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Lipid peroxidation and 4-hydroxy-2-nonenal formation by copper ion bound to amyloid-beta peptide

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Running title: Lipid peroxidation and HNE formation by Aβ-Cu^{2+}

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Abstract— The lipid peroxidation product 4-hydroxy-2-nonenal (HNE) is proposed to be a toxic factor in the pathogenesis of Alzheimer's disease. The primary products of lipid peroxidation are phospholipid hydroperoxides and degraded reactive aldehydes, such as HNE, as secondary peroxidation products. In this study, we investigated the role of amyloid-β peptide (Aβ) in the formation of phospholipid hydroperoxides and HNE by copper ion bound to Aβ. The $Aβ_{1-42}$-Cu^{2+} (1:1 molar ratio) complex showed an activity to form phospholipid hydroperoxides from phospholipid, 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC), through Cu^{2+} reduction in the presence of ascorbic acid. The phospholipid hydroperoxides were considered to be racemic mixture of 9-hydroperoxide and 13-hydroperoxide of linoleoyl residue. When Cu^{2+} was bound to two molar equivalents of $Aβ_{1-42}$ (2 $Aβ_{1-42}$-Cu^{2+}), lipid peroxidation was inhibited. HNE was generated from one of the phospholipid hydroperoxides, 1-palmitoyl-2-(13-hydroperoxy-cis-9, trans-11-octadecadienoyl) phosphatidylcholine (PLPC-OOH), by free Cu^{2+} in the presence of ascorbic acid through Cu^{2+} reduction and degradation of PLPC-OOH. HNE generation was markedly inhibited by equimolar concentrations of $Aβ_{1-40}$ (92%) and $Aβ_{1-42}$ (92%). However, $Aβ_{1-42}$ binding two or three molar equivalents of Cu^{2+} ($Aβ_{1-42}$-$2Cu^{2+}$, $Aβ_{1-42}$-$3Cu^{2+}$) acted as a pro-oxidant to form HNE from PLPC-OOH. These findings suggest that, at moderate concentrations of copper, Aβ acts primarily as an antioxidant to prevent Cu^{2+}-catalyzed oxidation of biomolecules, but that, in the presence of excess copper, pro-oxidant complexes of Aβ with Cu^{2+} are formed.

Keywords— amyloid-β peptide, copper, lipid peroxidation, 4-hydroxy-2-nonenal, phospholipid hydroperoxide, Alzheimer's disease, antioxidant

INTRODUCTION

Alzheimer's disease is characterized by deposition of aggregated amyloid-β peptide (Aβ) and oxidative stress marked by protein and RNA oxidation and lipid peroxidation (1-5). Lipid peroxidation *in vivo* involves mainly unsaturated fatty acyl residues esterified in phospholipids. The primary peroxidation products are phospholipid hydroperoxides having a hydroperoxy unsaturated fatty acyl residue (Fig. 1) (6,7). The phospholipid hydroperoxides are degraded to reactive aldehydes, such as 4-hydroxy-2-nonenal (HNE) (Fig.1), malondialdehyde and alkenals, as secondary peroxidation products (8-10), and the HNE has been demonstrated to cause neuronal death (11-14). The HNE-protein adducts are detected in the brain of patients with Alzheimer's disease (15-17). Therefore, HNE is considered to play a crucial role in oxidative

injury of biomolecules related to Alzheimer's disease but the role of Aβ in the formation of HNE is unknown.

The formation of HNE has been demonstrated by the reaction of one of the phospholipid hydroperoxides, 1-palmitoyl-2-(13-hydroperoxy-cis-9, trans-11-octadecadienoyl) phosphatidylcholine (PLPC-OOH), with hemoprotein (18). Lipid hydroperoxides are generally known to be degraded by transition metal ions and is known that Aβ shows a strong affinity for transition metal ions, especially Cu^{2+} such that Aβ is assumed to bind with Cu^{2+} physiologically (19-23). The Aβ-Cu^{2+} complex is reported to act as a pro-oxidant (24-27), an especially deleterious aspect since Aβ is rich in hydrophobic amino acids in the carboxyl-terminal region and is present in phospholipids with lipoproteins of both cerebrospinal fluid and plasma (28,29). Indeed, the lipoprotein of human cerebrospinal fluid is reported to be easily oxidized in vitro to generate phospholipid hydroperoxides (30,31) – an aspect that might account for the reduced concentrations of fatty acids in Alzheimer's disease. Therefore, we hypothesized that phospholipid hydroperoxides and HNE could be formed from phospholipids by the Aβ-Cu^{2+} complex. In support of this concept, in this study, we found that the Aβ-Cu^{2+} complex have an activity to generate phospholipid hydroperoxides from phospholipids. Moreover, we show that when Cu^{2+} concentration is higher than Aβ, in the presence of ascorbic acid (Fig.1), HNE could be detected as a reaction product from the phospholipid hydroperoxides with the Aβ-Cu^{2+} complex.

MATERIALS AND METHODS

Materials

Human $A\beta_{1-40}$ and $A\beta_{1-42}$ were purchased from Peptide Institute Inc. (Osaka, Japan); $A\beta_{1-28}$, $A\beta_{35-42}$, L-carnosine, trientine (triethylenetetramine), penicillamine, clioquinol (5-chloro-7-iodo-8-hydroxyquinoline), deferoxamine and glycyl-L-histidyl-L-lysine (GHL) from Sigma-Aldrich (St. Louis MO, USA); EDTA, oxine (8-hydroxyquinoline), histidine, histamine, spermine, spermidine and putrescine from Wako Pure Chemical Co. (Osaka, Japan); 4-hydroxy-2-nonenal (HNE) from Cayman chemical Co.; and 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC) from Avanti Polar Lipids, Inc. (Alabaster, USA). 1-palmitoyl-2-(13-hydroperoxy-cis-9, trans-11-octadecadienoyl) phosphatidylcholine (PLPC-OOH) was prepared from PLPC as described previously (18, 32, 33). Other chemicals were of the highest quality commercially available.

Determination of phospholipid hydroperoxides and HNE.

The determination of phospholipid hydroperoxide and HNE with high performance liquid chromatography (HPLC) was performed on a Hitachi model L-6000 liquid chromatograph. The HPLC conditions for the determination of HNE were described previously (18). An ODS

column (5μm, 4.6 × 250mm, TSK ODS-80Ts, Tosoh Co., Tokyo, Japan) was used. The mobile phase was acetonitrile/50 mM phosphate buffer (pH 2.7) (3:7; v/v), and the flow rate was 1.4 ml/min. The UV peaks were monitored at 222 nm, the absorption maximum of HNE. The chemiluminescence detection-high performance liquid chromatography (CL-HPLC) conditions for the determination of PLPC-OOH were described previously (18). An ODS column (5μm, 4.6 × 250mm, TSK ODS-80Ts, Tosoh Co., Tokyo, Japan) was used. The mobile phase was acetonitrile /methanol/water (75:23:2, v/v/v) containing 10 mM choline chloride and the flow rate was 1.5 ml/min. The eluent was mixed at post-column with chemiluminescence reagent which consisted of 0.15 μM cytochrome c, 10.0 μM luminol, 1.0 mM EDTA and 20 mM H_3BO_3-Na_2CO_3 buffer (pH 10), with a 2.5-ml/min flow rate. Phospholipid hydroperoxides were detected with the chemiluminescence detector (Shimadzu CLD-10A). PLPC-OOH was used as external standard to determine phospholipid hydroperoxides.

