Free Radical Research, April 2006; 40(4): 349–358

Broad-spectrum antioxidant peptides derived from His residue-containing sequences present in human paraoxonase 1

SU DUY NGUYEN[1], TAE-SOOK JEONG[2], MEE REE KIM[3], & DAI-EUN SOK[1]

[1]*College of Pharmacy, Chungnam National University, Yuseong-Ku, Taejon 305 764, South Korea,* [2]*Lipid metabolism Laboratory, Korea Research Institute of Bioscience and Biotechnology, Taejon 305 333, South Korea, and* [3]*Department of Food and Nutrition, Chungnam National University, Yuseong-Ku, Taejon 305 764, South Korea*

Accepted by Dr T. Grune

(Received 26 November 2005; in revised form 12 December 2005)

Abstract

Hydroxyl or peroxyl radicals and hypochlorous acid (HOCl) are known to cause the oxidation of lipoproteins. Here, we examined Cu^{2+}-binding property of paraoxonase 1 (PON1), and antioxidant actions of peptides, resembling His residue-containing sequences in PON1, against oxidations by Cu^{2+}, peroxyl radicals or HOCl. When Cu^{2+}-binding property of PON1 was examined spectrophotometrically, the maximal Cu^{2+} binding was achieved at 1:1 molar ratio of PON1: Cu^{2+}. Additionally, Cu^{2+}-catalyzed oxidative inactivation of PON1 was prevented by Ca^{2+}-depleted PON1 at 1:1 ratio, but not diethylpyrocarbonate (DEPC)-modified PON1, suggesting the participation of His residue in Cu^{2+}-binding. When His-containing peptides were examined for antioxidant actions, those with either His residue at N-terminal position 2 or 3, or His–Pro sequence at C-terminal remarkably prevented Cu^{2+}-mediated low density lipoprotein (LDL) oxidation and PON1 inactivation. Especially, FHKALY, FHKY or NHP efficiently prevented Cu^{2+}-induced LDL oxidation (24 h), indicating a tight binding of Cu^{2+} by peptides. In support of this, the peptide/Cu^{2+} complexes exhibited a superoxide-scavenging activity. Separately, in oxidations by 2,2′-azobis-2-amidinopropane hydrochloride or HOCl, the presence of Tyrosine (Tyr) or Cysteine (Cys) residue markedly enhanced antioxidant action of His-containing peptides. These results indicate that His-containing peptides with Tys or Cys residues correspond to broad spectrum antioxidants in oxidation models employing Cu^{2+}, 2,2′-azobis-2-amidinopropane hydrochloride (AAPH) or HOCl.

Keywords: *PON1, peptide, LDL, oxidation, Cu^{2+}, hydroxyl radicals*

Abbreviations: *LDL, low density lipoprotein; PON1, paraoxonase 1; ROS, reactive oxygen species; DPCH, diphenylcarbohydrazide; DEPC, diethylpyrocarbonate; HOCl, hypochlorous acid; Cys, Cysteine; Hys, Histdine; Tyr, Tyrosine; AAPH, 2,2′-azobis-2-amidinopropane hydrochloride; TBARS, thiobarbituric acid reactive substances*

Introduction

Paraoxonase1 (PON1), associated with high density lipoprotein (HDL) [1–3], was known to have the ability to hydrolyze organophosphates, aromatic carboxylic acid esters [1,4,5] and some lactone compounds [6,7]. Recent interest in the enzyme has arisen from the notion that the ability of PON1 to attenuate the oxidative modification of low density lipoprotein (LDL) or HDL may afford protection against atherosclerosis [3,8,9]. Animal model and clinical investigations have provided 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 [10–14]. Thus, PON1 has been considered to play an antiatherogenic role *in vivo*. Nonetheless, very recent studies [15–17] disapproved the lipase activity of PON1 and its antioxidant action against Cu^{2+} or

Correspondence: D.-E. Sok, College of Pharmacy, Chungnam National University, Yuseong-Ku, Taejon 305 764, South Korea.
Tel: 82 42 821 5940. Fax: 82 42 823 6566. E-mail: daesok@cnu.ac.kr

ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Taylor & Francis
DOI: 10.1080/10715760500534429

2,2′-azobis-2-amidinopropane hydrochloride (AAPH)-induced LDL oxidation. Therefore, the mechanism (s) for antiatherogenic action of PON1 has not been clarified.

According to previous studies [18–22], PON1 molecule has multiple functional properties; in addition to its hydrolytic activities, PON1 interacts with lipids such as phospholipids or fatty acids [18–21]. In addition, it possesses binding sites of metal ions such as Cd^{2+}, Hg^{2+} or Cu^{2+} [22]. Consistent with the above, PON1 was proposed to contain two Cu^{2+}-binding sites, based on the inactivation of PON1 by Cu^{2+}; a high affinity site showing a redox cycle of Cu^{2+}[23], and a low affinity site requiring $>5\,\mu M$ Cu^{2+}. Noteworthy, Cu^{2+}-catalyzed oxidative inactivation of PON1 was accompanied by the loss of His residues [23]. From this, it was supposed that His residue in PON1 molecule might be involved in the Cu^{2+}-binding in accordance with selective coordination of His residue with Cu^{2+} [24].

Meanwhile [25], human serum albumin has been known to play an antioxidant role in human blood as a chelator of metal ions as well as a free radical scavenger. In particular, the four amino acid peptide (Asp–Als–His–Lys, DAHK) occupying the N-terminus of human serum albumin constitutes a high-affinity binding site for Cu^{2+} [24,26]. A recent study [27] demonstrated that the synthetic peptide, DAHK, could prevent Cu^{2+}-induced oxidation of LDL. Such an antioxidant effect of DAHK was suggested to be ascribed to the Cu^{2+}-sequestrating action of DAHK as well as the superoxide dismutase-like activity of copper–DAHK complex. However, there has been no further study about antioxidant actions of DAHK against oxidations by peroxyl radicals or hypochlorous acid (HOCl), which had been reported to be scavenged by the peptides containing His residue [28,29]. Hereby, it was hypothesized that some His residue-containing sequences in PON1 molecule might be involved in the protections against various oxidation systems.

In this study, we synthesized the peptides resembling some His residue-containing sequences in PON1 molecule, and investigated their antioxidant actions against oxidation systems employing Cu^{2+}, AAPH or HOCl. Finally, we introduce representative antioxidant peptides expressing broad spectrum antioxidant activities.

Materials and methods

Materials

Asp–Ala–His–Lys (DAHK) was obtained from BACHEM Company (Bubendorf, Switzerland). Other peptides were synthesized by AnyGen Co., Ltd (Gwangju, Korea), and their structures were identified by LC/MS analyses. Other reagents including (AAPH), diphenylcarbohydrazide (DPCH) or diethylpyrocarbonate (DEPC) were obtained from Sigma-Aldrich Corp (St Louis, MO).

