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Oxidative Inactivation of Paraoxonase1, an Antioxidant Protein and its Effect on Antioxidant Action

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Paraoxonase1 (PON1), one of antioxidant proteins to protect low density lipoprotein (LDL) from the oxidation, is known to lose its activity in the oxidative environment. Here, we attempted to elucidate the possible mechanisms for the oxidative inactivation of PON1, and to examine the capability of hydroxyl radicals-inactivated PON1 to prevent against LDL oxidation. Of various oxidative systems, the ascorbate/Cu^{2+} system was the most potent in inactivating the purified PON1 (PON1) as well as HDL-bound PON1 (HDL-PON1). In contrast to a limited inactivation by Fe^{2+} (2.0 μM), the inclusion of Cu^{2+} (0.1–1.0 μM) remarkably enhanced the inactivation of PON1 in the presence of ascorbate (0.5 mM). A similar result was also obtained with the inactivation of HDL-PON1. The inactivation of PON1 by ascorbate/Cu^{2+} was pevented by catalase, but not general hydroxyl radical scavengers, supporting Cu^{2+}-catalyzed oxidative inactivation. In addition, Cu^{2+} alone inactivated PON1, either soluble or HDL-bound, by different mechanisms, concentration-dependent. Separately, there was a reverse relationship between the inactivation of PON1 and its preventive action against LDL oxidation during Cu^{2+}-induced oxidation of LDL. Noteworthy, ascorbate/Cu^{2+}-inactivated PON1, which was charaterized by the partial loss of histidine residues, expressed a lower protection against Cu^{2+}-induced LDL oxidation, compared to native PON1. Based on these results, it is proposed that metal-catalyzed oxidation may be a primary factor to cause the decrease of HDL-associated PON1 activity under oxidative stress, and radicals-induced inactivation of PON1 may lead to the decrease in its antioxidant action against LDL oxidation.

Keywords: PON1; Oxidative inactivation; Cu^{2+}; Hydroxyl radicals; Histidine; LDL oxidation

Abbreviations: LDL, low density lipoprotein; PON1, paraoxonase 1; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; DMSO, dimethylsulfoxide; PHMB, *p*-hydroxymercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid)

INTRODUCTION

Paraoxonase1 (PON1), associated with HDL,[1–3] was initially identified for its ability to hydrolyze 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,[4,7,8] and the ability to hydrolyze homocysteine thiolactone, which could pose a potential harm by homocysteinylation of some proteins.[9]

Recent interest in the enzyme has arisen from the idea that PON1 protects LDL and HDL from the lipid peroxidation.[1,3,10–12] This protection was proposed to be related to the peroxidase-like activity of PON1 on preexisting peroxides,[11,13] and the ability of PON1 to modify the proportion of oxidation products in oxidized LDL.[12,14,15] Animal model provides a support for this contention, 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 (KO) mice, PON1 was shown to be both necessary and sufficient for the *in vitro* protective effects of HDL on LDL oxidation.[8] Recent clinical investigations indicate that PON1 activity is lower in subjects with coronary heart diseases than control subjects.[17–19] Additionally, there are polymorphisms that affect the level of PON1 in blood, which may alter the propensity to develop the coronary vascular disease.[20]

Despite the association of PON1 activity with the prevention against LDL oxidation,[3,10,12–14]

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the mechanism by which PON1 inhibits the oxidation of LDL phospholipids is not clear. According to previous observations,[2,14] PON1 hydrolyzed oxidized phosphatidylcholines containing core aldehydes or isoprostanes, in addition to platelet activating factors.[21] However, such a hydrolytic function of PON1 is doubted by a recent report[22] that PON1 has no intrinsic phospholipase activity. Then, the prevention by PON1 against LDL oxidation might be due to antioxidant action of PON1 in the initial stage of LDL oxidation. This had been suggested by earlier observations[11–13] that the protection by PON1 against LDL oxidation was accompanied by a partial inactivation of the enzyme; the inactivation of PON1 was supposed to be due to the interaction between the oxidized lipids and sulfhydryl group at cysteine 283, which is not necessary for the hydrolytic activity of PON1.[11,15] Also, reactive oxygen species (ROS), generated from Cu^{2+}-oxidized oxidation of LDL,[23,24] could cause the decrease of PON1 activity as implied from the greater protective action of PON1 in the earlier stage of Cu^{2+}-oxidized LDL oxidation.[17] Presumably in support of this, hydrogen peroxide at millimolar concentrations was observed to partially inactivate PON1.[11,13] Recently, it was suggested that the enhanced inactivation of HDL-bound PON1 in glycoxidative condition[25] was ascribed to free radicals-induced oxidation. Besides, α,β-unsaturated lipid aldehydes,[26] stable decomposition products of lipid peroxides which gradually accumulate in the later stage of LDL oxidation, could also cause the inactivation of PON1 by modifying the cysteine residue of PON1 in the same way as had been observed with PHMB, a cysteine modifier.[15] Thus, PON1 is speculated to be one of antioxidant enzymes very susceptible to oxidative stress *in vivo* system. Nevertheless, there have been few attempts to define the *in vivo* conditions for oxidative inactivation of PON1, and the relationship between oxidative inactivation of PON1 and its antioxidant capacity. Here, we demonstrate that PON1 is highly susceptible to hydroxyl radicals produced from metal (Cu^{2+} or Fe^{2+})-catalyzed oxidation. And, it is proposed that Cu^{2+} -catalyzed oxidation of PON1, accompanied by the modification of some histidine residues, can result in the decrease of its antioxidant action.

MATERIALS AND METHODS

Chemicals and Reagents

L-ascorbic acid, cumene hydroperoxide, *tert*-butyl hydroperoxide, paraoxon, phenyl acetate, *p*-hydroxymercuribenzoate (PHMB), dimethylsulfoxide, 1,1,3,3,-tetraethoxypropane, catalase or mannitol were from Sigma Chemical Co. (St. Louis, MO, USA). 4-Hydroxy-2-nonenal and acrolein were from Calbiochem Co. (La Jolla, CA, USA) and Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), respectively. H_2O_2 (30%) were from Junsei Chemical Co. (Tokyo, Japan). Metal ions including cupric sulfate, cuprous chloride or ferrous sulfate were of analytical grade.

Assay of Paraoxonase1 (PON1)

PON1 activity was measured by adding enzyme solution to 0.5 ml of HEPES buffer (50 mM, pH 7.4) containing 2 mM $CaCl_2$ and 10 mM phenyl acetate, and the rate of generation of phenol was determined at 270 nm at room temperature.[5,7] PON1 activity towards paraoxon was quantified spectrophotometrically in 50 mM HEPES buffer (pH 7.4) containing 2 mM $CaCl_2$ and 1 mM paraoxon.[5] One unit of enzyme activity is expressed as one micromole of product generated per min.