Phospholipid hydroperoxide formation from PLPC by $A\beta_{1\text{-}42}$-Cu^{2+} complex.

The reaction mixture containing PLPC (1 mM) and ascorbic acid (2 mM) was incubated with Cu^{2+} (10 μM) or $A\beta_{1\text{-}42}$ (3.3~20 μM)-Cu^{2+} (10 μM) complex ($A\beta_{1\text{-}42}$/Cu^{2+}=2:1, 1:1, 1:2 and 1:3) in 5 mM HEPES buffer (pH 7.4) at room temperature. An aliquot of the reaction mixture was directly injected into a CL-HPLC to determine phospholipid hydroperoxides.

Effects of chelator on phospholipid hydroperoxide formation from PLPC by $A\beta_{1\text{-}42}$-Cu^{2+} complex.

The reaction mixture containing PLPC (1 mM), ascorbic acid (2 mM) and $A\beta_{1\text{-}42}$ (10 μM) -Cu^{2+} (10 μM) (1:1 molar ratio) complex was incubated with 10 μM chelator for 60 minutes in 5 mM HEPES buffer (pH 7.4) at room temperature. An aliquot of the reaction mixture was directly injected into the CL-HPLC to determine phospholipid hydroperoxides.

HNE formation from PLPC-OOH by Cu^{2+}.

The reaction mixture containing PLPC-OOH (500 μM), Cu^{2+} (1~3 μM) and ascorbic acid (500 μM) was incubated in 5 mM HEPES buffer (pH 7.4) at room temperature. An aliquot of the reaction mixture was directly injected into an HPLC and CL-HPLC to determine HNE and PLPC-OOH, respectively.

Effects of chelator on HNE formation by Cu^{2+}.

The reaction mixture containing PLPC-OOH (500 μM), Cu^{2+} (2 μM) and ascorbic acid (500 μM) was incubated with 2 μM chelator for 30 minutes in 5 mM HEPES buffer (pH 7.4) at room temperature. An aliquot of the reaction mixture was directly injected into an HPLC to determine HNE.

HNE formation from PLPC-OOH by $A\beta_{1\text{-}42}$-Cu^{2+} complex. The reaction mixture containing PLPC-OOH (500 μM) and ascorbic acid (500 μM) was incubated with Cu^{2+} (2-8 μM) or $A\beta_{1\text{-}42}$ (2μM)-Cu^{2+} (2-8 μM) complex for 30 minutes in 5 mM HEPES buffer (pH 7.4) at room

temperature. An aliquot of the reaction mixture was directly injected into an HPLC to determine HNE.

Ascorbate-oxidase activity of $A\beta_{1-42}$ complex.

Ascorbate-oxidase activity was followed optically at 260 nm. Reaction mixture contained 100 μM ascorbate, 22 μM $A\beta_{1-42}$ and various concentrations of Cu^{2+} in 20mM HEPES buffer (pH 7.4). Reactions were started by the addition of ascorbate.

Effects of chelator on HNE formation by $A\beta_{1-42}$-Cu^{2+} complex.

The reaction mixture containing PLPC-OOH (500 μM), ascorbic acid (500 μM) and $A\beta_{1-42}$ (2 μM)-Cu^{2+} (4 μM) (1:2 molar ratio) complex was incubated with 2 μM chelator for 60 minutes in 5 mM HEPES buffer (pH 7.4) at room temperature. An aliquot of the reaction mixture was directly injected into HPLC to determine HNE.

RESULTS

Phospholipid hydroperoxide formation from PLPC by $A\beta_{1-42}$-Cu^{2+} complex.

When synthetic phospholipid, PLPC, was incubated with both Cu^{2+} and ascorbic acid, the formation of phospholipid hydroperoxides was observed by CL-HPLC. The CL-HPLC chromatogram showed a main peak at 14 min. The main peak was considered to be racemic mixture of 9-hydroperoxide and 13-hydroperoxide of linoleoyl residue (data not shown). The formation of phospholipid hydroperoxides by Cu^{2+} is shown in Fig. 2. The Cu^{2+}-associated lipid peroxidation was inhibited by $A\beta_{1-42}$, when Cu^{2+} was bound to two molar equivalents of $A\beta_{1-42}$ (2 $A\beta_{1-42}$-Cu^{2+}). However, an equimolar complex of $A\beta_{1-42}$-Cu^{2+} (1:1 molar ratio) showed phospholipid hydroperoxide formation. When the Cu^{2+} concentration is higher than Aβ, the activity of phospholipid hydroperoxide formation by the complex ($A\beta_{1-42}$/Cu^{2+}=1:2, and 1:3) was higher than Cu^{2+}. The decrease of phospholipid hydroperoxides after a 60 minute incubation is consistent with the decomposition of phospholipid hydroperoxides.

Decomposition of PLPC-OOH and formation of HNE by Cu^{2+}.

When 500 μM PLPC-OOH was incubated with both 1-3 μM Cu^{2+} and 500 μM ascorbic acid for 5 minutes, a decrease of PLPC-OOH and formation of HNE was observed (Fig. 3). The rates of decomposition of PLPC-OOH and HNE formation were dependent on the Cu^{2+} concentration. In the absence of ascorbic acid, neither decomposition of PLPC-OOH nor formation of HNE was observed (data not shown). Thus, it is likely that Cu^{2+} is reduced to Cu^{+} by ascorbic acid, and that the Cu^{+} rapidly reacts with PLPC-OOH to generate HNE (Fig. 1). The reaction was further examined at 2 μM Cu^{2+} and the time courses of the decrease in PLPC-OOH and the formation of HNE are shown in Fig. 4. PLPC-OOH was rapidly and almost completely decomposed during the 20 minute incubation. However, HNE continued to increase after depletion of PLPC-OOH, reaching a peak after incubation for 80 min. The efficiency (mol %

yield) of the formation of HNE from decomposed PLPC-OOH after incubation for 80 minutes was 6.1 %.

Effects of chelators on HNE formation by Cu^{2+}.

Figure 5 shows the amount of HNE formed from PLPC-OOH by free Cu^{2+} in the presence of equimolar concentration of the copper chelators. The amount of HNE formed from 500 μM PLPC-OOH by incubation with 2 μM Cu^{2+} for 30 minutes (control) was 22.1 μM. The well-known Cu^{2+} chelator EDTA, deferoxamine, trientine, penicillamine, oxine and GHL, all inhibited the formation of HNE by 95, 88, 95, 73, 88 and 96 %, respectively. $Aβ_{1-40}$ and $Aβ_{1-42}$, which are also very efficient chelators for copper, also both inhibited the formation by 92 %. $Aβ_{1-28}$ and $Aβ_{35-42}$ inhibited by 84 and 10%, respectively. The metal chelator, clioquinol, a 5-chloro-7-iodo derivative of oxine, reported to induce a decrease in brain Aβ deposition (23, 34), was the least effect chelator only inhibiting formation by 25 %. Histidine and histamine inhibited by 36 and 17%, respectively. The biogenic polyamines such as putrescine, spermidine and spermine, inducers of cell growth and proliferation, reported to enhance Aβ-associated neurotoxicity (35), inhibited the formation by 11, 18 and 11%, respectively. Carnosine, a dipeptide having copper chelating property, has been reported to inhibit the oxidation of phosphatidylcholine by Cu^{2+} in the presence of ascorbic acid (36), but it did not show inhibitory effect.