Purification of paraoxonase1

PON1 was purified from human plasma according to a slight modification of the published procedures [5,19,21]. The purified enzyme (phenotype AB group) possessed a specific activity of approximately 1018 μmol/min/mg protein in the hydrolysis of phenyl acetate

Assay of paraoxonase1

PON1 activity towards phenyl acetate was determined in 0.5 ml of 50 mM Tris (pH 8.0) containing 1 mM $CaCl_2$ and 10 mM phenyl acetate [5,19]. The rate of phenol formation was monitored at 270 nm, and one unit of arylesterase activity is defined as 1 μmol of phenyl acetate hydrolyzed per min

Preparation of LDL

LDL isolation was performed using sequential ultracentrifugation as described [30]. Briefly, LDL (density, 1.019–1.063 g/ml) was isolated from fresh plasma of normolipidemic human volunteers at 4°C by sequential flotation of the lipoproteins in KBr solutions containing 1 mM sodium EDTA. Subsequently, LDL was exhaustedly dialyzed against 10 mM PBS buffer, pH 7.4 (150 mM NaCl + 2.7 mM KCl) containing 200 μM EDTA at 4°C. For LDL oxidation, LDL was dialyzed against 10 mM PBS buffer at 4°C to remove EDTA. The protein concentration of LDL was determined using BCA method [31].

Preparation of Ca^{2+}-depleted PON1, ascorbate/Cu^{2+}-inactivated PON1 or DEPC-modified PON1

Ca^{2+}-depleted PON1 was prepared by extensive dialysis of purified PON1 against distilled water. Ascorbate/Cu^{2+}-inactivated PON1 was prepared by incubating PON1 (0.1 μM) with 2 μM Cu^{2+} and 0.5 mM ascorbate in 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+}[23]. Separately, DEPC-modified PON1 was prepared by exposing PON1 (0.1 μM) to DEPC (0.1–10 mM) in 50 mM MES buffer (pH 6.0) containing 50 μM $CaCl_2$ at 37°C for 30 min. Finally, the enzyme preparations were concentrated by lyophilization after exhaustive dialysis against distilled water.

Effect of Ca^{2+}-depleted PON1 or ascorbate/Cu^{2+}-inactivated PON1 on ascorbate/Cu^{2+}-induced PON1 inactivation

Purified PON1 (5 U/ml, 0.1 μM) was incubated with 0.5 mM ascorbate and 2 μM Cu^{2+} in the presence

of either Ca^{2+}-depleted PON1 or ascorbate/Cu^{2+}-inactivated PON1 (0.5, 1.0, 2.0 or 3 μM) in 0.1 ml of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C for 10 min, and then 20 μl aliquot was taken for assay of PON1 activity.

Effect of either Ca^{2+}-depleted PON1 or ascorbate/Cu^{2+}-inactivated PON1 on formation of Cu^{2+}/DPCH complex

The interaction of Cu^{2+} with PON1 was assessed using DPCH as metal colorimeter detector [32]. Ca^{2+}-depleted PON1 or ascorbate/Cu^{2+}-inactivated PON1 at various concentrations was incubated with 2 μM Cu^{2+} in 0.5 ml of 10 mM PBS buffer (pH 7.4), and 10 min later, DPCH (0.5 mM) was included into the mixture. The absorbance at 495 nm, related to the DPCH–Cu^{2+} complex formation, was determined against an appropriate PON and DPCH-containing blank.

Effect of DEPC on either PON1 activity or Cu^{2+} binding capability of PON1

PON1 (0.1 μM) was exposed to DEPC (0.1–10 mM) in 50 mM MES buffer (pH 6.0 + 1 mM $CaCl_2$) at 38°C for 30 min, and then 20 μl aliquot was taken for assay of PON1 activity. Separately, PON1 (2 μM) modified with DEPC (0.1–10 mM) was preincubated with 2 μM Cu^{2+} in 0.5 ml of PBS buffer (pH 7.4), and 10 min later, DPCH (0.5 mM) was included into the mixture. The inhibitory effect of DEPC-modified PON1 on the formation of DPCH–Cu^{2+} complex was determined as described above.

Protective action of peptides against Cu^{2+} or AAPH-mediated LDL oxidation

LDL (0.1 mg protein/ml) was incubated with 5 μM Cu^{2+} in the presence of each peptide (5 μM) in 10 mM PBS buffer at 37°C, and the formation of conjugated diene formation [33] was assessed by monitoring the change of absorbance at 234 nm for 24 h. Separately, LDL (0.1 mg protein/ml) was incubated with 5 μM Cu^{2+} or 5 mM AAPH in the presence of each peptide in 10 mM PBS buffer at 37°C for 3 h, and the production of thiobarbituric acid reactive substances (TBARS) was measured as previously described [34,35]. Additionally, the time-dependent effect of peptide on AAPH-induced LDL oxidation was examined by incubating LDL with 5 mM AAPH in the presence of each peptide (50 μM) in 0.2 ml PBS buffer for the designated time.

Effect of peptides on ascorbate/Cu^{2+}-induced inactivation of PON1

PON1 (5 U/ml, 0.1 μM) was incubated with 0.5 mM ascorbate and 2 μM Cu^{2+} in the presence of peptide (1–8 μM) in 0.1 ml HEPES buffer (pH 7.4) containing 50 μM $CaCl_2$ at 38°C, and 10 min later, 20 μl aliquot was taken for the assay of PON1 activity [23].

Superoxide-scavenging activity of peptide/Cu^{2+} complex

The assay was performed in 0.5 ml of 50 mM sodium carbonate (pH 10.2) containing 0.1 mM xanthine and 25 μM nitroblue tetrazolium (NBT) as described [36]. The reaction was initiated by adding xanthine oxidase (20 nM) to the above mixture containing each peptide/Cu^{2+} complex, which was prepared from preincubation of peptide (1 mM) with Cu^{2+} (1 mM) in 0.1 ml of HEPES buffer (pH 7.4) at 25°C. The increase in absorbance at 560 nm was monitored for 5 min.

Prevention by peptides against HOCl-induced inactivation of acetylcholinesterase

Eel acetylcholinesterase (0.25 U/ml) was incubated with 10 μM HOCl in 0.1 ml PBS buffer (pH 7.4) at 38°C in the presence of each peptide (10 μM), and 15 min later, 20 μl aliquot was taken for the remaining activity of acetylcholinesterase as described [37].

Statistical analyses

All statistical analyses were performed using a SPSS program for window. Statistical assessments were performed using ANOVA for the initial demonstration of significance at $p < 0.05$, followed by *post-hoc* Duncan's multiple-range test [38].