Purification of PON1

PON1 was purified from human plasma through (pseudo) affinity chromatography using Cibacron Blue 3GA, anion-exchanger chromatography using DEAE Sephacel column, gel chromatography employing Sephacryl S-200-HR column, and finally affinity chromatography using concanavalin-A sepharose column according to a slight modification of the published procedures.[5,7] The identification of PON1 from human plasma had been reported previously.[12] The purified enzyme, which showed a M.W. of ~45 kDa, homogeneous on SDS-PAGE, and a mass (m/z) of 45161.88 in MALDI-TOF mass spectrometry (Voyager DE-RP mass spectrometer; Framingham, MA, USA), possessed a specific activity of approximately 721 and 0.391 μmol/min/mg protein in hydrolysis of phenyl acetate and paraoxon, respectively. Separately, when the phenotype of PON1 was determined by the dual substrate method as described previously,[27] the purified PON1 was found to belong to phenotype AB group.

Preparation of LDL or HDL

Serum LDL and HDL were isolated from fasted normolipidemic human volunteers by ultracentrifugation at a density ranging between 1.019–1.063 g/ml as described,[11] and dialyzed overnight against 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl at 4°C. Prior to the use, LDL (1 mg protein/ml) and HDL (6.2 mg protein/ml) were dialyzed against the above buffer and the same buffer containing 1 mM Ca^{2+}, respectively, at 4°C.

Effect of Various Oxidants on the Activity of Purified Paraoxonase1 (PON1)

PON1 (0.5 unit) was preincubated with each oxidant system at 38°C in 0.1 ml of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} for 10 min, and the aliquot (20 μl) was taken for the assay of remaining activity in the hydrolysis of phenylacetate. Separately, the oxidative inactivation was performed in PBS buffer (pH 7.4) containing 10 mM phosphate buffer and 150 mM NaCl. In this experiment, HEPES buffer was passed through Chelex 100 resin before use.

Inactivation of PON1 by Ascorbate/Cu^{2+} or Ascorbate/Fe^{2+}

PON1 (0.5 unit) was preincubated with ascorbate of various concentrations (0.01–0.5 mM) in the presence or absence of 1 μM Cu^{2+} at 38°C in 0.1 ml of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C for 10 min. Separately, the concentration-dependent effect of Cu^{2+} or Fe^{2+}, in combination with ascorbate, was examined by including Cu^{2+} (0.1–1.0 μM) and Fe^{2+} (0.25–2.0 μM) in the reaction mixture containing 0.5 mM ascorbate for 10 and 30 min, respectively.

Inactivation of PON1 by H_2O_2 in Combination with Cu^{2+} or Fe^{2+}

PON1 (0.5 unit) was incubated with H_2O_2 of various concentrations (0.1–1 mM) in the presence of 1 μM Cu^{2+} and 2 μM Fe^{2+} in 0.1 ml of HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C for 10 and 30 min, respectively.

Protective Effect of Antioxidant Enzyme, Radical Scavengers or Other Candidate Compounds on the Inactivation of PON1 by Ascorbate/Cu^{2+}

PON1 (0.5 unit) was preincubated with 0.5 mM ascorbate/1 μM Cu^{2+} in the presence of each radical scavenger or antioxidant enzyme at 38°C in 0.1 ml of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+}, and 10 min later an aliquot (20 μl) was taken for the assay of remaining activity. Separately, the ascorbate/Cu^{2+}-induced inactivation was performed in the presence of each candidate compound.

Oxidative Inactivation of HDL-PON1

HDL-PON1 was exposed to various oxidant systems as done with purified PON1. Additionally, 0.5 units of HDL-PON1 (0.05 mg protein) was incubated with ascorbate (0.5 mM) and Cu^{2+} (2 μM) in 0.1 ml of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C for various times (0–30 min). Similarly, HDL-PON1 was incubated with ascorbate (0.5 mM) in combination with each metal ion of various concentrations (0.5–4 μM) as described in Figure legends. Separately, HDL-PON1 was incubated with Cu^{2+} (1–10 μM) as described above.

Inactivation of PON1 by Hydroperoxides or Lipid Aldehydes

PON1 (0.5 unit) was preincubated with each hydroperoxide in 0.1 ml of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C for various times (0–60 min). Separately, 4-hydroxy-2-nonenal or acrolein was incubated with PON1 in the same buffer containing 0.3 mM Ca^{2+} for various times (0–15 h), and an aliquot was taken for the assay of remaining activity. The concentration-dependent effect of 4-hydroxy-2-nonenal was examined by incubating PON1 with various concentrations (0.1–1 mM) of 4-hydroxy-2-nonenal for 15 h.

Change of LDL Oxidation and PON1 Activity During the Incubation of LDL with Cu^{2+} in the Presence of PON1 of Different Amounts

LDL (0.1 mg protein/ml) was exposed to Cu^{2+} (10 μM) in the presence of PON1 (5–40 units/ml) in 0.2 ml of 10 mM PBS buffer (pH 7.4) containing 0.3 mM Ca^{2+} at 38°C for 3 h, and then an aliquot was taken for the assay of remaining activity and for the determination of TBARS value as reported previously.[11,15]

Preparation of Native PON1, Ascorbate/Cu^{2+}-inactivated PON1, or PHMB-inactivated PON1

Ascorbate/Cu^{2+}-inactivated PON1 and PHMB-inactivated PON1 were prepared by exposing of PON1 (25 μg/ml) to ascorbate (0.5 mM)/Cu^{2+} (1 μM) or PHMB (0.5 mM), respectively, in 10 ml of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C until PON1 activity decreased to $<10\%$ level of control. Then, the inactivated enzyme and native enzyme were extensively dialyzed in 25 mM Tris buffer (pH 7.4) containing 1 mM Ca^{2+} at 4°C for 20 h, and then freeze-dried.

Protective Action of Native PON1, Ascorbate/Cu^{2+}-inactivated PON1, or PHMB-inactivated PON1 Against LDL Oxidation

LDL (0.1 mg protein/ml) was exposed to Cu^{2+} (10 μM) in the presence of each inactivated PON1 or native PON1 (28 or 41 μg protein/ml) in 0.2 ml of 10 mM PBS buffer (pH 7.4) containing 0.3 mM Ca^{2+} at 38°C for 3 h, and then an aliquot was taken for the determination of TBARS value.

