (pH 7.4) containing 50 $\mu$M $Ca^{2+}$ for 12 h at 38 °C. An aliquot (20 $\mu$l) was taken for the assay of remaining activity employing phenylacetate as a substrate. Data are expressed as means ± S.D. from triplicate determinations.

| Phospholipid | Concentration ($\mu$M) | Enzyme activity (%) |
|---|---|---|
| Control | – | 45.9 ± 3.4 |
| Dioleoyl-PG | 0.3 | 105.1 ± 4.6 |
| | 1.0 | 134.5 ± 7.7 |
| | 3.0 | 139.9 ± 10.6 |
| Dioleoyl-PC | 0.3 | 64.5 ± 4.4 |
| | 3.0 | 87.7 ± 5.7 |
| | 10.0 | 93.1 ± 3.6 |
| $\gamma$-Myristoyl-$\beta$-oleoyl-PC | 1.0 | 49.0 ± 4.5 |
| | 3.0 | 63.7 ± 3.9 |
| | 10.0 | 95.6 ± 2.4 |

in contrast, neither linoleic acid nor arachidonic acid showed any remarkable stabilization action at the concentrations used.

Separately, the requirement of $Ca^{2+}$ for the stabilization of PON1 by oleic acid was investigated (Figure 9B). In contrast to a negligible remaining activity in the absence of $Ca^{2+}$, the inclusion of $Ca^{2+}$ alone demonstrated a partial stabilization of PON1 with a level of 45 and 52 % at 0.05 and 1 mM, respectively. Although oleic acid (10 $\mu$M) failed to show remarkable stabilization in the presence of $Ca^{2+}$ at 0.3 or 0.6 $\mu$M, it expressed a full stabilization in a concert with $Ca^{2+}$ at 0.05 or 1 mM.

In an additional experiment (Table 2), where phospholipids were tested for the stabilization of PON1 in combination with 50 $\mu$M $Ca^{2+}$, dioleoyl-PG (0.3 $\mu$M) accomplished full protection, while dioleoyl-PC and $\beta$-oleoyl-$\gamma$-myristoyl-PC (3 $\mu$M) showed partial protection. However, neither Triton X-100 nor deoxycholate at 0.1 %, in combination with $Ca^{2+}$, demonstrated a remarkable stabilization effect.