Results

Implication of His residue in Cu^{2+}-binding by PON1

Despite reports [22,23] that PON1 has Cu^{2+}-binding sites, PON1 (< 1 μM) failed to prevent Cu^{2+}-induced LDL oxidation. To further explore Cu^{2+}-binding property of PON1, we employed protein rather than LDL as target of Cu^{2+} oxidation. First, when inactive PON1, Ca^{2+}-depleted, was examined for the protection against Cu^{2+} (2 μM)/ascorbate (0.5 mM)-induced inactivation of PON1 in 50 mM HEPES buffer (pH 7.4 + 50 μM Ca^{2+}), it prevented oxidative inactivation of PON1 dose-dependently (Figure 1), achieving a maximal protection at 1:1 molar ratio of Cu^{2+}/inactive PON1. In contrast, ascorbate/Cu^{2+}-inactivated PON1, with some His residues being destructed [23], failed to show a significant protection. To provide a positive support for the above, the ability of PON1 to associate with Cu^{2+} was examined using DPCH, which was known to form a complex with free Cu^{2+} [32]. As shown in Figure 1, Ca^{2+}-depleted PON1 interfered with the formation of Cu^{2+}/DPCH complex with a maximal

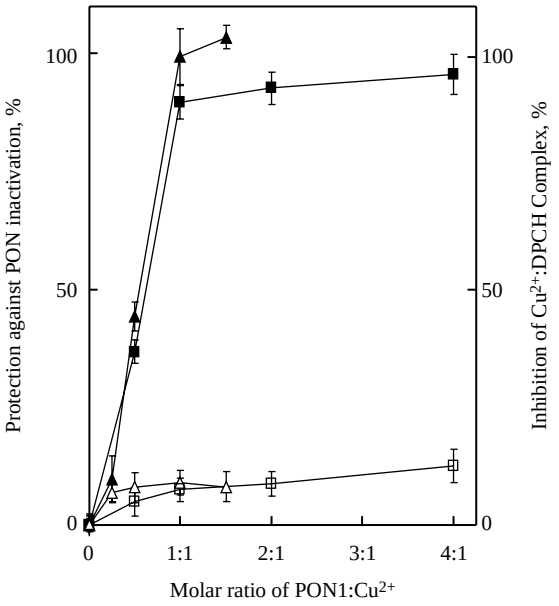

Figure 1. Effect of Ca^{2+}-depleted or ascorbate/Cu^{2+}-inactivated PON1 on ascorbate/Cu^{2+}-inactivated PON1 and formation of Cu^{2+}/DPCH complex. Purified PON1 (5 U/ml, 0.1 μM) was incubated with 0.5 mM ascorbate and 2 μM Cu^{2+} in the presence of Ca^{2+}-depleted PON1 (closed symbol) or ascorbate/Cu^{2+}-inactivated PON1 (open symbol) at various concentrations (0.5–3 μM) in 0.1 ml of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C for 10 min, and then 20 μl aliquot was withdrawn for assay of PON1 activity. Separately, Ca^{2+}-depleted PON1 or ascorbate/Cu^{2+}-inactivated PON1 (1–8 μM) was preincubated with 2 μM Cu^{2+} in PBS buffer, pH 7.4 for 10 min, and then the inhibitory effect of PON1 on Cu^{2+}/DPCH complex formation was determined as described in "Materials and methods" section. Results are expressed as a mean ± SD (bar) value of triplicate experiments. Triangle, protection against PON1 inactivation; Square, inhibition of Cu^{2+}/DPCH complex.

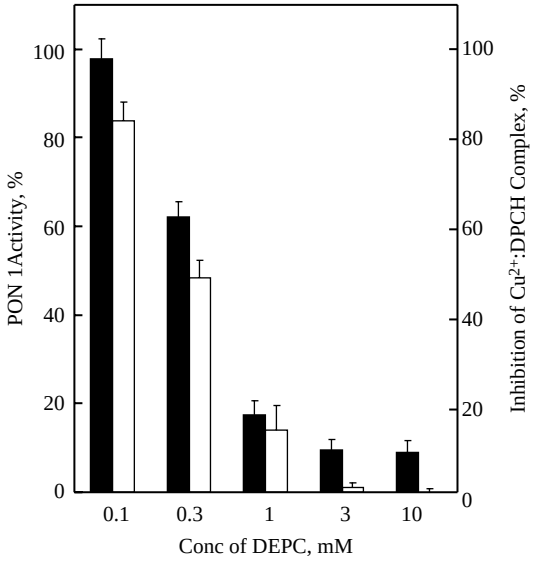

Figure 2. Effect of DEPC modification on cataltytic activity or Cu^{2+}-binding capability of PON1. PON1 (0.1 μM) was exposed to DEPC of various concentration (0.1–10 mM) in 50 mM MES buffer pH 6.0 containing 1 mM $CaCl_2$ at 38°C for 30 min, and then 20 μl aliquot was taken for assay of PON1 activity. Separately, DEPC-modified PON1 (2 μM) were preincubated with 2 μM Cu^{2+} in PBS buffer, pH 7.4 for 10 min and then the inhibition of Cu^{2+}/DPCH complex formation was determined as described in "Material and methods" section. Results are expressed as a mean ± SD (bar) value of triplicate experiments. □, PON1 activity; ■, inhibition of Cu^{2+}/DPCH complex.

inhibition at 1:1 molar ratio of Cu^{2+}/PON1. Again, ascorbate/Cu^{2+}-treated PON1 lost the capability of associating with Cu^{2+}. All these support, the idea that His residue is implicated in Cu^{2+}-binding. To further address this, PON1 was exposed to DEPC, a His residue modifier and then Cu^{2+}-binding ability and catalytic activity of PON1 were assessed. As shown in Figure 2, DEPC reduced Cu^{2+}-binding ability and catalytic activity of PON1 in a dose-dependent manner.

Antioxidant action of His-containing peptides against Cu^{2+}-induced LDL oxidation

From the above, it was supposed that the peptides of some His residue-containing sequence in PON1, rather than PON1 molecule, would be efficient in preventing Cu^{2+}-induced LDL oxidation. In this regard, the peptides, resembling some His residue-containing sequences in PON1 or their analogues were considered for the design of antioxidant peptides against LDL oxidation. First, when Cu^{2+}-induced LDL oxidation was performed in the presence of His-containing peptides with longer sequences (Table I), peptide I (VNHPDAK), II (LHLKTIRHKLL), III (PEHFYGTDHYFIDPYL) or peptide V (LAHKIHVYEKHA) showed a remarkable protection; I (72%), II (90%), III (85%) and V (53%). In contrast, the protective action of peptide IV (DPYLRSWEMYLG), VI-1 (LWVGCHPFGMKIFYD) or VII (TVFHKALY) was negligible (<10%). Thus, the peptides (I–III or V) with His residue at second or third position were more protective than those (VI-1 or VII) retaining His residue at other positions, similar to the observation with analogues of DAHK (84% protection), a representative antioxidant peptide [27]. Consistent with this, the removal of N-terminal Thr residue from peptide VII (TVFHKALY) and peptide VII-1 (TVFHKAL), generating peptide VII-2 (VFHKALY, 88%) and VII-3 (VFHKAL, 95%), respectively, led to a remarkable increase of antioxidant action as presented in Table I. Meanwhile, a similar potency between VFHKALY (88%) and VII-4 (FHKALY, 89%) indicates that His residue at position 2 is as important as that at position 3. Similarly, a remarkable protection by LHLKT, LWVGCHP, FHKALY, FHKY or DAHK was also reproduced when LDL oxidation was assessed (data not shown), based on HPLC assay of cholesteryl ester oxidation [39].