Amino Acid Analyses of Ascorbate/Cu^{2+} -inactivated PON1

Ascorbate/Cu^{2+} -inactivated PON1 was prepared as described above, and after extensive dialysis against cold distilled water, was subjected to amino acid analysis. Amino acid analyses were done by Pico-Tag method using automated amino acid analyzer (Waters Pico Tag HPLC system, Milford, MA) at the Korea Basic Science Center (Taejon, Korea). For the determination of cysteine content, the sample was oxidized with a mixture of formic acid/H_2O_2 (19:1, v/v), and for the tryptophan determination, the sample digestion was done in the presence of 4 M methanesulfonic acid.[28,29]

Quantitative Determination of Cysteine Residue in Ascorbate/Cu^{2+}- or PHMB-inactivated PON1

Ascorbate/Cu^{2+}-inactivated PON1 and PHMB-inactivated PON1 were prepared as described above, extensively dialyzed in 25 mM Tris buffer (pH 7.4) containing 1 mM Ca^{2+} for 24 h, and then freeze-dried. The respective PON1 (final cnoncentration, 13 μM) was included in 50 mM Tris buffer (pH 7.8) containing 0.5% Tergitol, 2 M guanidine, and 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) [DTNB]. The amount of thiol was determined by measuring the optical density at 412 nm as described previously.[30]

RESULTS

Susceptibility of PON1 to Oxidants

In an experiment to examine the possible oxidative inactivation of the enzyme, PON1 was subjected to various oxidation systems in 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C for 10 min, and the remaining activity was determined on the basis of the hydrolysis of phenylacetate as a substrate (Table I). First, the 10 min-exposure of PON1 to 1 mM H_2O_2, 1 mM 3-morpholinosydnonimine (peroxynitrite generator), or 1 mM sodium hypochlorite (HOCl donor) caused a slight inactivation (5–15% inactivation). Noteworthy, the combination of Cu^{2+} (1 μM) and ascorbate (0.5 mM) showed > 90% inactivation whereas ascorbate (0.5 mM) and Cu^{2+} (1 μM) exhibited approximately 16 and 8% inactivation of PON1, respectively. These indicate that PON1 is susceptible to Cu^{2+}-catalyzed oxidation. Separately, when the oxidative inactivation of PON1 was performed in PBS buffer, pH 7.4 (10 mM phosphate + 0.15 M NaCl) containing 50 μM Ca^{2+}, a similar inactivation degree was observed with each oxidant system except sodium hypochlorite, which demonstrated approximately 42 and 76% inactivation of PON1 at 0.3 and 1 mM, respectively. Additionally, the inactivation of PON1 by ascorbate/Cu^{2+} was smaller in PBS buffer containing 50 mM phosphate than 10 mM phosphate. Taken together, it is suggested that among the oxidation systems tested, ascorbate/Cu^{2+} system is the most potent in inactivating PON1.

TABLE I Effect of various oxidants on PON1 activity

Treatment	Concentration	Enzyme activity (%)
Control		100
Ascorbate	0.5 mM	81.2 ± 2.1
Ascorbate/Cu^{2+}	0.5 mM/1 μM	8.7 ± 1.7
	0.5 mM/1 μM	11.2 ± 3.7*
Dehydroascorbate/Cu^{2+}	1 mM	86.2 ± 3.5
Hydrogen peroxide	2 mM	95.0 ± 3.5
Sodium hypochlorite	1 mM	85.2 ± 2.3
	1 mM	24.3 ± 3.6*
	0.3 mM	58.6 ± 3.9*
	0.1 mM	81.4 ± 4.6*
3-Morpholinosydnonimine	1 mM	90.3 ± 3.8

PON1 (5 units/ml) was incubated with each oxidant system at 38°C in 0.1 ml of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+}, and 10 min later, an aliquot was taken for the assay of remaining activity. Results are the mean ± SD of three assays. *Oxidative inactivation in the PBS buffer.

Metal Ion-catalyzed Oxidative Inactivation of PON1

In the subsequent experiment, the metal-catalyzed oxidative inactivation of PON1 was extensively examined. When PON1 at 5 units/ml (0.156 μM) was incubated with ascorbate (0.5 mM) and Cu^{2+} (1 μM) at 38°C, the activity decreased in a time-dependent pattern; after 10 min, the remaining activity was below 10% of control value (Fig. 1A). Further study showed that the inactivation of PON1 by ascorbate/Cu^{2+} differed according to the amount of PON1 protein used; the increase of PON1 amount to 20 units/ml (0.62 μM) and 40 units/ml (1.25 μM) led to 56 and 22% inactivation, respectively. Since the maximal inactivation of PON1 by ascorbate (0.5 mM) alone was limited to ~18% (Fig. 1A), it was supposed that Cu^{2+} might play a catalytic role in the generation of inactivating species. In this regard, the concentration-dependent effect of Cu^{2+} on PON1 was examined by exposing PON1 to various concentrations (1–10 μM) of Cu^{2+}. Because the inactivation of PON1 by Cu^{2+} at low concentrations (1 or 2 μM) during 10 min incubation was small (<10%), the incubation time was extended to 20 min. As Fig. 1 indicates, PON1 was inactivated by Cu^{2+} in a concentration-dependent manner with a biphasic pattern of inactivation, suggesting that the mechanism for the inactivation of PON1 by Cu^{2+} alone may differ according to the concentration of Cu^{2+}. From the interpolation of the Cu^{2+}-inactivation curve, the IC_{50} values for higher affinity phase and lower affinity phase of inactivation were estimated to be approximately 2 and 7 μM, respectively.

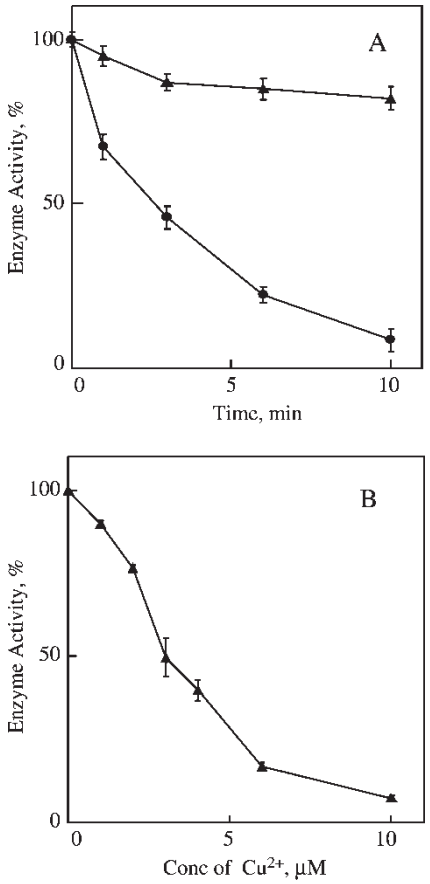

FIGURE 1 Time-dependent inactivation of PON1 by ascorbate/Cu^{2+}, ascorbate or Cu^{2+}. A. PON1 (5 units/ml) was incubated with 0.5 mM ascorbate in the presence or absence of 1 μM Cu^{2+} at 38°C in 0.1 ml of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+}, and 1, 3, 6, or 10 min later, an aliquot was taken for the assay of remaining activity: ▲, Ascorbate alone; ●, Ascorbate/Cu^{2+}. B. PON1 (5 units/ml) was incubated with various concentrations (1–10 μM) of Cu^{2+} for 20 min at 38°C in 0.1 ml of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+}. Data are expressed as a mean ± SD (bar) value of triplicate assays, presented as a percentage of control activity.