HNE formation by $Aβ_{1-42}$-Cu^{2+} complex and ascorbate-oxidase activity of $Aβ_{1-42}$-Cu^{2+} complex.

It has been reported that $Aβ_{1-42}$ has an ability to bind up to 3.5 molar equivalents of Cu^{2+} (37), and $Aβ_{1-42}$ binding two molar equivalents of Cu^{2+} ($Aβ_{1-42}$-$2Cu^{2+}$) generates hydrogen peroxide in the presence of reductant (27). $Aβ_{1-42}$ with equimolar Cu^{2+} decreased HNE formation by 91% as compared to free Cu^{2+} (Fig. 6A), and ascorbate-oxidase activity was also markedly inhibited (Fig. 6B). However, $Aβ_{1-42}$ with two molar equivalents of Cu^{2+} provided a 36% inhibition of the formation by free Cu^{2+}, and showed ascorbate-oxidase activity. The $Aβ_{1-42}$ binding three molar equivalents of Cu^{2+} showed marked ascorbate-oxidase activity and formation of HNE, the same as shown for the formation by free Cu^{2+}.

Effects of chelators on lipid peroxidation and HNE formation by $Aβ_{1-42}$-Cu^{2+} complex.

Table 1 shows the inhibitory effect of chelators on phospholipid hydroperoxide and HNE formation by $Aβ_{1-42}$-Cu^{2+} complex. phospholipid hydroperoxide formed from PLPC by $Aβ_{1-42}$-Cu^{2+} (1:1) in the presence of copper chelator was determined. The total amounts of $Aβ_{1-42}$ and copper chelators were two times higher than Cu^{2+}. EDTA, trientine, penicillamine and GHL, markedly inhibited the formation of phospholipid hydroperoxide by 96, 92, 93 and 92 %, respectively.

HNE formed from PLPC-OOH by $Aβ_{1-42}$-$2Cu^{2+}$ (1:2) in the presence of copper chelator was

determined. The total amounts of $A\beta_{1-42}$ and copper chelators were equimolar to Cu^{2+}. EDTA, trientine, deferoxamine, and GHL, markedly inhibited the formation of HNE by 93, 87, 82 and 87 %, respectively.

DISCUSSION

Lipid peroxidation is induced under conditions of oxidative stress, such as Alzheimer's disease, ischemia-reperfusion, atherosclerosis, cancer, and various other disorders. The lipid peroxidation process involves the formation of lipid hydroperoxides, HNE, and malondialdehyde (6-9). Among these products, lipid hydroperoxides, which are mainly generated from phospholipids, cholesterol esters and unsaturated fatty acids, are the intermediates of peroxidation reactions. Phospholipids are the main components of cell membranes and lipoproteins, and these are converted to phospholipid hydroperoxides under peroxidative conditions. Incubation of PLPC with free Cu^{2+} in the presence of ascorbic acid generated phospholipid hydroperoxides through Cu^{2+} reduction and peroxidation of PLPC (Fig. 2). The Cu^{2+}-associated reactions are generally inhibited by copper chelators. Because the $A\beta_{1-42}$ when binding equimolar Cu^{2+}, forms a square-planar complex including His^{6}, His^{13}, His^{14}, and N-terminal amino group (21), the equimolar complex ($A\beta_{1-42}$-Cu^{2+}) was expected to be redox-inactive. However, the $A\beta_{1-42}$-Cu^{2+} was not redox-inactive, and showed phospholipid hydroperoxide formation (Fig. 2), and this is the first evidence for phospholipid hydroperoxide formation by $A\beta_{1-42}$-Cu^{2+} complex. Two molar equivalents of $A\beta_{1-42}$ showed Cu^{2+}-associated lipid peroxidation but to a lesser extent. On the other hand, when Cu^{2+} concentration is higher than $A\beta_{1-42}$, the complexes ($A\beta_{1-42}$-$2Cu^{2+}$ and $A\beta_{1-42}$-$3Cu^{2+}$) showed higher lipid peroxidation compared to free Cu^{2+}, and the formed phospholipid hydroperoxide was reduced after incubation for 60 minutes (Fig. 2). The decrease in formed phospholipid hydroperoxide was caused by the reaction of phospholipid hydroperoxides with the complexes as mentioned below.

The reaction of one of the phospholipid hydroperoxides, PLPC-OOH, with hemoprotein has demonstrated the rapid formation of HNE (18). Reaction of ferric ion with PLPC-OOH is very slow and no HNE formation has been observed (18). In the presence of ascorbic acid as reductant, the reaction was also very slow (unpublished result). In the absence of ascorbic acid, reaction of Cu^{2+} with PLPC-OOH proceeds also very slowly (data not shown). On the other hand, in the presence of ascorbic acid, Cu^{2+} was reduced to Cu^{+}, and rapidly reacted with PLPC-OOH, followed by a generation of HNE (Figs. 3 and 6). The reaction was dependent on both the concentration of Cu^{2+} and the ascorbate-oxidase activity.

The time-courses of the decomposition of PLPC-OOH and HNE formation by 2 μM Cu^{2+} are shown in Fig. 4. In this reaction, over 99% of the added PLPC-OOH decomposed after incubation for 20 minutes; however, the increase in HNE concentration continued for more than

20 minutes. The increase in HNE after depletion of PLPC-OOH has been previously observed in the case of HNE formation by methemoglobin (18). These data suggest the operation of more than one pathway that generates HNE from PLPC-OOH — one pathway that rapidly generates a burst of product and another pathway that generates HNE more slowly, probably from a stable unidentified intermediate. The maximum efficiency (mol% yield) of the formation of HNE from decomposed PLPC-OOH, shown after incubation for 80 minutes, was 6.1%, and was higher than that by methemoglobin (1.6%) (18). Cu^{2+}-associated decomposition of phospholipid hydroperoxides may be an important mechanism for HNE formation *in vivo*.

Transition metal-associated reactions are generally inhibited by metal chelators. GHL, known as a growth-modulating tripeptide isolated from human serum, is very efficient chelator for copper (38, 39). Because the GHL-Cu^{2+} forms a planar tridentate complex involving the imino nitrogen of the histidyl imidazole ring (38), Cu^{2+}-associated HNE formation from PLPC-OOH was considered to be markedly inhibited (Fig. 5). The $A\beta_{1-28}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ when binding equimolar Cu^{2+}, forms a square-planar complex (21). The formation of HNE by these equimolar complexes was also markedly inhibited. In contrast, $A\beta_{35-42}$ having no Cu^{2+}-binding site did not inhibit the formation of HNE. These data indicate that the formation of equimolar complexes of Cu^{2+} with Aβ is necessary for inhibition of Cu^{2+}-associated HNE formation from PLPC-OOH.