Table I. Effect of peptides on Cu^{2+}-mediated LDL oxidation or ascorbate/Cu^{2+}-induced PON1 inactivation.

No.	Sequence of peptide*	Protection (%)	
		LDL oxidation†	PON1 inactivation‡
	DAHK	84.2 ± 5.7^{c}	68.6 ± 5.8^{c}
I-1	NHP	88.8 ± 2.6^{c}	75.5 ± 1.5^{cde}
II-1	LHLKT	83.4 ± 4.7^{c}	79.6 ± 6.2^{de}
V-1	LAHKIHV	71.0 ± 2.9^{b}	71.5 ± 3.7^{cd}
VI-2	LWVGCHP	87.9 ± 1.8^{c}	78.0 ± 7.8^{de}
VI-3	GCHP	85.6 ± 3.1^{c}	88.9 ± 4.5^{fg}
VII-1	TVFHKAL	2.6 ± 0.2^{a}	11.1 ± 2.9^{a}
VII-2	VFHKALY	88.4 ± 0.4^{c}	43.2 ± 2.9^{b}
VII-3	VFHKAL	94.9 ± 6.8^{c}	50.3 ± 7.5 ^{b}
VII-4	FHKALY	89.2 ± 2.4^{c}	91.4 ± 4.7 ^{g}
VII-5	FHKAL	94.4 ± 5.6^{c}	90.8 ± 5.3^{fg}
VII-6	FHKY	89.9 ± 3.5^{c}	81.9 ± 5.6^{ef}
VII-7	FHK	72.3 ± 1.4^{b}	67.8 ± 4.6^{c}

* The above peptide fragments are derived from the following peptides with positions of amino acid sequence in human PON1; Peptide I (131–137), VNHPDAK/Peptide II (153–163), LHLKTIRHKLL/Peptide III (174–190), PEHFYGTDHYFIDPYL/Peptide IV (187–198), DPYLRSWEMYLG/Peptide V (240–251), LAHKIHVYEKHA/Peptide VI (279–294), LWVGCHPNGMKIFFYD/Peptide VII (344–361), TVFHKALY. † LDL (0.1 mg protein/ml) was incubated with 5 μM Cu^{2+} in the presence of peptide (5 μM) in 200 μl of 10 mM PBS buffer at 38°C for 3 h. LDL oxidation was measured by TBARS assay as described in "Materials and methods" section. ‡ PON1 (5 U/ml, 0.1 μM) was incubated with 0.5 mM ascorbate and 2 μM Cu^{2+} in the presence of peptide (5 μM) in 0.1 ml of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C for 10 min. Results are expressed as a mean ± SD value of triplicate experiments. Means with same letter are not significantly different ($p < 0.05$).

Separately, the removal of FGMKIFYD from peptide VI-1 (LWVGCHPFGMKIFYD), generating peptide VI-2 (LWVGCHP), also enhanced the antioxidant activity dramatically; peptide VI-1 (7%) vs peptide VI-2 (88%). However, the removal of LWV from peptide VI-2 (LWVGCHP), generating VI-3 (GCHP), did not further augment the antioxidant activity; GCHP (86%) vs LWVGCHP (88%). Thus, it is suggested that His–Pro sequence at C-terminal is as important as His residue at position 2 or 3 at N-terminal. This may be supported by higher activity of (VNHP, 97%), compared to (NPHG, 10%). Moreover, in case of tripeptide, His–Pro sequence is more contributory than His Lys sequence; I-1 (NHP, 89%) vs VII-7 (FHK, 72%). When the dose-dependent effect of representative peptides (I-1, VI-2, VII-2, VII-4, VII-5 and V-6) was investigated (data not shown), the molar ratio of peptide/Cu^{2+} for a remarkable protection (>85%) against LDL oxidation was estimated to be close to 1:1, indicative of a stoichiometric interaction between Cu^{2+} and peptides. In further study (Figure 3) monitoring the oxidation of LDL for 24 h, the peptides (I-1, V-1, VII-4 or VII-6) maintained antioxidant action (Figure 1). However, peptide VI-2 started to lose antioxidant action after 3 h, accompanied by the oxidation of Cys residue.

Protective action of peptides against ascorbate/Cu^{2+}-induced inactivation of PON1

Previously [20,23], PON1 activity was shown to be inactivated during Cu^{2+}-catalyzed oxidation. When Cu^{2+} (2 μM)/ascorbic acid (0.5 mM)-induced inactivation of PON1 was performed in the presence of each peptide (5 μM) for 10 min, a prominent protection (>43%) was shown by most of peptides containing His residue at position 2 or 3 (Table I).

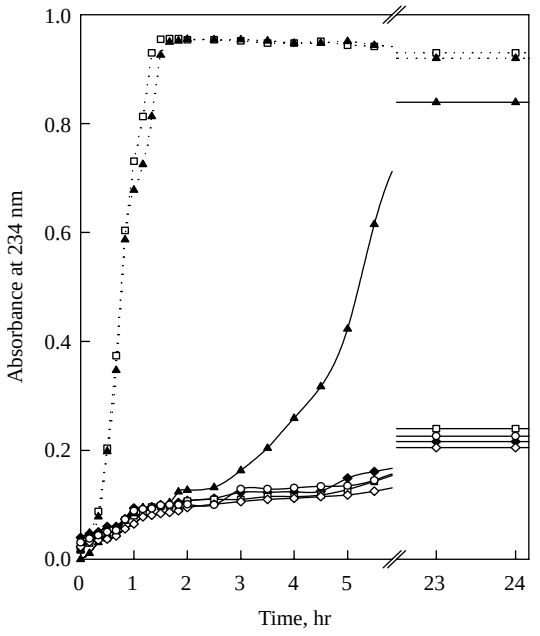

Figure 3. Effect of peptide on time-dependent oxidation of LDL by Cu^{2+}. LDL (0.1 mg protein/mg) was incubated with 5 μM Cu^{2+} in the presence of each peptide (5 μM) in 10 mM PBS buffer (pH 7.4). The time course for conjugated diene formation was monitored at 234 nm for 24 h. Results are the representative of duplicate experiments. □--□, control; ◆—◆, peptide I-1; ▲--▲, peptide IV; □—□, peptide V-1; ▲—▲, peptide VI-2; ◇—◇, peptide VII-4; ○—○, peptide VII-6.