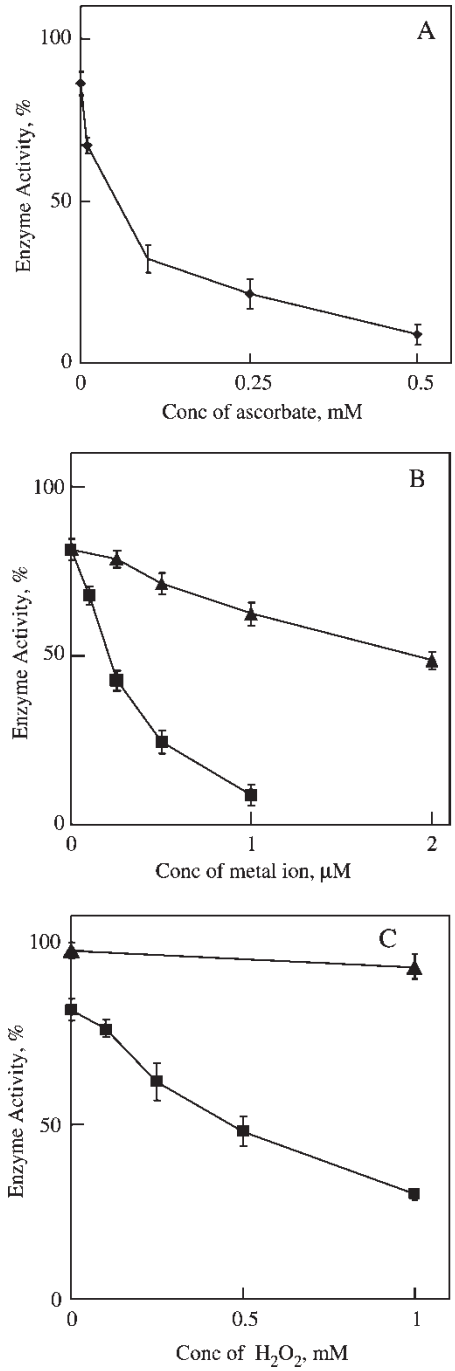

FIGURE 2 Oxidative inactivation of PON1 by ascorbate/metal ion or H_2O_2/metal ion. A. PON1 (5 units/ml) was incubated with various concentrations (0.01–0.5 mM) of ascorbate in the presence of 1 μM Cu^{2+} in 0.1 ml of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C for 10 min. B. PON1 (5 units/ml) was incubated with 0.5 mM ascorbate in combination with Cu^{2+} (0.1–1 μM) or Fe^{2+} (0.25–2 μM) as described above; ▲, 30 min incubation with Fe^{2+}; ■, 10 min incubation with Cu^{2+}. C. PON1 (5 units/ml) was incubated with various concentrations (0.1–1 mM) of H_2O_2 in combination with 1 μM Cu^{2+} or 2 μM Fe^{2+} as described above; ▲, 30 min incubation with Fe^{2+}; ■, 20 min incubation with Cu^{2+}. Data are expressed as a mean ± SD (bar) value of triplicate sets.

Next, we attempted to find the minimal concentration of ascorbate, in combination with Cu^{2+}, required for the efficient inactivation of PON1 during 10 min incubation. When the concentration of ascorbate was varied from 0.01 to 0.5 mM with the Cu^{2+} concentration being fixed at 1 μM, a concentration-dependent effect of ascorbate was expressed (Fig. 2A); in combination with Cu^{2+}, ascorbate at 10 and 100 μM showed 30 and 70% inactivation, respectively. Thus, ascorbate at relatively low concentrations was successful to promote the redox cycle of copper ions. However, the effect of ascorbate could not be replaced by thiols such as mercaptoethanol. To see the selectivity of metal ions in enhancing the ascorbate action, Cu^{2+} or Fe^{2+} was incubated with PON1 in the presence of ascorbate (0.5 mM) during 10 min incubation. As exhibited in Fig. 2B, Cu^{2+} (0.01–1 μM), in combination with ascorbate (0.5 mM), demonstrated a concentration-dependent inactivation of PON1 with a maximal inactivation of >90%. Meanwhile, at most 50% inactivation was achievable with 2 μM Fe^{2+} in combination with 0.5 mM ascorbate.

TABLE II Protective effect of radical scavengers, antioxidant enzyme or other compounds on the inactivation of PON1 by ascorbate/Cu^{2+}

Treatment	Concentration	Enzyme activity (%)
Control		100
Ascorbate / Cu^{2+}	0.5 mM/1μM	8.6 ± 2.2
+ Catalase	326 unit/ ml	97.1 ± 2.8
+ Benzoate	1.0 mM	11.9 ± 0.5
+ Mannitol	0.2 M	11.6 ± 0.8
+ Dimethyl sulfoxide	0.2 M	73.6 ± 2.9
+ Phenyl acetate	5.0 mM	13.6 ± 1.1
+ Paraoxon	1.0 mM	11.2 ± 2.3
+ Ca^{2+}	5.0 mM	21.5 ± 2.5
+ Zn^{2+}	20 μM	12.2 ± 4.8
+ Mercaptoethanol	50 μM	82.9 ± 4.2
+ Imidazole	1.0 mM	65.4 ± 3.5

PON1 (5 units/ml) was incubated with 0.5 mM ascorbate and 1 μM Cu^{2+} in the presence of each radical scavenger, antioxidant enzyme or candidate protectant at 38°C in 0.1 ml of HEPES buffer (pH 7.4) containing 50 μM Ca^{2+}, and 10 min later, an aliquot was taken for the assay of remaining activity. Results are the mean ± SD of three assays.