It has been reported that $A\beta_{1-42}$ has an ability to bind up to 3.5 molar equivalents of Cu^{2+} (37). $A\beta_{1-42}$ binding two molar equivalents of Cu^{2+} ($A\beta_{1-42}$-$2Cu^{2+}$) showed reduced ascorbate-oxidase activity inhibited the formation of HNE, corresponding to about 60 % of the formation by free Cu^{2+} (Fig. 6). Ascorbate radicals were observed during ascorbate-oxidase reaction by $A\beta_{1-42}$-$2Cu^{2+}$ (data not shown). On the other hand, three molar equivalents of Cu^{2+} ($A\beta_{1-42}$-$3Cu^{2+}$) was equivalent to free Cu^{2+} suggesting an overload of inhibition. In this regard, Opazo et al. reported that $A\beta_{1-42}$ purified from Alzheimer's disease plaque binds two molar equivalents of Cu^{2+}, and it acts as pro-oxidant to generate hydrogen peroxide in the presence of reductant (27). The $A\beta_{1-42}$-$2Cu^{2+}$ and $A\beta_{1-42}$-$3Cu^{2+}$ generated hydrogen peroxide in the presence of ascorbate much the same as ascorbate-oxidase activity and HNE formation (unpublished result). These data indicate that the complex of Cu^{2+} with Aβ acts as a pro-oxidant when Cu^{2+} concentration is higher than $A\beta_{1-42}$. On the other hand, the generation of hydrogen peroxide by free Cu^{2+} in the presence of ascorbate as reductant was inhibited by equimolar $A\beta_{1-42}$ (unpublished result) and lipid peroxidation by Cu^{2+} was inhibited by two molar equivalent of $A\beta_{1-42}$ (Fig. 2). These data indicate that $A\beta_{1-42}$ acts as antioxidant when Cu^{2+} concentrations are lower than $A\beta_{1-42}$. Such an antioxidant effect of Aβ is not completely surprising. Kontush et al. documented the *in vitro* effect of Aβ as antioxidant on Cu^{2+}-catalyzed oxidation of low density lipoprotein when added in molar excess of Cu^{2+} (40). Nunomura et al. reported that increases of Aβ *in vivo* are associated with decreased oxidative damage (41). Based on data presented here,

we suspect that Aβ acts primarily as antioxidant to prevent Cu^{2+}-catalyzed oxidation of biomolecules in the brain at low concentrations of Cu^{2+}. On the other hand, at higher concentrations of Cu^{2+} than $A\beta_{1-42}$, $A\beta_{1-42}$-Cu^{2+} (1:1) shows phospholipid hydroperoxide formation, and furthermore, $A\beta_{1-42}$-$2Cu^{2+}$ (1:2) and $A\beta_{1-42}$-$3Cu^{2+}$ (1:3) show both phospholipid hydroperoxide and HNE formations. As such, both antioxidant and pro-oxidant properties of the Aβ complex with Cu^{2+} are dependent on the ratio of Cu^{2+} to Aβ.

The phospholipid hydroperoxide formation by $A\beta_{1-42}$-Cu^{2+} (1:1) was markedly inhibited by copper chelators such as EDTA, trientine, penicillamine and GHL (Table 1). The HNE formation was also markedly inhibited by copper chelators such as EDTA, trientine, deferoxamine and GHL. These data indicated that the oxidative stress by the complex of Aβ with Cu^{2+} in the brain of Alzheimer's disease may be abrogated by copper chelator treatment.

The copper ion is considered to be mainly bound to copper-chelating protein such as ceruloplasmin and metallothionein *in vivo* (42). Bound copper, similar to Cu^{2+} of $A\beta_{1-42}$-$2Cu^{2+}$ (Fig. 6), carnosine-Cu^{2+} and polyamines-Cu^{2+} (Fig. 5) in this study, is assumed to be redox-active, and may induce HNE formation from lipid hydroperoxides *in vivo*. The mechanism of the formation of HNE *in vivo* has been a long-standing question. The reaction of Cu^{+}, generated by reduction of binding Cu^{2+}, with lipid hydroperoxides may be one of a possible route of HNE formation *in vivo*.

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REFERENCES

[1] Smith, M. A.; Perry, G.; Richey. P. L.; Sayre, L. M.; Anderson, V. E.; Beal M. F.; Kowall, N. Oxidative damage in Alzheimer's. *Nature* **382**:120-121; 1996.

[2] Nunomura, A.; Perry, G.; Aliev, G.; Hirai K.; Takeda, A.; Balraj, E. K.; Jones, P. K.; Ghanbari, H.; Wataya, T.; Shimohama, S.; Chiba, S.; Atwood, C. S.; Petersen, R. B.; Smith, M. A. Oxidative damage is the earliest event in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* **60**:759-767; 2001.

[3] Varadarajan, S.; Yatin, S.; Aksenova, M.; Butterfield, D. A. Review: Alzheimer's amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity. *J. Struct. Biol.* **130**:184-208; 2000.

[4] Kontush, A. Amyloid-beta: an antioxidant that becomes a pro-oxidant and critically contributes to Alzheimer's disease. *Free Radic. Biol. Med.* **31**:1120-1131; 2001.

[5] Atwood, C. S.; Obrenovich, M. E.; Liu, T.; Chan, H.; Perry, G.; Smith, M. A.; Martins, R. N.

Amyloid-beta: a chameleon walking in two worlds: a review of the trophic and toxic properties of amyloid-beta. *Brain Res. Brain Res. Rev.* **43**:1-16; 2003.

[6] Girotti, A.W. Lipid hydroperoxide generation, turnover, and effector action in biological systems. *J. Lipid Res.* **39**:1529-1542; 1998.

[7] Miyazawa, T.; Fujimoto, K.; Suzuki, T.; Yasuda, K. Determination of phospholipid hydroperoxides using luminol chemiluminescence--high-performance liquid chromatography. *Methods Enzymol.* **233**:324-332; 1994.

[8] Esterbauer, H.; Schaur, R. J.; Zollner, H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic. Biol. Med.* **11**:81-128; 1991.

[9] Parola, M.; Bellomo, G.; Robino, G.; Barrera, G.; Dianzani, M. U. 4-Hydroxynonenal as a biological signal: molecular basis and pathophysiological implications. *Antioxid. Redox Signal.* **1**:255-284; 1999.

[10] Spiteller, P.; Kern, W.; Reiner, J.; Spiteller, G. Aldehydic lipid peroxidation products derived from linoleic acid. *Biochim. Biophys. Acta* **1531**:188-208; 2001.

[11] Mark, R. J.; Lovell, M. A.; Markesbery, W, R.; Uchida, K.; Mattson, M. P. A role for 4-hydroxynonenal, an aldehydic product of lipid peroxidation, in disruption of ion homeostasis and neuronal death induced by amyloid beta-peptide. *J. Neurochem.* **68**:255-264; 1997.

[12] Butterfield, D.A.; Castegna. A.; Lauderback, C. M.; Drake, J. Evidence that amyloid beta-peptide-induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death. *Neurobiol. Aging.* **23**:655-664; 2002.

[13] Rabacchi, S. A.; Friedman, W. J.; Shelanski, M. L.; Troy, C. M. Divergence of the apoptotic pathways induced by 4-hydroxynonenal and amyloid beta-protein. *Neurobiol Aging.* **25**:1057-1066; 2004.

[14] Keller, J. N.; Hanni, K. B.; Markesbery, W. R. 4-hydroxynonenal increases neuronal susceptibility to oxidative stress. *J. Neurosci. Res.* **58**:823-830; 1999.

[15] Ando, Y.; Brannstrom, T.; Uchida, K.; Nyhlin, N.; Nasman, B.; Suhr, O.; Yamashita, T.; Olsson, T.; El Salhy, M.; Uchino, M.; Ando, M. Histochemical detection of 4-hydroxynonenal protein in Alzheimer amyloid. *J. Neurol. Sci.* **156**:172-176; 1998.