Overall, the antioxidant effect of peptides in ascorbate/Cu^{2+}-induced inactivation of PON1 was similar to the finding with Cu^{2+}-induced oxidation of LDL. Noteworthy, the removal of Val residue at N-terminal of VFHKALY (VII-2) or VFHKAL (VII-3) augmented the protective action of peptides markedly; VFHKALY (43%) vs FHKALY (91%), and VFHKAL (50%) vs FHKAL (91%), somewhat different from the findings with Cu^{2+}-induced LDL oxidation. Thus, Val residue at N-terminal was a negative factor in the protection against PON1 inactivation. Again, the contributory role of His–Pro sequence was evidenced from higher activity of LWVGCHP (78%), compared to LWVGCHPFG-MKIFYD (16%). The Cys effect was also evidenced from greater efficacy of LWVGCHP than LWVGAHP (64%), and GCHP (89%) than GAHP (51%). Further, when the dose-dependent effect of peptides on Cu^{2+} (2 μM)/ascorbic acid-induced inactivation of PON1 was examined (Table II), the 50% protection (EC_{50}) value of LAHKIHV, LWVGCHP, FHKALY, FHKY or NHP ranged from 2.5–3.1 μM, close to that of DAHK (Table II), and a maximal protection (80–90%) was observed at approximately 1:2 molar ratio of Cu^{2+}/peptide. Separately, when the dose-dependent effect of peptides on the formation of complex between DCPH and Cu^{2+} (5 μM) was examined (Table II), 50% inhibitory concentration (IC_{50}) of peptides was between 3.6 and 5.3 μM, and the maximal inhibition (approximately 95%) was also found at 1:2 molar ratio of Cu^{2+}/peptide.

Superoxide dismutase-like activity of peptide/Cu^{2+} complex

The copper/peptide complexes had been observed to show superoxide dismutase activity [27]. In this respect, the copper ion complexes of representative peptides, which exhibited a prominent protection against Cu^{2+}-induced LDL oxidation were examined for superoxide-scavenging activity. As shown in Table III, at either 0.5 or 1 μM, each complex of Cu^{2+}/peptide (VI-2, VII-2 or VII-4) demonstrated greater superoxide-scavenging activities than Cu^{2+}: DAHK complex, which had been previously reported to show superoxide dismutase-like activity [27].

Table II. Effect of each peptide on ascorbate/Cu^{2+}-induced inactivation of PON1 and formation of Cu^{2+}/DPCH complex.

No.	Sequence of peptide	EC_{50} (μM) PON inactivation	IC_{50} (μM) Cu^{2+}/DPCH
	DAHK	2.8 ± 0.3	4.0 ± 0.3
I-1	NHP	2.7 ± 0.4	4.0 ± 0.2
V-I	LAHKIHV	3.1 ± 0.3	5.3 ± 1.6
VI-2	LWVGCHP	2.5 ± 0.2	3.9 ± 0.2
VII-4	FHKALY	2.6 ± 0.5	3.6 ± 0.1
VII-6	FHKY	2.9 ± 0.4	3.8 ± 0.2

Protective action of peptide against ascorbate/Cu^{2+}-induced inactivation of PON1 and their inhibitory effect on the formation of Cu^{2+}/DPCH complex was determined as described in Figure 1. EC_{50} and IC_{50} values are expressed as mean ± SD. EC_{50} and IC_{50} are expressed as concentrations to show 50% protection against ascorbate/Cu^{2+}-inactivated PON1 and 50% inhibitory effect on formation of Cu^{2+} DPCH complex, respectively.

Table III. Superoxide-scavenging activity of peptide/Cu^{2+}complex.

No.	Sequence of peptide	Concentration of peptide:Cu^{2+} complex, (μM)	Inhibition of NBT reduction (%)
	DAHK	0.5	$0.1 \pm 4.9^{a\star}$
		1.0	$22.1 \pm 1.0^{a\dagger}$
VI-2	LWVGCHP	0.5	$29.1 \pm 2.4^{b\star}$
		1.0	$50.6 \pm 4.6^{c\dagger}$
VII-2	VFHKALY	0.5	$21.2 \pm 5.1^{b\star}$
		1.0	$39.2 \pm 3.7^{b\dagger}$
VII-4	FHKALY	0.5	$28.6 \pm 5.8^{b\star}$
		1.0	$54.4 \pm 1.4^{c\dagger}$

Xanthine oxidase (20 nM) was incubated with 0.1 mM xanthine and 25 μM nitroblue tetrazolium (NBT) in the presence of peptide/Cu^{2+}complex in 0.5 ml of 50 mM sodium carbonate (pH 10.2). The NBT reduction was determined by monitoring the increase of absorbance at 560 nm for 5 min. Inhibition (%) of NBT reduction was determined as described in materials and methods. Means with same letter are not significantly different ($p < 0.05$). ⋆Compared with DAHK at 0.5 μM. †Compared with DAHK at 1 μM.

Antioxidant effect of peptides on AAPH-induced oxidation of LDL

Next, to see the inhibitory effect of peptides on AAPH-induced generation of lipid peroxyl radicals [40], each peptide (50 μM) was incubated with LDL in the presence of AAPH (5 mM) for 3 h. As displayed in Table IV, the protective action of the peptide differed according to amino acid sequence. In contrast to DAHK showing at most 13.3% protection, the peptide (VI-2, VII-2, VII-4 or VII-6) with Tyrosine (Tyr) or Cysteine (Cys) residue had a substantial (>35%) protection, consistent with the subsidiary role of tyrosine or cysteine residue as a scavenger of peroxyl radicals [41–43]. The positive role of Cys residue is suggested from the higher protection by LWVGCHP (38%), compared to LWVGAHP (23%). Nonetheless, GCHP (26%) was less protective than LWVGCHP, implying the subsidiary role of non-polar amino acid residues. In a related study (Figure 4), the protective action of each peptide against AAPH-induced LDL oxidation decreased time-dependently, probably suggesting a gradual oxidative degradation of peptide. The sustained protective action, dependent on the peptide structure, was the greatest with VI-2 (LWVGCHP), followed by VII-6 (FHKY) and VII-4 (FHKALY), but DAHK was much less protective, reaffirming the role of Tyr or Cys residue.

Table IV. Protection by peptides against AAPH-induced LDL oxidation and HOCl-induced AChE inactivation.

No.	Sequence of peptide	Protection (%)	
		LDL oxidation*	AChE inactivation†
	DAHK	13.3 ± 2.3[a]	27.8 ± 3.7[ab]
I-1	NHP	9.1 ± 2.5[a]	17.2 ± 3.8[a]
II-1	LHLKT	9.7 ± 2.3[a]	26.5 ± 7.7[ab]
V-1	LAHKIHV	12.3 ± 4.5[a]	29.3 ± 6.6[ab]
VI-2	LWVGCHP	37.7 ± 2.6[d]	72.5 ± 6.9[d]
VI-3	GCHP	26.1 ± 0.4[c]	45.9 ± 10.4[c]
VII-2	VFHKALY	36.8 ± 1.1[d]	48.3 ± 3.7[c]
VII-4	FHKALY	39.3 ± 3.1[d]	104.1 ± 3.1[f]
VII-5	FHKAL	20.5 ± 2.2[b]	32.5 ± 8.1[b]
VII-6	FHKY	40.3 ± 4.3[d]	85.1 ± 13.0[e]

*LDL (0.1 mg protein/ml) was incubated with 5 mM AAPH in the presence of each peptide (50 μM) in 200 μl of 10 mM PBS buffer at 38°C for 3 h. LDL oxidation was measured by TBARS assay. †Eel acetylcholinesterase (AChE) at 0.25 U/ml was incubated with 10 μM HOCl in the presence of each peptide (10 μM) in 0.1 ml PBS buffer (pH 7.4) at 38°C for 15 min, and 20 μl aliquot was withdrawn for assay of remaining activity. Results are expressed as a mean ± SD value of triplicate experiments. Means with same letter are not significantly different ($p < 0.05$).