The enhancement of Fe^{2+} concentration beyond 2 μM failed to further augment the inactivation degree. Thus, Cu^{2+} was more effective than Fe^{2+} in a concert with ascorbate. Next, in an attempt to define the mechanism by which ascorbate, in combination with Cu^{2+}, inactivates the PON1, catalase was tested for the protective action. As indicated in Table II, catalase expressed a great (>90%) protection, suggesting that the ascorbate/Cu^{2+}-mediated inactivation may involve the generation of H_2O_2. In an endeavor to substantiate the above suggestion, PON1 was incubated with H_2O_2 at various concentrations (0.1–1.0 mM) in the presence of Cu^{2+} (1 μM) or Fe^{2+} (2 μM). Figure 2C demonstrates that Cu^{2+}, in combination with H_2O_2, inactivated PON1 remarkably in a concentration-dependent manner, in a good agreement with Cu^{2+}-catalyzed oxidation employing hydroxyl radicals as inactivating species. Meanwhile, H_2O_2/Fe^{2+} system demonstrated no significant inactivation under the present condition. In related study, where hydroxyl radical scavengers[31,32] such as mannitol, benzoate, or ethanol were tested for the prevention against the ascorbate/Cu^{2+}-induced inactivation of PON1, no significant protection was exhibited by those scavengers. Meanwhile, dimethylsulfoxide,[32] which is known to be oxidized by metal ions-bound hydroxyl radicals, expressed approximately 70% protection at 0.2 M (Table II). Taken together, these might support the view[31,32] that Cu^{2+}-bound hydroxyl radicals may be responsible for the oxidative inactivation of PON1.

Based on these data, it was assumed that the inactivation of PON1 by Cu^{2+} alone (Fig. 1B) might be due to metal-catalyzed oxidation employing some amino acid residues as reducing agent. To verify this notion, PON1 was exposed to Cu^{2+} (1 μM) in the presence of catalase (326 units/ml) or DMSO (200 mM). Of note, the inactivation by Cu^{2+} at 1 μM was prevented almost fully (> 90%) by catalase or dimethylsulfoxide (data not shown), suggesting that the inactivation of PON1 by Cu^{2+} at low concentrations may occur according to the mechanism for H_2O_2/Cu^{2+}-catalyzed oxidation. Meanwhile, the inactivation by Cu^{2+} at a higher concentration (10 μM) was prevented partially (<20%) by either catalase or DMSO.

Generally, metal-catalyzed oxidative inactivation of some enzymes, where the metal-binding sites exist at the active site, is prevented by their substrates or competitive inhibitors. In this respect, phenylacetate and paraoxon, substrates of PON1, were tested for the protective action against the ascorbate/Cu^{+2}-induced inactivation. However, phenylacetate and paraoxon exhibited no remarkable protection (<10%) at concentrations used (Table II), implying that the Cu^{2+}-binding site may be somewhat away from the active center of PON1. Although Ca^{2+}, necessary for the catalysis of PON1,[5] showed a slight protection at 5 mM, the effective concentration of Ca^{2+} was far above that required for the catalysis, and moreover, the maximal protection was less than 20%. In addition, Zn^{2+}, which is known to compete with Cu^{2+} in some proteins,[33,34] was without effect. Next, when mercaptoethanol or imidazole, which can interact with ROS, was tested for the prevention against the inactivation of PON1 by ascorbate/Cu^{2+}, both mercaptoethanol (50 μM) and imidazole (1 mM) were found to show a prominent protection. These results led to the surmise that the primary target amino acid for Cu^{2+}-catalyzed oxidation might be histidine or cysteine residue.

To identify the amino acid residue, in PON1 molecule, susceptible to the oxidation by ascorbate/Cu^{2+}, PON1 was first exposed to ascorbate/Cu^{2+}, and then after extensive dialysis and freeze-drying, subjected to amino acid analyses. Table III indicates that the treatment with 0.5 mM ascorbate and 2 μM Cu^{2+} caused the loss of approximately 31%, corresponding to at least three histidine residues, of total 12 histidine residues present in human PON1. Meanwhile, the amount of the other amino acid residues, generally known

TABLE III Amino acid analyses of ascorbate/Cu^{2+}-inactivated PON1

Amino acid residue	Ratio of oxidized PON1/Native PON1 (%)
Arginine	106.0
Lysine	98.4
Tyrosine	99.5
Methionine	111.8
Tryptophan	113.6
Histidine	69.2
Cysteine	111.1

TABLE IV Quantitative determination of cysteine residue in ascorbate/Cu^{2+} - or PHMB-inactivated PON1

Type of PON1	Relative amount of cysteine residue (%)
Native PON1	100
Ascorbate/Cu^{2+}- inactivated PON1	112.1 ± 10.7
PHMB-inactivated PON1	< 5

Native PON1, ascorbate/Cu^{2+}-inactivated PON1 and PHMB-inactivated PON1 were prepared, and subjected to thiol determination as described in Materials and Methods. To the respective PON1 (final concentration, 13 μM) in 50 mM Tris buffer (pH 7.8) was added DTNB (final concentration, 1 mM). The amount of thiol was determined by measuring the absorbance at 412 nm.

to be sensitive to oxidation, was not reduced significantly. In a separate analysis, there was no evidence for the alteration in the amount of cysteine residue, reduced form, present in PON1 molecule. To provide more supportive data, the cysteine residue of either ascorbate/Cu^{2+} -inactivated PON1 or PHMB-inactivated PON1 was quantitated as described previously.[30] Table IV indicates that the amount of cysteine residue of ascorbate/Cu^{2+}-inactivated PON1 was almost the same as that of native PON1, while the cysteine residue of PHMB-inactivated PON1 was found to be almost fully modified.

Oxidative Inactivation of HDL-PON1

After establishing the oxidative inactivation of purified PON1, we turned to the oxidative inactivation of HDL-PON1. First, when HDL-PON1 (5 units/ml) was incubated with 0.5 mM ascorbate and 2 μM Cu^{2+} at 38°C, the PON1 activity decreased in a time-dependent pattern; the activity decreased to approximately 30 and 10% of control value after 10 min and 30 min, respectively (Fig. 3A), in contrast to ~ 82% activity remaining after 30 min exposure to ascorbate (0.5 mM) alone. Thus, HDL-PON1 was also found to be sensitive to Cu^{2+}-catalyzed oxidation, although to a lower extent than purified PON1. In an independent experiment, where the susceptibility of HDL-PON1 to Cu^{2+} alone was examined, HDL-PON1 (5 units/ml) was incubated with Cu^{2+} of various concentrations (0–10 μM) for 30 min. As demonstrated in Fig. 3B, the dose-dependent inactivation of HDL-PON1 by Cu^{2+} was found to be similar to the observation with purified PON1 (Fig. 1B). In comparison, HDL-PON1 seems to be less susceptible to Cu^{2+}, compared to purified PON1, since the former took a longer incubation time than the latter. To see the mechanism responsible for the inactivation of HDL-PON1 by Cu^{2+} alone, Cu^{2+}-mediated inactivation was examined in the presence of catalase or DMSO. Of note, the inactivation by Cu^{2+} at a low concentration (2 μM) was prevented almost fully (>90%) by either catalase (326 units/ml) or DMSO (200 mM), suggesting that the inactivation of HDL-PON1 by Cu^{2+} at low concentrations follows the same mechanism as proposed for the inactivation of purified PON1. In related experiment, HDL-PON1 was exposed to Cu^{2+} or Fe^{2+} in combination with 0.5 mM ascorbate for 30 min (Fig. 3 C). Again, Cu^{2+} (1 or 2 μM), combined with acorbate, was sufficient to inactivate HDL-associated PON1, but Fe^{2+} was less efficient than Cu^{2+}. Separately, when HDL-PON was exposed to other oxidants, HDL-PON1 was observed to be as sensitive to HOCl (1 mM) as purified PON1 (data not shown). However, hydrogen peroxide (1 mM) or peroxynitrite generator (1 mM) failed to inactivate HDL-PON1.