## DISCUSSION

Concerning the interaction of PON1 with lipids, earlier studies showed a stimulating effect of some phospholipids on PON1 activity [32,33]. Recently [38], phospholipids together with apolipoprotein A1 were observed to participate in the stabilization of PON1 activity in HDL. In addition, PON1 was found to associate with HDL through direct binding of its N-terminal to HDL phospholipid [45]. Our present results provide lines of evidence for the direct effect of lipids on PON1 activity. One is the inhibition of PON1 by polyunsaturated fatty acids, especially linoleic acid. The greater inhibitory action of linoleic acid, compared with other polyunsaturated fatty acids, and moreover, the higher inhibitory potency of $\alpha$-linolenic acid, compared with $\gamma$-linolenic acid, appear to underscore the importance of the number and position of double bond for the inhibitory action. Noteworthy is that the competitive inhibition of PON1 by linoleic acid reflects that the binding site for linoleic acid may overlap with the active centre of PON1 containing the $Ca^{2+}$ site [46]. Also of interest is the remarkable protection by saturated ($C_8$–$C_{14}$) or monounsaturated ($C_{16}$–$C_{22}$) fatty acids against ascorbate/$Cu^{2+}$-induced oxidative inactivation of PON1. The protective action of saturated fatty acids ($C_6$–$C_{18}$), relying on the length of acyl chain, and the higher protection by monounsaturated fatty acids, compared with polyunsaturated fatty acids, may support the binding of these protective fatty acids to a specific site, so-called the protective binding site. Thus the protective binding site seems to recognize the structural geometry of fatty acids, determined by the type or size of acyl group. The possible link of their protective action to micelle formation is unlikely, since there is no relationship between the protective efficacy of saturated fatty acids and their c.m.c. values [42]. Furthermore, polyunstaturated fatty acids showed no protective action despite their lower c.m.c. values [42,43]. In addition, concentration-dependent protection by monoenoic acids showed a saturation pattern well below c.m.c. values (60–100 $\mu$M), a characteristic feature expressed by a monomer portion during the micelle formation [41]. However, a broad specificity of the protective binding site towards monoenoic acids is suggested by a similar protection by monoenoic acids of different acyl-chain lengths ($C_{16}$–$C_{20}$), and there is no positional requirement of a double bond among $C_{18:1}$ fatty acids. The suppressive effects of detergents, above their c.m.c. values, on the protective action of oleic acid might result from the incorporation of oleic acid monomer to micellar form of detergents. Similarly, this might account for the inhibition of the oleic acid action by linoleic acid or arachidonic acid above c.m.c. values [42,43]. However, the strong inhibition of the oleate action by linoleic acid at low concentrations (5 or 10 $\mu$M) and moreover, the non-competitive properties of the inhibition, may coincide with the idea that the antagonism between two lipids may be mediated through different binding sites; probably the protective binding site for oleic acid and the active centre for linoleic acid. The study employing derivatives of oleic acid reveals that the protective binding site may contain a cationic subsite in addition to a non-polar subsite. Then, the cationic subsite in the protective binding site could be the $Ca^{2+}$-stabilization site [46], different from the $Ca^{2+}$ site in the active centre [33,46], as alluded to by the similarity between the $EC_{50}$ value (0.6 $\mu$M) of $Ca^{2+}$ for the protective action of oleic acid and the $K_D$ value (0.66 $\mu$M) of $Ca^{2+}$ reported for the stabilization site of PON1 [33,46]. Phospholipids also expressed a remarkable protection against oxidative inactivation of PON1. The greater protection by phospholipids with oleoyl group, compared with those bearing saturated or polyunsaturated acyl groups, and moreover, the higher efficacy of dioleoyl-PC than mono-oleoyl-PC may comply with the selective recognition of oleoyl group by protective binding site of PON1. In addition, the greater protection by dioleoyl-PG, compared with dioleoyl-PC, may be ascribed to its preferential association to the $Ca^{2+}$ site in the protective binding site. This might be supported by the low $EC_{50}$ values (0.3–1 $\mu$M) of $Ca^{2+}$ for the protective action of phospholipids, similar to the observation with that of oleic acid. Therefore, it is supposed that two protective lipids, oleic acid and oleoylated phospholipid, may share the same protective binding site. However, since the c.m.c. values of phospholipids are very low, the micelle form might be more responsible for the protection by phospholipids. Nonetheless, the dose-dependent protection by dioleoyl-PG at low concentrations may emphasize the protective action of the monomeric dioleoyl-PG form. Another common property is that the action of protective lipids is inhibited by linoleic acid. The non-competitive inhibition might be explained by the assumption that the prior binding of inhibitory linoleic acid at active centre may interfere with the association of protective lipids at the protective binding site. Further, such a contrasting action between linoleic acid and protective lipids may well be extended to the stabilization of PON1, as manifested from the opposing effects of linoleic acid and protective lipid on the stability of PON1. Moreover, the requirement of $Ca^{2+}$ for the stabilizing effect of lipids, and the superiority of negatively charged lipids to neutrally charged lipids

in the stabilization support the idea that the site responsible for the stabilization of PON1 by lipids may overlap with the protective binding site.