Antioxidant effect of peptides on HOCl-induced oxidation of acetylcholinesterase

Recently, myeloperoxidase HOCl-induced modification of lipoproteins has been of attention in respect of the atherosclerosis progress. To examine HOCl-scavenging activity of peptides, their protective action against HOCl-induced inactivation of acetylcholinesterase, sensitive to HOCl [44], was investigated.

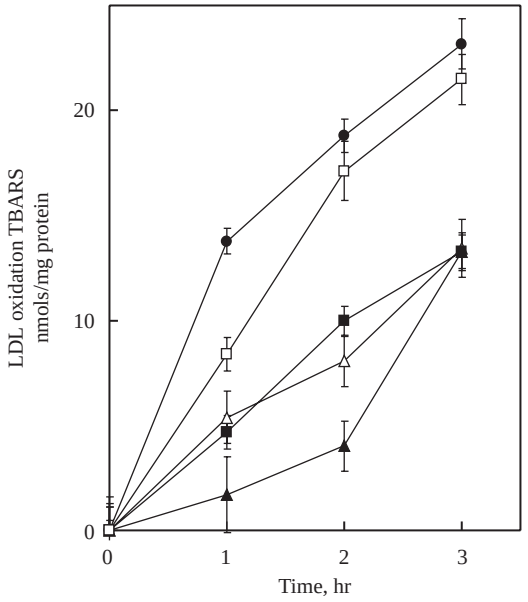

Figure 4. Time-dependent protection by peptide against AAPH-induced LDL oxidation. LDL (0.1 mg protein/ml) was incubated with 5 mM AAPH in 200 μl of 10 mM PBS buffer at 38°C in the presence of each peptide (50 μM) for indicated time, and 0.2 ml solution was used for determination of TBARS value as given in materials and methods. Results are expressed as a mean ± SD (bar) value of triplicate experiments. •, Control; □, DAHK; ▲, Peptide VI-2, ■, Peptide VII-4; △, Peptide VII-6.

Table IV indicates that peptide VII-4 and VII-6 containing Tyr residue, and peptide VI-2 bearing Trp and Cys residue showed a remarkable protection (73–104%) at a 1:1 molar ratio of peptide/HOCl. Thus, it is supposed that the protective action of these peptides may be mainly due to the HOCl-removing activity. In contrast, the peptides (I-1, II-1, V-1 or VII-5) lacking HOCl-reactive Tyr, Trp or Cys residue [29], had relatively lower protections (17–33%).

Discussion

Some part of PON1 action has been proposed to be related to its promoting effect on the reverse cholesterol transport [45] as well as its antioxidant action [9,45,46]. However, the antioxidant role of PON1 in Cu^{2+}-induced oxidation of LDL was not supported by a recent report [17]. Nonetheless, our present results suggest a possible role of PON1 as Cu^{2+} scavenger in preventing Cu^{2+}-catalyzed inactivation of PON1 as well as in competing with DCPH. This discrepancy may be explained by the notion that Cu^{2+}-scavenging activity of PON1 may differ according to oxidation targets; PON1 as a macromolecule may be inefficient in interrupting the redox cycling of copper ion in apolipoprotein B of LDL [47,48]. Alternatively, Cu^{2+}-scavenging activity of PON1 might be diminished in the presence of LDL lipid as implied from the interfering effect of lipids on Cu^{2+}-catalyzed inactivation of PON1 [19]. Besides, Cu^{2+}-binding site of PON1 molecule might be oxidatively degraded during Cu^{2+}-induced oxidation of LDL as had been demonstrated with ascorbate/Cu^{2+}-treated PON1 [23]. PON1 was supposed to contain at least two Cu^{2+} sites [23], one of which was suggested to be a Cu^{2+} site of high affinity, similar to the Cu^{2+} site of apolipoprotein B [47]. In support of this, Ca^{2+}-depleted PON1 showed

a complete protection against Cu^{2+}-mediated inactivation of PON1 at a 1:1 molar ratio of inactive PON1/Cu^{2+}. A further support comes from the 1:1 molar ratio of PON1/Cu^{2+} for the inhibition of formation of Cu^{2+}/DPCH complex. The involvement of His residue in Cu^{2+} binding by PON1 had been suggested from the partial loss of His residues in Cu^{2+}-catalyzed oxidative inactivation of PON1 [23,49]. A further support may come from the inverse relationship between DEPC modification and Cu^{2+}-binding capability of PON1. From these, it was supposed that the peptides of some His residue-containing sequences in PON1 might tightly bind to Cu^{2+} as had been demonstrated with DAHK [26,27]. Noteworthy, the peptides, resembling some His-containing sequences in PON1 molecule, exerted a remarkable protection against Cu^{2+}-catalyzed oxidation of LDL. The position of His residue in the peptides seemed to be a primary factor in preventing against Cu^{2+}-mediated oxidation of LDL or PON1. The presence of His residue at second or third position was crucial for the antioxidant action, similar to the finding [26,27] with DAHK analogues. Additionally, His–Pro sequence at C-terminal apparently contributed to the antioxidant action as demonstrated with LWVGCHP. In particular, NHP was the smallest peptide displaying a potent antioxidant action against Cu^{2+} oxidation of LDL. Antioxidant effect of some His-containing peptides appeared to depend on the oxidation model; for example, FHKALY was more protective than VFHKALY in preventing ascorbate/Cu^{2+}-induced inactivation of PON1, a polar oxidation target, while this was not true for Cu^{2+}-induced oxidation of LDL. In preventing LDL oxidation, antioxidant action of peptides such as LAHKIHV, FHKALY, FHKY or NHP was maintained for >24 h, implying that they can sequestrate copper ions, responsible for the oxidation of LDL. However, the stoichiometry for the interaction between Cu^{2+} and peptides seems to differ according to Cu^{2+} oxidation system; the protective action of the peptides in Cu^{2+}-mediated LDL oxidation was maximal at 1:1 molar ratio of peptide/Cu^{2+}, contrasting with 2:1 ratio of peptide/Cu^{2+} for the protection against Cu^{2+}-induced inactivation of PON1. In either case, the sequestration of Cu^{2+} is likely the underlying mechanism for the suppressive effect of peptides on Cu^{2+}-mediated oxidation. Furthermore, the Cu^{2+} complex of representative peptides exhibited higher superoxide-scavenging activity than Cu^{2+}/DAHK complex [27]. Additionally, these peptides also exhibited a remarkable protection against Fe^{2+}-catalyzed inactivation of PON1, where non-specific hydroxyl radicals would be predominant reactive species (data not shown). In this respect, those peptides may correspond to general inhibitors of metal ions-catalyzed oxidation. Another system for oxidation of lipoprotein would be alkylperoxyl radicals [9,40]; lipoproteins were susceptible to alkylperoxyl radicals and hydroperoxides generated from AAPH [40,41]. Previous data [28,41–43,50] showed that Lys, Tyr, Cys or His residues of protein or peptide were susceptible to peroxyl radicals generated from AAPH oxidation. This might explain why LWVGCHP and FHKALY, containing Cys or Tyr residue were more efficient than DAHK in preventing AAPH-induced oxidation of LDL. Further, the positive role of Cys residue is manifested from higher protection by LWVGCHP than LWVGAHP, and the contributory role of Tyr residue from greater protection by FHKALY than FHKAL. Additional oxidant for lipoprotein oxidation *in vivo* is myeloperoxidase HOCl as suggested from the detection of chlorotyrosine in atherosclerotic plaque [51,52]. Further, HOCl causes the oxidative modification of HDL apolipoprotein A-I [53]. Therefore, the compounds capable of scavenging HOCl could be of a therapeutic value. Again, the peptides, such as LWVGCHP, FHKALY or FHKY, characterized by the presence of Cys or Tyr residue were more protective than DAHK. The great protection by LWVGCHP may be addressed by the existence of Cys or Trp residue, highly reactive with HOCl [29,54] or chlorinated amines formed from Lys or His side chains [53,55]. Meanwhile, the positive role of Tyr residue might be explained by the chlorine transfer from chlorinated amines, formed from Lys or His side chains, to Tyr side chain, as suggested from a recent publication [55].