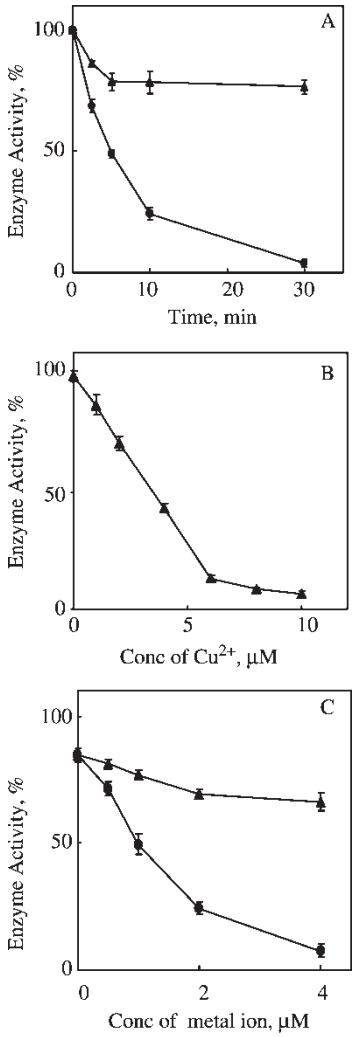

FIGURE 3 Oxidative inactivation of HDL-PON1. A. HDL -PON1 (5 units/ml) was incubated with 0.5 mM ascorbate and 2 μM Cu^{2+} in 0.1 ml of HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C, and 2.5, 5, 10 or 30 min later, an aliquot was taken for the assay of remaining activity: ▲, Ascorbate alone; ●, Ascorbate/Cu^{2+}. B. HDL-PON1 (5 units/ml) was incubated with various concentrations (1–10 μM) of Cu^{+2} for 30 min as described above. C. HDL-PON1 (5 units/ml) was incubated with 0.5 mM ascorbate in combination with Cu^{2+} or Fe^{2+} (0.5–4 μM) in 0.1 ml of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C for 30 min: ▲, $Fe^{2+;}$ ●, Cu^{2+}. Data are expressed as a mean ± SD (bar) value of triplicate sets.

Inactivation of PON1 by Hydroperoxides or α, β-Unsaturated Aldehydes

Subsequently, we turned to the study on the inactivation of PON1 by hydroperoxides, such as cumene hydroperoxide or *tert*-butyl hydroperoxide. As demonstrated in Fig. 4A, cumene hydroperoxide at 2 mM showed a time-dependent inactivation of PON1 during incubation at 38°C; after 1 h exposure to cumene peroxide, the PON1 activity decreased to approximately 74% of control, similar to the activity (68%) remaining after the exposure to H_2O_2 (2 mM). Meanwhile, *tert*-butyl hydroperoxide at 2 mM expressed no inactivation of PON1. Thus, the inactivating potency differed according to the structure of hydroperoxides. Additionally, the inclusion of Cu^{2+} at 2 μM failed to enhance cumene hydroperoxide-induced inactivation of PON1 after 1 h exposure, excluding the possibility that alkoxyl radicals may be responsible for the inactivation of PON1. Next, we turned to the possible inactivation of PON1 by lipid aldehydes, which are produced during Cu^{2+}-mediated lipid peroxidation, since some electrophilic lipid aldehydes were reported to participate in the protein modification.[35,36] When PON1 was exposed to various aldehydes (1 mM), such as acrolein, *trans*-2-hexenal, 4-hydroxy-2-nonenal, or malondialdehyde, for 12 h at 38°C, a remarkable inactivation was observed with only 4-hydroxynonenal and acrolein, which exhibited approximately 83 and 20% inactivation, respectively. In further study, 4-hydroxy-2-nonenal (1 mM) expressed a time-dependent inactivation of PON1 (Fig. 4B). Under the same condition, the 50% inactivating concentration of 4-hydroxy-nonenel was estimated to be around 0.3 mM.

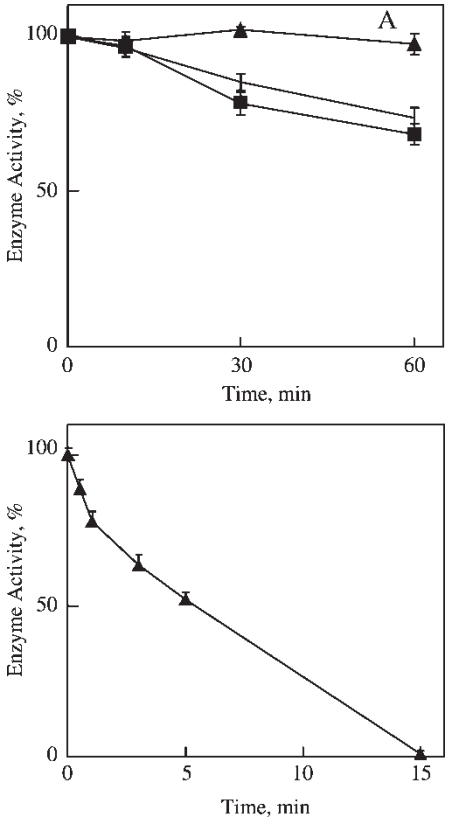

FIGURE 4 Inactivation of PON1 by hydroperoxides or 4-hydroxy-2-nonenal. A. PON1 (5 units/ml) was incubated with 2 mM hydrogen peroxide (■), cumene hydroperoxide (◆) or *tert*-butyl hydroperoxide (▲) in 0.1 ml of HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C, and 10, 30 or 60 min late, an aliquot was taken for the assay of remaining activity. B. PON1 (5 units/ml) was exposed to 4-hydroxy-2-nonenal (1 mM) for a long incubation time (1–15 h) in 0.1 ml of HEPES buffer (pH 7.4) containing 0.3 mM Ca^{2+} at 38°C. Data are expressed as a mean ± SD (bar) value of triplicate sets.