[16] Sayre, L. M.; Zelasko, D. A.; Harris, P. L.; Perry, G.; Salomon, R. G.; Smith, M. A. 4-Hydroxynonenal-derived advanced lipid peroxidation end products are increased in Alzheimer's disease. *J. Neurochem.* **68**:2092-2097; 1997.

[17] Takeda, A.; Smith, M. A.; Avila, J.; Nunomura, A.; Siedlak, S. L.; Zhu, X.; Perry, G.; Sayre, L. M. In Alzheimer's disease, heme oxygenase is coincident with Alz50, an epitope of tau induced by 4-hydroxy-2-nonenal modification. *J. Neurochem.* **75**:1234-1241; 2000.

[18] Hayashi, T.; Uchida, K.; Takebe, G.; Takahashi, K. Rapid formation of 4-hydroxy-2-nonenal, malondialdehyde, and phosphatidylcholine aldehyde from phospholipid

hydroperoxide by hemoproteins. *Free Radic. Biol. Med.* **36**:1025-1033; 2004.

[19] Dong, J.; Atwood, C. S.; Anderson, V. E.; Siedlak, S. L.; Smith, M. A.; Perry, G.; Carey, P. R. Metal binding and oxidation of amyloid-beta within isolated senile plaque cores: Raman microscopic evidence. *Biochemistry* **42**:2768-2773; 2003.

[20] Atwood, C. S.; Moir, R. D.; Huang, X.; Scarpa, R. C.; Bacarra, N. M.; Romano, D. M.; Hartshorn, M. A.; Tanzi, R. E.; Bush, A. I. Dramatic aggregation of Alzheimer abeta by Cu(II) is induced by conditions representing physiological acidosis. *J. Biol. Chem.* **273**:12817-12826; 1998.

[21] Syme, C. D.; Nadal, R. C.; Rigby, S. E.; Viles, J. H. Copper binding to the amyloid-beta (Abeta) peptide associated with Alzheimer's disease: folding, coordination geometry, pH dependence, stoichiometry, and affinity of Abeta-(1-28): insights from a range of complementary spectroscopic techniques. *J. Biol. Chem.* **279**:18169-18177; 2004.

[22] Miura, T.; Suzuki, K.; Kohata, N.; Takeuchi, H. Metal binding modes of Alzheimer's amyloid beta-peptide in insoluble aggregates and soluble complexes. *Biochemistry* **39**:7024-7031; 2000.

[23] Bush, A. I. The metallobiology of Alzheimer's disease. *Trends Neurosci.* **26**:207-214; 2003.

[24] Rottkamp, C. A.; Raina, A. K.; Zhu, X.; Gaier, E.; Bush, A. I.; Atwood, C. S.; Chevion, M.; Perry, G.; Smith, M. A. Redox-active iron mediates amyloid-beta toxicity. *Free Radic. Biol. Med.* **30**:447-450; 2001.

[25] Huang, X.; Atwood, C. S.; Hartshorn M. A.; Multhaup, G.; Goldstein, L. E.; Scarpa, R. C.; Cuajungco, M. P.; Gray, D. N.; Lim, J.; Moir, R. D.; Tanzi, R. E.; Bush, A. I. The A beta peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction. *Biochemistry* **38**:7609-7616; 1999.

[26] Huang, X.; Cuajungco, M. P.; Atwood, C. S.; Hartshorn, M. A.; Tyndall, J. D.; Hanson, G. R.; Stokes, K. C.; Leopold, M.; Multhaup, G.; Goldstein, L. E.; Scarpa, R. C.; Saunders, A. J.; Lim, J.; Moir, R. D.; Glabe, C.; Bowden, E. F.; Masters, C. L.; Fairlie, D. P.; Tanzi, R. E.; Bush, A. I. Cu(II) potentiation of alzheimer abeta neurotoxicity. Correlation with cell-free hydrogen peroxide production and metal reduction. *J. Biol. Chem.* **274**:37111-37116; 1999.

[27] Opazo, C.; Huang, X.; Cherny, R. A.; Moir, R. D.; Roher, A. E.; White, A. R.; Cappai, R.; Masters, C. L.; Tanzi, R. E.; Inestrosa, N. C.; Bush, A. I. Metalloenzyme-like activity of Alzheimer's disease beta-amyloid. Cu-dependent catalytic conversion of dopamine, cholesterol, and biological reducing agents to neurotoxic H2O2. *J. Biol. Chem.* **277**:40302-40308; 2002.

[28] Koudinov, A. R.; Koudinova, N. V.; Kumar, A.; Beavis, R. C.; Ghiso, J. Biochemical characterization of Alzheimer's soluble amyloid beta protein in human cerebrospinal fluid: association with high density lipoproteins. *Biochem. Biophys. Res. Commun.* **223**:592-597; 1996.

[29] Koudinov, A.; Matsubara, E.; Frangione, B.; Ghiso, J. The soluble form of Alzheimer's amyloid beta protein is complexed to high density lipoprotein 3 and very high density lipoprotein in normal human plasma. *Biochem. Biophys. Res. Commun.* **205**:1164-1171; 1994.

[30] Prasad, M. R.; Lovell, M. A.; Yatin, M.; Dhillon, H.; Markesbery, W. R. Regional membrane phospholipid alterations in Alzheimer's disease. *Neurochem. Res.* **23**:81-88; 1998.

[31] Schippling, S.; Kontush, A.; Arlt, S.; Buhmann, C.; Sturenburg, H. J.; Mann, U.; Muller-Thomsen, T.; Beisiegel, U. Increased lipoprotein oxidation in Alzheimer's disease. *Free Radic. Biol. Med.* **28**:351-360; 2000.

[32] Hayashi, T.; Miyazawa, T. Age-associated oxidative damage in microsomal and plasma membrane lipids of rat hepatocytes. *Mech. Ageing Dev.* **100**:231-242; 1998.

[33] Saito, Y.; Hayashi, T.; Tanaka, A.; Watanabe, Y.; Suzuki, M.; Saito, E.; Takahashi, K. Selenoprotein P in human plasma as an extracellular phospholipid hydroperoxide glutathione peroxidase. Isolation and enzymatic characterization of human selenoprotein P. *J. Biol. Chem.* **274**:2866-2871; 1999.

[34] Cherny, R. A.; Atwood, C. S.; Xilinas, M. E.; Gray, D. N.; Jones, W. D.; McLean, C. A.; Barnham, K. J.; Volitakis, I.; Fraser, F. W.; Kim, Y.; Huang, X.; Goldstein, L. E.; Moir, R. D.; Lim, J. T.; Beyreuther, K.; Zheng, H.; Tanzi, R. E.; Masters, C. L.; Bush, A. I. Treatment with a copper-zinc chelator markedly and rapidly inhibits beta-amyloid accumulation in Alzheimer's disease transgenic mice. *Neuron* **30**:665-676; 2001.

[35] Yatin, S. M.; Yatin, M.; Varadarajan, S.; Ain, K. B.; Butterfield, D. A. Role of spermine in amyloid beta-peptide-associated free radical-induced neurotoxicity. *J. Neurosci. Res.* **63**:395-401; 2001.

[36] Decker, E. A.; Livisay, S. A.; Zhou, S. A re-evaluation of the antioxidant activity of purified carnosine. *Biochemistry (Mosc)* **65**:766-770; 2000.