Collectively, it is shown that some His residue-containing peptides, derived from the structure of PON1 molecule, can express antioxidant actions against various oxidations mediated by Cu^{2+}, AAPH or HOCl. In particular, VWLGCHP, FHKALY and FHKY have an advantage over DAHK in that they express a broad spectrum of antioxidant actions. In future study, a design of broad spectrum antioxidant peptides, resistant to enzymatic or non-enzymatic degradation *in vivo*, would be explored to find potential antiatherosclerosis agents.

Acknowledgements

This work was financially supported by a grant from KRIBB Research Initiative Program, Korea.

References

[1] La Du BN. Structural and functional diversity of paraoxonase. Nat Med 1996;2:1186–1187.

[2] Berliner JA, Navab M, Fogelman AM, Frank JS, Demer LL, Edwards PA, Watson AD, Lusis AJ. Atherosclerosis: Basic mechanism, oxidation, inflammation and genetics. Circulation 1995;9:2488–2496.

[3] Mackness MI, Mackness B, Durrington PA, Connelly PW, Hegele RA. Paraoxonase: Biochemistry, genetics and relationship to plasma lipoproteins. Curr Opin Lipidol 1996;7:69–76.

[4] Davies HG, Richter RJ, Keifer M, Broomfield CA, Sowalla J, Furlong CE. The effect of the human serum paraoxonase polymorphism is reversed with diazoxon, soman and sarin. Nat Genet 1996;14:334–336.

[5] Gan KN, Smolen A, Eckerson HW, La Du BN. Purification of human serum paraoxonase/arylesterase. Drug Metab Dispos 1991;19:100–106.

[6] Billecke SD, Draganov R, Counsell P, Stetson C, Watson C, La Du BN. Human serum paraoxonase (PON1) isozymes Q and R hydrolyze lactones and cyclic carbonate esters. Drug Metab Dispos 2000;28:1335–1342.

[7] Teiber JF, Draganov DI, La Du BN. Lactonase and lactonizing activities of human serum paraoxonase (PON1) and rabbit serum PON3. Biochem Pharmacol 2003;66:887–896.

[8] Watson AD, Berliner JA, Hama SY, La Du BN, Fauli KF, Fogelman AM, Mohamad N. Protective effect of high density lipoprotein associated paraoxnase: Inhibition of the biological activity of minimally oxidized low density lipoprotein. J Clin Investig 1995;96:2882–2891.

[9] Aviram M, Rosenblat M, Billecke S, Erogul J, Sorenson R, Bisgaier CL, Newton RS, La Du BN. Human serum paraoxonase (PON 1) is inactivated by oxidized low density lipoprotein and preserved by antioxidants. Free Radic Biol Med 1999;26:892–904.

[10] Shih DM, Gu L, Xia YR, Navab M, Li WF, Hama S, Castellani LW, Furlong CE, Costa LG, Fogelman AM, Lusis AJ. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. Nature 1998;394:284–287.

[11] Shih DM, Gu L, Hama S, Xia YR, Navab M, Fogelman AM, Lusis AJ. Genetic-dietary regulation of serum paraoxonase expression and its role in atherogenesis in a mouse model. J Clin Investig 1996;97:1630–1639.

[12] Aviram M, Hardak E, Vaya J, Mahmood S, Milo S, Hoffman A, Billicke S, Draganov D, Rosenblat M. Human serum paraoxnase (PON 1) Q and R selectively decrease lipid peroxides in human coronary and carotid atherosclerotic lesions: PON1 esterase and peroxidase-like activities. Circlulation 2000;101:2510–2517.

[13] Ruiz J, Blanche H, James RW, Garin MC, Vaisse C, Charpentier G, Cohen N, Morabia A, Passa P, Froguel P. Gln–Arg192 polymorphism of paraoxonase and coronary heart disease in type 2 diabetes. Lancet 1995;30:869–872.

[14] Mackness B, Davies GK, Turkie W, Lee E, Roberts DH, Hill E, Roberts C, Durrington PN, Mackness MI. Paraoxonase status in coronary heart disease: Are activity and concentration more important than genotype? Arterioscler Thromb Vasc Biol 2001;21:1451–1457.

[15] Marathe GK, Zimmerman GA, McIntyre TM. PAF acetylhydrolase, and not paraoxonase-1, is the oxidized phospholipid hydrolase of high density lipoprotein particles. J Biol Chem 2003;278:3937–3947.

[16] Connelly PW, Draganov D, Maguire GF. Paraoxonase-1 does not reduce or modify oxidation of phospholipids by peroxynitrite. Free Radic Biol Med 2005;38:164–174.

[17] Teiber JF, Draganov DI, La Du BN. Purified human serum PON1 does not protect LDL against oxidation in the *in vitro* assays initiated with copper or AAPH. J Lipid Res 2004;45:2260–2268.

[18] Sorenson RC, Bisgaier CL, Aviram M, Hsu C, Billecke S, La Du BN. Human serum paraoxonase/arylesterase's retained hydrophobic N-terminal leader sequence associates with HDLs by binding phospholipids: Apolipoprotein A-I stabilizes activity. Arterioscler Thromb Vasc Biol 1999;19:2214–2225.

[19] Nguyen SD, Sok DE. Beneficial effect of oleoylated lipids on paraoxonase1: Protection against oxidative inactivation and stabilization. Biochem J 2003;375:275–285.

[20] Nguyen SD, Sok DE. Preferential inhibition of paraoxonase activity of human paraoxonase 1 by negatively charged lipids. J Lipid Res 2004;45:2211–2220.

[21] Kuo CL, La Du BN. Comparison of purified human and rabbit serum paraoxonases. Drug Metab Dispos 1995; 23:935–944.

[22] Debord J, Bollinger JC, Merle L, Dantoine T. Inhibition of human serum arylesterase by metal chlorides. J Inorg Biochem 2003;94:1–4.