Previously, there have been reports that the modification of cysteine residues, unnecessary for catalysis, in PON1 led to a loss of antioxidant action. In the present study, the dose-dependent protection by oleic acid against PHMB-induced inactivation supports the idea that the cysteine residue (Cys-283), a target for PHMB [15,47,48], may exist in the protective binding site rather than the active centre. Further support for this may come from a similar pattern of protection, which is chain-length-dependent, by saturated fatty acids against PHMB inactivation and ascorbate/$Cu^{2+}$ inactivation. The strict requirement of acyl-chain length ($C_{12}$) for the maximal protection by saturated fatty acids against PHMB inactivation, in contrast with a broad range of chain lengths ($C_8$–$C_{12}$) for ascorbate/$Cu^{2+}$ inactivation, may agree with the notion that, in contrast with one cysteine target for PHMB inactivation, there may be multiple amino acid targets for ascorbate/$Cu^{2+}$ in the protective binding site of PON1. The possible targets for ascorbate/$Cu^{2+}$ inactivation could be conserved histidine residues in the active site, because histidine residues, sensitive to $Cu^{2+}$-catalysed oxidation [29], are essential for the catalysis [44]. In support of the above, a separate study showed that the decrease of the activity of PON1 exposed to ascorbate/$Cu^{2+}$ was accompanied by the loss of histidine residues, and such a loss of histidine residues was prevented by the presence of oleic acid (Su, N.D. and Sok, D.-E., unpublished work). Taken together, it is proposed that the primary target locus sensitive to ascorbate/$Cu^{2+}$ inactivation may be a specific site containing both histidine and cysteine residues. In addition, oleic acid also prevented against the inactivation of PON1 by oxidative systems such as ascorbate/$Fe^{2+}$ or $H_2O_2$. In this respect, oleic acid may correspond to a broad-spectrum protective lipid, capable of protecting PON1 from various oxidative systems. Furthermore, since oleic acid successfully prevented HDL-PON1 from oxidative inactivation in the presence of $Ca^{2+}$ at physiological concentrations, the beneficial effect of oleic acid might be practical in protecting HDL-PON1 from the oxidative inactivation system *in vivo*, where cellular pro-oxidant activity with regard to LDL oxidation is expressed [49].

Although the histidine residue of PON1 is suggested to be a target for oxidative modification, the role of the histidine residue as an antioxidant may be less than that of the cysteine residue in a state tightly associated with HDL membrane *in vivo*; the susceptibility of HDL-PON1 to ascorbate/$Cu^2$, compared with PHMB, was diminished to a much greater extent in the presence of $Ca^{2+}$ at physiological concentrations. Thus histidine residues of PON1 associated with HDL might not participate directly in the removal of oxidants *in vivo*. Instead, it is conceivable that histidine residues proximal to the cysteine residue might contribute to enhancing the reactivity of cysteine residues by stabilizing the thiolate anion according to the mechanism for the charge interaction between cysteine residue and positively charged amino acid residue in some proteins sensitive to oxidation [50]. Support for this may come from the p$K$ value ($\approx$7.5) of cysteine residue in PON1, lower than that of normal cysteine residues [50]. Alternatively, histidine residues, in concert with cysteine residue, could efficiently scavenge reactive oxygen species or copper ions [29,30]. In this regard, the protection of histidine residues from oxidative modification would be necessary for the maximal antioxidant function of the cysteine residue in PON1.

In particular, the protective action by oleic acid against oxidative inactivation of PON1 and its stabilizing action might be of physiological relevance, since the concentration for its beneficial actions is below the c.m.c. level. Further, the incorporation of oleic acid into phospholipids could induce the same effect as demonstrated by the beneficial role of oleoylated phospholipids. The positive effect of oleate supplementation in elevating PON1 activity [34,51] in animal experiments might be in part due to the protective effect of oleic acid or oleoylated phospholipids on oxidative inactivation of PON1 as well as the stability of PON1. Also, it is possible that either the resistance of membrane phospholipids rich in oleoyl groups to oxidation [35,36] or the positive effect of oleic acid on the formation of very-high-density lipoprotein particles [34] contributed to maintaining the activity of PON1 through indirect mechanisms.

Further work on the dietary and pharmacological approaches to the maintenance of PON1 activity by keeping the protective binding site intact may prove to have an application in the prevention of atherosclerosis as well as in the treatment of organophosphate or lactone intoxication.

We express gratitude to Dr Byoung Chul Park (Proteome Research Laboratory, Korea Research Institute of Bioscience and Biotechnology, Taejon, South Korea) for the matrix-assisted laser-desorption ionization–time-of-flight mass analysis. This work was financially supported by a research grant (R05-2001-000-00518-0) from the Korea Science and Engineering Foundation, South Korea.

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Received 6 May 2003/1 July 2003; accepted 18 July 2003
Published as BJ Immediate Publication 18 July 2003, DOI 10.1042/BJ20030663