[37] Atwood, C. S.; Scarpa, R. C.; Huang, X.; Moir, R. D.; Jones, W. D.; Fairlie, D. P.; Tanzi, R. E.; Bush, A. I. Characterization of copper interactions with alzheimer amyloid beta peptides: identification of an attomolar-affinity copper binding site on amyloid beta1-42. *J. Neurochem.* **75**:1219-1233; 2000.

[38] Pickart, L.; Freedman, J. H.; Loker, W. J.; Peisach, J.; Perkins, C. M.; Stenkamp, R. E.; Weinstein, B. Growth-modulating plasma tripeptide may function by facilitating copper uptake into cells. *Nature* **288**:715-717; 1980.

[39] Freedman, J. H.; Pickart, L.; Weinstein, B.; Mims, W. B.; Peisach, J. Structure of the Glycyl-L-histidyl-L-lysine--copper(II) complex in solution. *Biochemistry* **21**:4540-4544; 1982.

[40] Kontush, A.; Berndt, C.; Weber, W.; Akopyan, V.; Arlt, S.; Schippling, S.; Beisiegel, U. Amyloid-beta is an antioxidant for lipoproteins in cerebrospinal fluid and plasma. *Free Radic. Biol. Med.* **30**:119-128; 2001.

[41] Nunomura, A.; Perry, G.; Aliev, G.; Hirai, K.; Takeda, A.; Balraj, E. K.; Jones, P. K.; Ghanbari, H.; Wataya, T.; Shimohama, S.; Chiba, S.; Atwood, C. S.; Petersen, R. B.; Smith, M. A. Oxidative damage is the earliest event in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* **60**:759-767; 2001.

[42] Koizumi, M.; Fujii, J.; Suzuki, K.; Inoue, T.; Inoue, T.; Gutteridge. J. M.; Taniguchi, N. A marked increase in free copper levels in the plasma and liver of LEC rats: an animal model for Wilson disease and liver cancer. *Free Radic. Res.* **28**:441-450; 1998.

ABBREVIATIONS

HNE — 4-hydroxy-2-nonenal

Aβ— amyloid-beta peptide

PLPC — 1-palmitoyl-2-linoleoyl phosphatidylcholine

PLPC-OOH— 1-palmitoyl-2-(13-hydroperoxy-cis-9, trans-11-octadecadienoyl) phosphatidylcholine

GHL — glycyl-L-histidyl-L-lysine

CL-HPLC — chemiluminescence detection-high performance liquid chromatography

Figure legends

Fig. 1. **HNE formation from PLPC**.

Fig. 2. **Phospholipid hydroperoxide formation from PLPC by $A\beta_{1-42}$-Cu^{2+} complex.** The reaction mixture containing PLPC (1 mM) and ascorbic acid (2 mM) was incubated with Cu^{2+} (10 μM) or $A\beta_{1-42}$ (3.3~20 μM) - Cu^{2+} (10 μM) complex in 5 mM HEPES buffer (pH 7.4) at room temperature. An aliquot of the reaction mixture was directly injected into a CL-HPLC to determine phospholipid hydroperoxide. Each point represents the mean ± S.D. of three experiments.

Fig. 3. **Cu^{2+}-dependent decomposition of PLPC-OOH and the formation of HNE from PLPC-OOH in the presence of ascorbic acid.** 500 μM PLPC-OOH and 500 μM ascorbic acid was incubated with 1-3 μM Cu^{2+} for 5 minutes in 5 mM HEPES buffer (pH 7.4). An aliquot of the reaction mixture was directly injected into a CL-HPLC and HPLC to determine PLPC-OOH and HNE, respectively. Each point represents the mean ± S.D. of three experiments.

Fig. 4. T**ime courses of the decomposition of PLPC-OOH and the formation of HNE**

from PLPC-OOH by Cu^{2+} in the presence of ascorbic acid. 500 μM PLPC-OOH and 500 μM ascorbic acid was incubated with 2 μM Cu^{2+} in 5 mM HEPES buffer (pH 7.4). An aliquot of the reaction mixture was directly injected into a CL-HPLC and HPLC to determine PLPC-OOH and HNE, respectively. Each point represents the mean ± S.D. of three experiments.

Fig. 5. **Effects of chelators on the formation of HNE from PLPC-OOH by Cu^{2+} and ascorbic acid.** 500 μM PLPC-OOH, 500 μM ascorbic acid and 2 μM Cu^{2+} was incubated with 2 μM chelator for 30 minutes in 5 mM HEPES buffer (pH 7.4). An aliquot of the reaction mixture was directly injected into an HPLC to determine HNE. Each point represents the mean ± S.D. of three experiments.

Fig. 6. **Effects of ratio of Cu^{2+} to $Aβ_{1-42}$ on Cu^{2+}-dependent formation of HNE and ascorbate-oxidase activity.** (A) 500 μM PLPC-OOH and 500 μM ascorbic acid was incubated with $Aβ_{1-42}$ (2 μM) - Cu^{2+} (2-8 μM) (○) for 30 minutes in 5 mM HEPES buffer (pH 7.4). Closed circle (●) represent HNE formation by Cu^{2+} alone (2-8 μM) which concentration are the same as those of $Aβ_{1-42}$-Cu^{2+} complex. An aliquot of the reaction mixture was directly injected into an HPLC to determine HNE. Each point represents the mean ± S.D. of three experiments. (B) Reaction mixture contained 100 μM ascorbate, 22 μM $Aβ_{1-42}$ and various concentrations of Cu^{2+} (○) in 20mM HEPES buffer (pH 7.4). Reactions were started by the addition of ascorbate. Ascorbate-oxidase activity was followed optically at 260 nm. Each point represents the mean of three experiments.

Table 1.

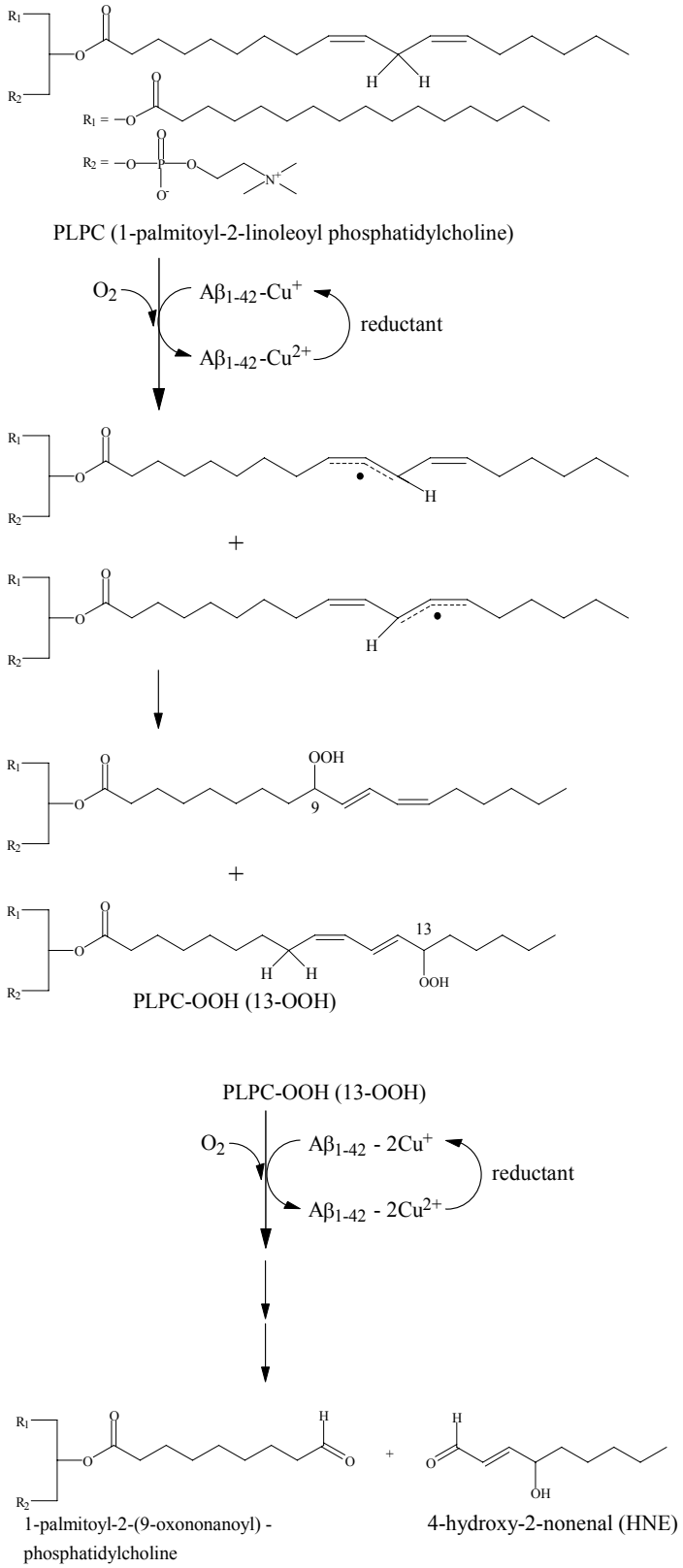
Effects of chelators on phospholipid hydroperoxide and HNE formations by $Aβ_{1-42}$-Cu^{2+} complex.