[23] Nguyen SD, Sok DE. Oxidative inactivation of paraoxnase1, an antioxidant protein and its effect on antioxidant action. Free Radic Res 2003;37:1319–1330.

[24] Bar-Or D, Thomas GW, Rael LT, Lau EP, Winkler JV. Asp–Ala–His–Lys (DAHK) inhibits copper-induced oxidative DNA double strand breaks and telomere shortening. Biochem Biophys Res Commun 2001;28:356–360.

[25] Halliwell B. Albumin—an important extracellular antioxidant? Biochem Pharmacol 1988;37:569–571.

[26] Bar-Or D, Curtis G, Rao N, Bampos N, Lau E. Characterization of the Co^{2+} and Ni^{2+} binding amino-acid residues of the N-terminus of human albumin: An insight into the mechanism of a new assay for myocardial ischemia. Eur J Biochem 2001;268:42–47.

[27] Bar-Or D, Rael LT, Lau EP, Rao NK, Thomas GW, Winkler JV, Yukl RL, Kingston RG, Curtis CG. An analog of the human albumin N-terminus (Asp–Ala–His–Lys) prevents formation of copper-induced reactive oxygen species. Biochem Biophys Res Commun 2001;284:856–862.

[28] Kohen R, Yamamoto Y, Cundy KC, Ames BN. Antioxidant activity of carnosine, homocarnosine, and anserine present in muscle and brain. Proc Natl Acad Sci USA 1988;85:3175–3179.

[29] Hawkins CL, Pattison DI, Davies MJ. Hypochlorite-induced oxidation of amino acids, peptides and proteins. Amino Acids 2003;25:259–274.

[30] Schumaker VN, Puppione DL. Sequential flotation ultracentrifugation. Methods Enzymol 1986;128:155–170.

[31] Bollag DM, Michael DR, Stuart JE. Protein methods. 2nd ed. New York: Wiley-Liss; 1996.

[32] Turkington RW, Tracy FM. Spectrophotometric determination of ultramicro amounts of copper with 1,5-diphenylcarbohydrazide. Anal Chem 1958;30:1699–1701.

[33] Esterbauer H, Striegl G, Puhl H, Rotheneder M. Continuous monitoring of *in vitro* oxidation of human low-density lipoprotein. Radic Res Commun 1989;6:67–75.

[34] Morel DW, Hessler JR, Chisolm GM. Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. J Lipid Res 1983;8:1070–1076.

[35] Esterbauer H, Cheeseman KH. Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. Methods Enzymol 1990;186:407–421.

[36] Beauchamp C, Fridovich I. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Anal Biochem 1971;44:276–287.

[37] Ellman GL, Courtney KD, Andres VJ, Feather-stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.

[38] Steel RGD, Torrie JH. Principles and procedures of statistics. New York: McGraw-Hill; 1960. p 99–132.

[39] Sattler W, Mohr D, Stocker R. Rapid isolation of lipoproteins and assessment of their peroxidation by high-performance liquid chromatography postcolumn chemiluminescence. Methods Enzymol 1994;233:469–489.

[40] Lass A, Witting P, Stocker R, Esterbauer H. Inhibition of copper- and peroxyl radical-induced LDL lipid oxidation by ebselen: Antioxidant actions in addition to hydroperoxide-reducing activity. Biochim Biophys Acta 1996;1303:111–118.

[41] Ma YS, Chao CC, Stadtman ER. Oxidative modification of glutamine synthetase by 2,2′-azo bis (2-amidinopropane)

dihydrochloride: Loss of 2 of 16 histidine residues, 6 of 17 tyrosine residues, 5 of 16 methionine residues, and all of the tryptophan residues (2 residues). Arch Biochem Biophys 1999;363:129–134.

[42] Dean RT, Hunt JV, Grant AJ, Yamamoto Y, Niki E. Free radical damage to proteins: The influence of the relative localization of radical generation, antioxidants, and target proteins. Free Radic Biol Med 1991;11:161–168.

[43] Gebicki S, Gebicki JM. Formation of peroxides in amino acids and proteins exposed to oxygen free radicals. Biochem J 1993;289:743–749.

[44] Den Hartog GJ, Vegt E, Van der Vijgh WJ, Haenen GR, Bast A. Hypochlorous acid is a potent inhibitor of acetylcholinesterase. Toxicol Appl Pharmacol 2002;181:228–332.

[45] Rosenblat M, Vaya J, Shih D, Aviram M. Paraoxonase 1 (PON1) enhances HDL-mediated macrophage cholesterol efflux via the ABCA1 transporter in association with increased HDL binding to the cells: A possible role for lysophosphatidylcholine. Atherosclerosis 2005;179:69–77.

[46] Aviram M, Rosenblat M. Paraoxonases 1, 2, and 3, oxidative stress, and macrophage foam cell formation during atherosclerosis development. Free Radic Biol Med 2004;37:1304–1316.

[47] Burkitt MJ. A critical overview of the chemistry of copper-dependent low density lipoprotein oxidation: Roles of lipid hydroperoxides, alpha-tocopherol, thiols, and ceruloplasmin. Arch Biochem Biophys 2001;394:117–135.

[48] Wagner P, Heinecke JW. Copper ions promote peroxidation of low density lipoprotein lipid by binding to histidine residues of apolipoprotein B100, but they are reduced at other sites on LDL. Arterioscler Thromb Vasc Biol 1997;17:3338–3346.

[49] van Himbergen TM, van Tits LJ, Hectors MP, de Graaf J, Roest M, Stalenhoef AF. Paraoxonase-1 and linoleic acid oxidation in familial hypercholesterolemia. Biochem Biophys Res Commun 2005;333:787–793.

[50] Kwon HY, Choi SY, Won MH, Kang T, Kang JH. Lysine, histidine, proline, and valine residues were sensitive to AAPH. Biochim Biophys Acta 2000;1543:69–76.

[51] Podrez EA, Abu-Soud HM, Hazen SL. Myeloperoxidase-generated oxidants and atherosclerosis. Free Radic Biol Med 2000;28:1717–1725.

[52] Hazell LJ, Arnold L, Flowers D, Waeg G, Malle E, Stocker R. Presence of hypochlorite-modified proteins in human atherosclerotic lesions. J Clin Investig 1996;97:1535–1544.

[53] Pattison DI, Hawkins CL, Davies MJ. Hypochlorous acid-mediated oxidation of lipid components and antioxidants present in low-density lipoproteins: Absolute rate constants, product analysis, and computational modeling. Chem Res Toxicol 2003;16:439–449.

[54] Bergt C, Fu X, Huq NP, Kao J, Heinecke JW. Lysine residues direct the chlorination of tyrosines in YXXK motifs of apolipoprotein A-I when hypochlorous acid oxidizes high density lipoprotein. J Biol Chem 2004;279:7856–7866.

[55] Pattison DI, Davies MJ. Kinetic analysis of the role of histidine chloramines in hypochlorous acid mediated protein oxidation. Biochemistry 2005;44:7378–7387.