Relationship between PON1 Inactivation and Prevention against LDL Oxidation

To see how the oxidative inactivation of PON1 affects its protective action against LDL oxidation, Cu^{2+}-catalyzed oxidation of LDL was performed in the presence of PON1. In this experiment, LDL was exposed to 5 μM Cu^{2+} in the presence of PON1 (5–40 units/ml), and 3 h later an aliquot was taken for the determination of LDL oxidation and PON1 inactivation. As shown in Fig. 5, native PON1 at 5 units/ml (0.156 μM) showed a slight protection (~10%) against LDL oxidation, and the remaining PON1 activity was less than 5% of control. Meanwhile, the enhancement of PON1 amount up to 40 units/ml (1.25 μM) led to an almost full (>90%)

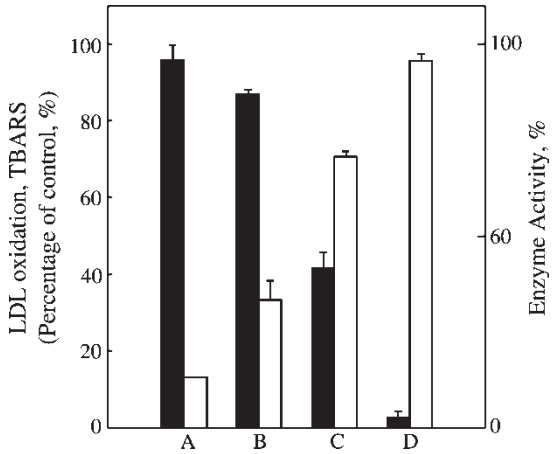

FIGURE 5 Change of LDL oxidation and PON1 activity during exposure of LDL to Cu^{2+} in the presence of PON1 of various amounts. LDL (0.1 mg protein/ml) was exposed to Cu^{2+} (10 μM) in the presence of PON1 (5–40 units/ml) in 0.2 ml of 10 mM PBS buffer (pH 7.4) containing 0.3 mM Ca^{2+} at 38°C for 3 h, and then an aliquot was taken for the assay of remaining activity and for the determination of TBARS value. Data are expressed as a mean ± SD (bar) of triplicate sets, presented as percentage of control value. LDL oxidation (closed bars); Remaining PON1 activity (open bars). A, 4 units/ml; B, 10 units/ml; C, 20 units/ml; D, 40 units/ml.

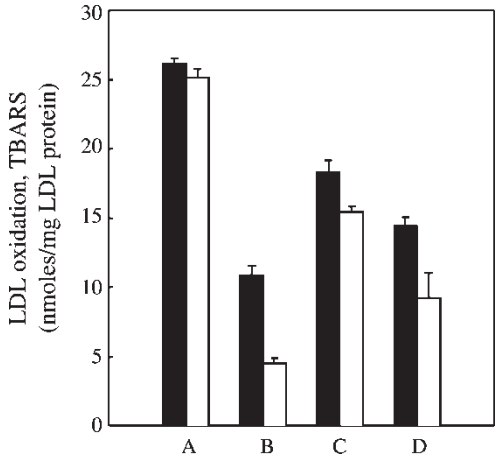

FIGURE 6 Protective action of native PON1, ascorbate/Cu^{2+}-inactivated PON1, or PHMB-inactivated PON1 against LDL oxidation. LDL (0.1 mg protein/ml) was exposed to Cu^{2+} (10 μM) in the presence of each type of PON1 (28 or 41 μg protein/ml) in 0.2 ml of 10 mM PBS buffer (pH 7.4) containing 0.3 mM Ca^{2+} at 38°C for 3 h, and then an aliquot was taken for the determination of TBARS value. A, without PON1; B, native PON1; C, ascorbate/Cu^{2+}-inactivated PON1; D, PHMB-inactivated PON1. Data are expressed as a mean ± SD (bar) of triplicate sets, presented as percentage of control value: 28 μg protein/ml (closed bars); 41 μg protein/ml (open bars).

protection against LDL oxidation, while the inactivation of PON1 being below 10%. Thus, there is a reverse relationship between the PON1 inactivation and the protection against LDL oxidation. These results led to the notion that once PON1 is inactivated during Cu^{2+}-catalyzed oxidation, it might exert a lower protective action against LDL oxidation. To test this possibility, three types of PON1, native PON1, ascorbate/Cu^{2+}-inactivated PON1, or PHMB-inactivated PON1, were prepared in sufficient amount, and tested for the protective action against Cu^{2+}-mediated LDL oxidation. As exhibited in Fig. 6, native PON1 at 30 units/ml, corresponding to 41 μg protein/ml (about 0.93 μM), showed a remarkable (approximately 82%) protection against Cu^{2+} (10 μM)-induced oxidation of LDL whereas ascorbate/Cu^{2+}-inactivated PON1 and PHMB-inactivated PON1 demonstrated about 39 and 63% protection against the oxidation of LDL, respectively. Noteworthy, ascorbate/Cu^{2+}-inactivated PON1 was much less effective than native PON1 in preventing against Cu^{2+}-induced LDL oxidation, suggesting that ascorbate/Cu^{2+}-induced inactivation of PON1 led to the diminishment of its antioxidant action.

DISCUSSION

The present study indicates that PON 1 may be among the enzymes highly susceptible to ascorbate/metal ion -mediated oxidation;[37–39] the concentration of ascorbic acid and Cu^{2+}, concomitantly required for effective inactivation of PON 1, is relatively low, and moreover, close to that in some pathological conditions.[40,41] The promoting action of ascorbate on the Cu^{2+}-mediated inactivation of PON1, and the prevention by catalase or DMSO against the inactivation of PON1 by ascorbate/Cu^{2+} are well consistent with the notion that Cu^{2+} -bound hydroxyl radical is a reactive oxidant intermediate responsible for the inactivation as well exemplified[37,38] in the Cu^{2+}-catalyzed oxidation system; the exposure of oxygen to Cu^{1+} produces superoxide radical, which is further converted to H_2O_2 in the presence of reducing agent, and then the reaction of H_2O_2 with Cu^{1+} generates Cu^{2+}-bound hydroxyl radicals.[37,38] This is further supported by the accelerating effect of Cu^{2+} on the H_2O_2-mediated inactivation of PON1. From these, it is supposed that PON1 possesses a Cu^{2+} -binding site of a high affinity. Cu^{2+}-catalyzed oxidation may also apply to the inactivation of PON1 by a low concentration (2 μM) of Cu^{2+} alone as evidenced from the protective action of either catalase or DMSO. Then, the higher affinity site of Cu^{2+} may be responsible for the redox cycle in the Cu^{2+}-catalyzed oxidation, probably employing some amino acid residues with reducing power. In addition, the ascorbate/Fe^{2+} system also showed a significant inactivation of PON1, suggesting that hydroxyl radicals, generated through redox cycle at sites other than Cu^{2+} -binding site, could also inactivate PON1, although to a lower extent. Furthermore, HDL-bound PON1 was inactivated by either ascorbate/Cu^{2+} or ascorbate/Fe^{2+}. Therefore, ROS, such as H_2O_2 or superoxide anions generated in the presence of Cu^{2+} or Fe^{2+} at submicromolar- or micromolar concentrations could cause the oxidative inactivation of HDL-PON1, which results in the reduction of antioxidant function of HDL *in vivo* system. The possible implication of copper ions in the atherosclerosis was suggested,[24] and an evidence for the possible role of iron ions in the promotion of atherosclerosis has been provided.[38,42]