a. The reaction mixture containing PLPC (1 mM), ascorbic acid (2 mM) and $Aβ_{1-42}$ (10 μM) - Cu^{2+} (10 μM) (1:1 molar ratio) complex was incubated with 10 μM chelator for 60 minutes in 5 mM HEPES buffer (pH 7.4) at room temperature. An aliquot of the reaction mixture was directly injected into a CL-HPLC to determine phospholipid hydroperoxide.

b. The reaction mixture containing PLPC-OOH (500 μM), ascorbic acid (500 μM) and $Aβ_{1-42}$ (2 μM) - Cu^{2+} (4 μM) (1:2 molar ratio) complex was incubated with 2 μM chelator for 60 minutes in 5 mM HEPES buffer (pH 7.4) at room temperature. An aliquot of the reaction mixture was directly injected into an HPLC to determine HNE.

Each value represents the mean of three experiments.

Fig. 1. **HNE formation from PLPC**.



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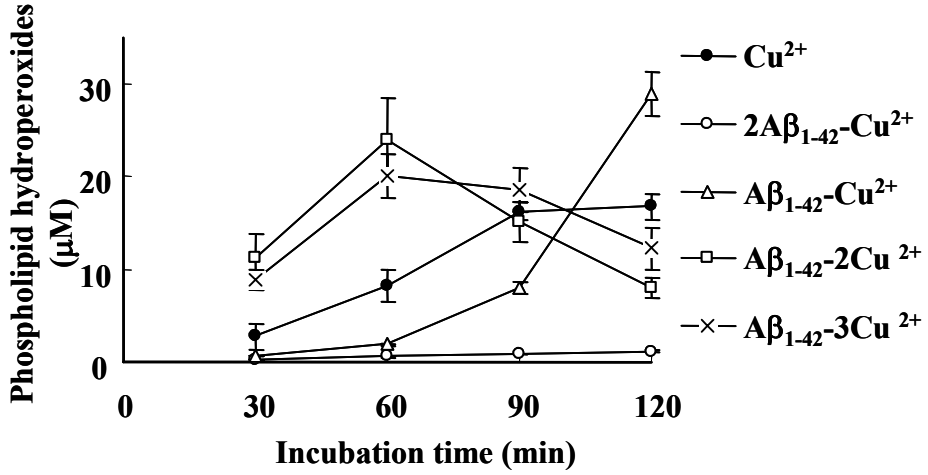
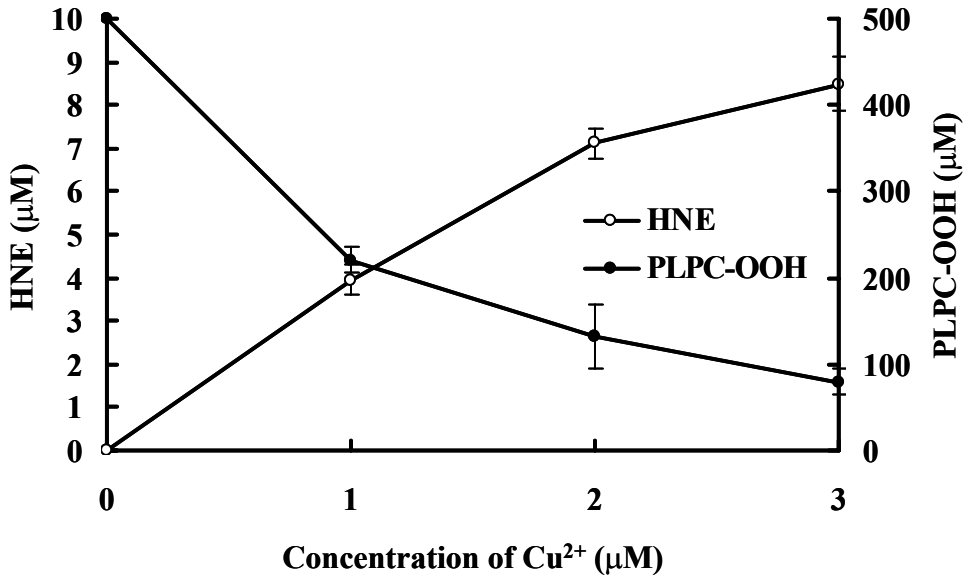


Fig. 3. **Cu^{2+}-dependent decomposition of PLPC-OOH and the formation of HNE from PLPC-OOH in the presence of ascorbic acid.** 500 μM PLPC-OOH and 500 μM ascorbic acid was incubated with 1-3 μM Cu^{2+} for 5 minutes in 5 mM HEPES buffer (pH 7.4). An aliquot of the reaction mixture was directly injected into a CL-HPLC and HPLC to determine PLPC-OOH and HNE, respectively. Each point represents the mean ± S.D. of three experiments.