The plausible targets for the inactivation by ascorbate/Cu^{2+} would be conserved histidine residues, responsible for the hydrolytic mechanism of PON 1.[43] Histidine residue had been reported to be target susceptible to Cu^{2+}-catalyzed oxidation owing to the high affinity of its imidazole ring for copper ions.[44,45] This may be supported by our result that some part of histidine residues in PON1 molecule was lost after the exposure to ascorbate/Cu^{2+} system. Meanwhile, the possibility that tryptophan residue, necessary for substrate binding in active center,[46] might be a target for Cu^{2+}-oxidation is not likely, since the inactivation of PON1 by ascorbate/Cu^{2+} was not prevented by phenyl acetate. Moreover, there was no change in the quantity of tryptophan residue after the exposure of

PON1 to ascorbate/Cu^{2+}. Although the cysteine residue is also known to be susceptible to the oxidation, the cysteine residue in PON1 was resistant to Cu^{2+}-catalyzed oxidation as revealed by amino acid analysis as well as thiol determination.

The partial inactivation of PON1 by cumene hydroperoxide seems to be in support of the previous report[11] that PON1 activity decreased partially during the incubation with phospholipid peroxide or cholesteryl ester hydroperoxide. However, the concentration of cumene hydroperoxide or hydrogen peroxide, required for a remarkable inactivation of PON1, is relatively high, and moreover, their inactivation degree is limited to at most 40%. Therefore, the inactivation of PON1 by peroxides *in vivo* system is less likely. It is well known that metal-catalyzed break-down of lipid peroxide leads to the formation of alkoxy radicals or stable lipid aldehydes.[47] Our present results suggest that α,β-unsaturated aldehydes such as 4-hydroxy-2-nonenal or acrolein, rather than alkoxy radicals, may be more likely involved in the loss of PON1 activity. Generally, cysteine or histidine residues, corresponding to nucleophiles, are good targets for electrophilic α,β-unsaturated aldehydes.[35] The higher inactivation by 4-hydroxy-2-nonenal, less electrophilic than acrolein, might be explained by the assumption that once bound to active site, 4-hydroxy-2-nonenal, more bulky than acrolein, might pose the greater steric hindrance than acrolein. Alternatively, 4-hydroxy-2-nonenal, more non-polar than acrolein, might show a higher affinity toward active site of PON1. Although the inactivating concentration of 4-hydroxy-2-nonenal was relatively high, it is possible that α,β-unsaturated aldehydes such as 4-hydroxy-2-nonenal, diffusible, could accumulate in lipoprotein membranes in a sufficient concentration to inactivate PON1.

Taken together, our data indicate that PON 1 may be inactivated in at least three ways; attack by hydroxyl radicals, direct oxidation by peroxides and alkylation by α,β-unsaturated aldehydes. In other words, PON1, present in relatively high concentration (~5 μM) in physiological system,[48] might scavenge these reactive compounds at least according to stoichiometric removal mechanism. However, most (~90%) of PON1 in blood belongs to HDL-associated form, which was observed to be resistant to peroxides or α,β-unsaturated aldehydes. Therefore, it is likely that hydroxyl radicals may be active species primarily responsible for the oxidative inactivation of PON1 *in vivo* system.

Previous data[11–13] showed that the prevention by PON1 against Cu^{2+}-catalyzed oxidation of LDL was accompanied by the inactivation of PON1. Reaffirming this, present study employing PON1 of various amounts indicates that there is a relationship between the inactivation of PON1 and the protection against LDL oxidation. Accordingly, at least some part of protective action of PON1 might be related to the removal of inactivating species. However, the concentration of PON1 used here corresponds to 0.16–1.3 μM, insufficient to scavenge ROS or lipid aldehydes on a molar basis. Then, this discrepancy might be explained by the assumption that there could be multiple scavenging sites in PON1 molecule. Alternatively, the action of PON1 may be mainly due to the interference of initial stage in LDL oxidation, where ROS, such as H_2O_2 or superoxide anions,[23,24] are produced.

Previous reports[11,13] showed that iodoacetamide- or PHMB-modified PON1, with cysteine residue modified, showed a diminished protection against LDL oxidation, suggesting the involvement of cysteine residue in antioxidant action of PON1.[11,13] This was confirmed by our present result that compared to native PON1, PHMB-inactivated PON1 showed a lower protection against Cu^{2+}-catalyzed LDL oxidation. In particular, the lower protection by ascorbare/Cu^{2+}-inactivated PON1, compared to native PON1, against Cu^{2+}-catalyzed LDL oxidation indicates that the histidine residue, a primary target of Cu^{2+}-catalyzed oxidation, in PON1 may be also important for the protective action of PON1 against LDL oxidation. Moreover, there are several histidine residues, compared to one reduced cysteine residue in the active site of PON1 molecule.[43,46] Although it is supposed that histidine residue and cysteine residue participate in the prevention against LDL oxidation through different mechanisms, it is also possible that both amino acid residues may cooperate with each other in exerting antioxidant action. In this regard, the exposure of PON1 to oxidative systems, which can modify both histidine residue as well as cysteine residue, may accelerate the loss of antioxidant action. Therefore, the oxidative stress, involving multiple oxidative processes, *in vivo* system might more likely promote the atherosclerosis as demonstrated with atherosclerotic apolipoprotein E-deficient mice[49] or coronary disease patients.[18] All these might be consistent with recent reports that it is crucial to preserve the PON1 activity to maintain the antioxidant action of PON1 *in vitro* as well as *in vivo* systems.[50–52] A further study remains to be performed on to find effective compounds capable of maintaining PON1 activity in the environment of oxidative stress.

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