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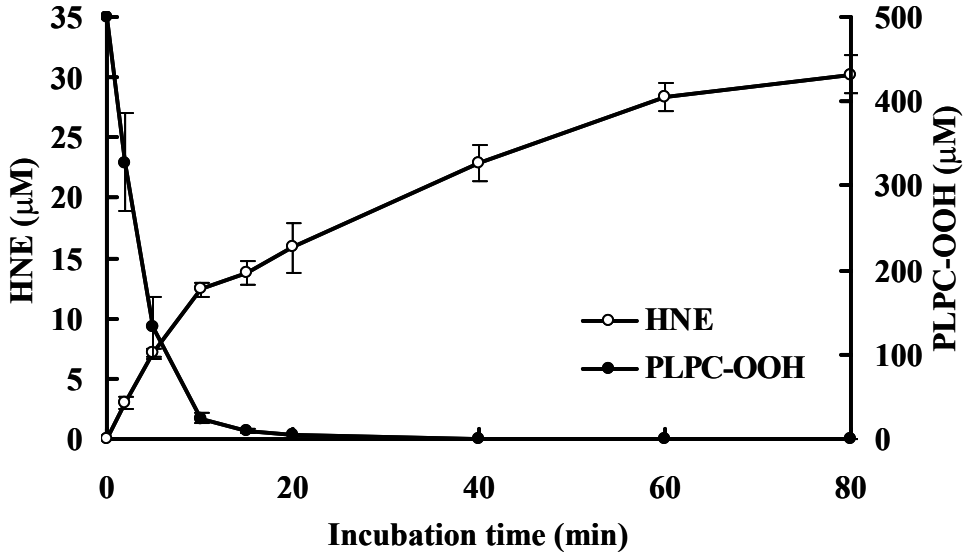

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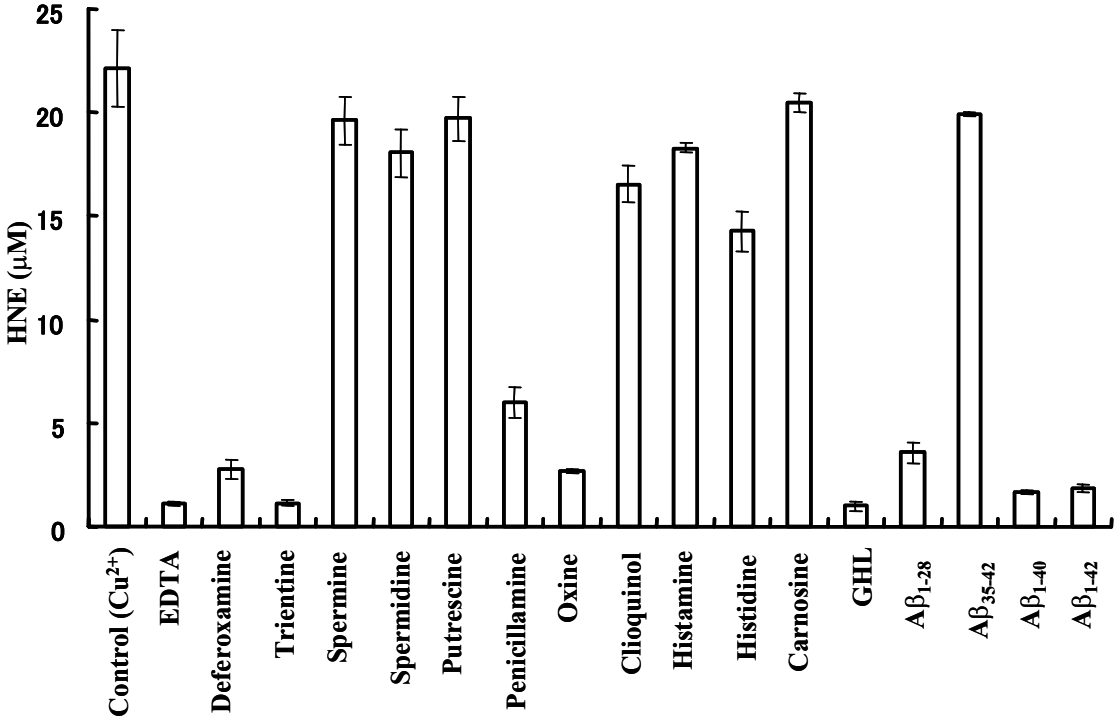

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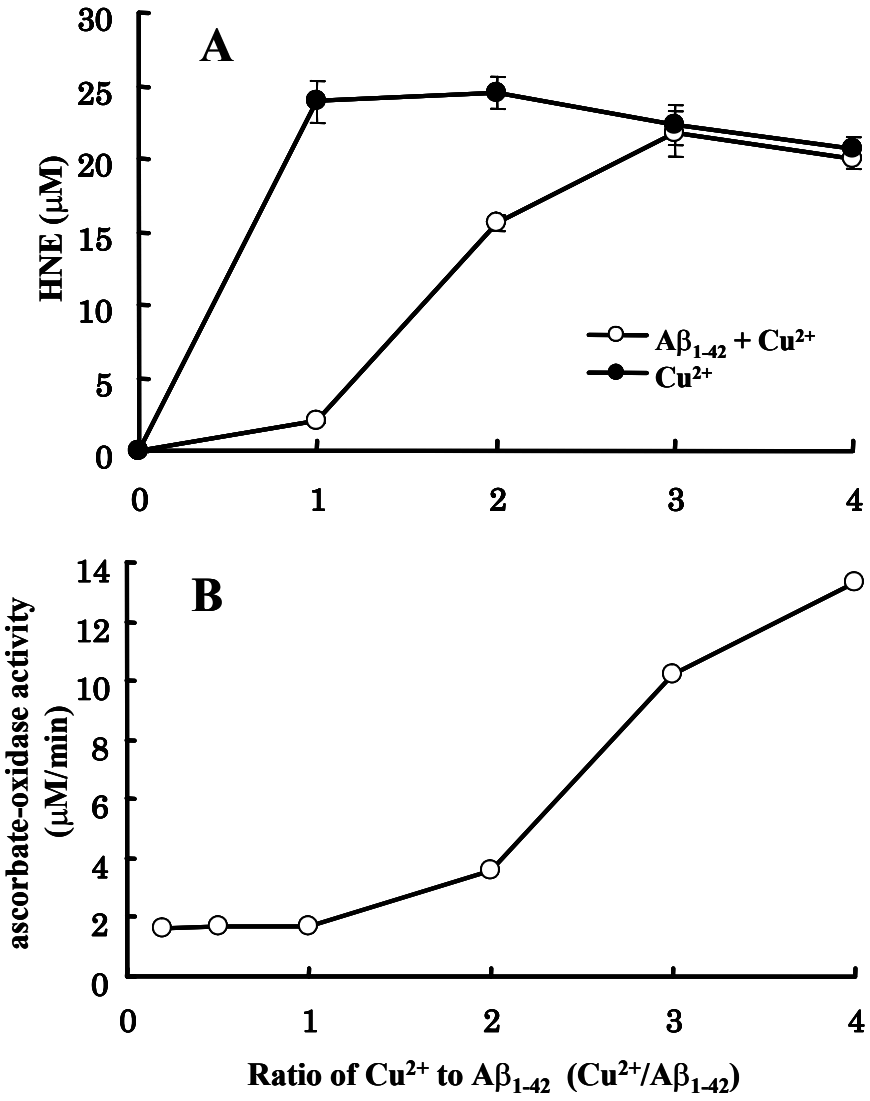

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b. The reaction mixture containing PLPC-OOH (500 μM), ascorbic acid (500 μM) and $A\beta_{1-42}$ (2 μM) - Cu^{2+} (4 μM) (1:2 molar ratio) complex was incubated with 2 μM chelator for 60 minutes in 5 mM HEPES buffer (pH 7.4) at room temperature. An aliquot of the reaction mixture was directly injected into an HPLC to determine HNE.

Each value represents the mean of three experiments.

Chelators	Inhibition of phospholipid hydroperoxide formation (%)	Inhibition of HNE formation (%)
EDTA	96	93
Oxine	61	64
Trientine	92	87
Penicillamine	93	56
Carnosine	52	6
Deferoxamine	61	82
GHL	92	87