Doctoral Thesis

Studies on inhibitory effect of oolong tea polyphenol on oxidative stress

（ウーロン茶ポリフェノールの酸化ストレス抑制効果に関する研究）

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Contents

Abstract

Oolong tea contains unique polymerized polyphenols including oolonghomobisflavan A (OFA) that is generated from green tea leaves during the semi-fermentation process. It has been reported that intake of oolong tea has beneficial effects on health, although the antioxidant effects of OFA remains unclear.

Oxidation of low-density lipoprotein (LDL) by reactive oxygen species (ROS) and reactive nitrogen species (RNS) has been suggested to be involved in the onset of atherosclerosis. We studied effect of OFA on LDL oxidation by ROS and RNS *in vitro*. LDL oxidation was induced by a peroxyl radical-generating reagent (AAPH), transition-metal ions (Cu^{2+}), or a peroxynitrite generator (SIN-1) in the presence of OFA. Cholesterol ester hydroperoxide (CE-OOH) and thiobarbituric acid reactive substances (TBARS) were determined by HPLC as the indices of lipid peroxidation of LDL, while protein fragmentation, protein carbonyl formation, and nitrotyrosine formation were analyzed by SDS-PAGE and western blotting as the indices of oxidative modification of apolipoprotein B-100 (apo B-100) in LDL. OFA suppressed CE-OOH formation in LDL oxidized by AAPH or SIN-1, and formation of TBARS in LDL oxidized by Cu^{2+}. In addition, OFA inhibited fragmentation, carbonylation, and nitration of apo B-100, in which the heparin-binding activity of apo B-100 was protected by OFA. These results suggest that OFA has antioxidant activity to suppress both lipid peroxidation and oxidative modification of apo B-100 in LDL oxidized by ROS and RNS. Polymerized polyphenols in oolong tea may contribute to prevent atherosclerosis by reducing oxidative stress.

Keywords

oolong tea, polyphenols, antioxidant, low-density lipoprotein, oxidation

Abbreviations

AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride; Apo B-100, apolipoprotein B-100; CBB, Coomassie Brilliant Blue; CE-OOH, cholesteryl ester hydroperoxide; DNPH, 2,4-dinitrophenylhydrazine; DPPH; 2,2-diphenyl-1-picrylhydrazyl; DTPA, dietylene triamine pentaacetic acid; ECL, enhanced chemiluminescence; ECG, epicatechin-3-gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; HPLC, high-performance liquid chromatograph; HNE, 4-hydroxy-2-nonenal; HDL, high-density lipoprotein; $L^{\cdot}$, lipid radical; LDL, low-density lipoprotein; LH, polyunsaturated fatty acid side chain; $LOO^{\cdot}$, lipid peroxyl radical; LOOH, lipid hydroperoxide; OFA, oolonghomobisflavan A; $ONOO^{-}$, peroxynitrite; ONOOH, peroxynitrous acid; OTTP, oolong tea polymerized polyphenol; oxLDL, oxidized LDL; IDL, intermediate-density lipoproteins; PBS, phosphate-buffered saline; RNS, reactive nitrogen species; ROS, reactive oxygen species; SIN-1, 3-(4-Morpholinyl) sydnonimine; SDS-PAGE, sodium dodecyl sulfatepolyacrylamide gel electrophoresis; TBARS, 2-thiobarbituric acid reactive substances; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TBS, tris-buffered saline; VLDL, very low-density lipoprotein.

Chapter 1

General introduction

Tea is one of the most widely consumed plant-based beverage for its specific aroma, taste and healthy effects. Freshly harvested tea leaves are processed differently to produce specific types of tea. Oolong is a semifermented tea prepared from leaves of *Camellia sinensis*, while green tea is an unfermented tea. The fermentation of oolong tea is not mediated by microbes, but instead, it is mediated by oxidative enzymes such as polyphenol oxidase and peroxidase in the leaves.[1)] The fermentation process induces browning and generates unique flavors and tastes. Depending on the method of preparation including degree of oxidation, various oolong teas are available in Asian countries. Several studies have been reported that oolong tea has a wide range of beneficial health effects including antihyperglycemic effects,[2)] mitigation of the risk of cardiovascular disease.[3)] The major components in oolong tea are alkaloids, saponins, polysaccharides, L-theanines, and polyphenols.[4,5)] The degree of oxidation in fermentation is affected to polyphenols of oolong tea. Oolong tea contains polymerized polyphenols derived from the oxidative polymerization of catechins such as oolonghomobisflavans.[6)] The polymerized polyphenols are not presented in green tea (Table 1.1).[7)] Oolonghomobisflavan A (OFA, Figure 1.1 A) are most abundant oolong tea polymerized polyphenol (OTPP), has a unique structure as a dimer of epigallocatechin gallate (EGCG, Figure 1.1 B).[8)] The biological effects of oolong tea polyphenols have been attracted considerable attention in recent years, although the mechanism remains unclear.

Plasma lipoproteins are water-soluble particles that are divided into seven classes depending on sizes, lipids, and apolipoproteins (chylomicrons, chylomicron remnants, very low density lipoprotein (VLDL), intermediate density lipoproteins (IDL), low density lipoprotein (LDL), high density lipoprotein (HDL), and lipoprotein (a).[9,10)] LDL is derived from VLDL that is produced by the liver. Lipoprotein lipase hydrolyzes triglycerides in VLDL, increasing the proportion of cholesterol and changing it to LDL.[10)] LDL is composed core of cholesterol esters and triacylglycerols, which is covered by phospholipid

monolayer containing unesterified cholesterol and apolipoprotein B-100 (apo B-100) (Figure 1.2).[11,12] LDL is major cholesterol transporters,[9] it has been suggested that the increasing of LDL in blood and the oxidation of LDL are positively associated with the onset of cardiovascular diseases.[13] A high level of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in human body can inflict damage to the structure and function of LDL (Figure 1.3),[14] which may lead to atherosclerosis.[14-16] LDL oxidation involves lipid peroxidation and modification of apolipoprotein.[17] Several studies indicate that the process of LDL oxidation *in vitro* occur in two main step, which is minimally oxidation and extensively oxidation of LDL (Figure 1.4).[18,19] During the minimally oxidation of LDL occur little modification in apo B-100, which has low affinity to macrophages scavenger receptors.[19] Subsequently, unsaturated fatty acid moieties of lipids and apo B-100 are further oxidized resulting to recognition by macrophages through the scavenger receptors.[19] Unsaturated fatty acid moieties of lipids can be oxidized by ROS such as hydroxyl radical (·OH) and RNS such as peroxynitrite ($ONOO^-$), and generating lipid hydroperoxides[17,20] that process conducts by free radical chain reaction (Figure 1.5). Polyunsaturated fatty acid side chain (LH) containing multiple double bonds in between which lie methylene bridges reactive hydroxyl radical (·OH) (reaction 1).[21] The resulting, lipid radical ($L^{\cdot}$) adds rapidly to molecular oxygen (O_2) to generate a lipid peroxyl radical ($LOO^{\cdot}$) (reaction 2).[21] That itself can propagate the chain by reacting with another polyunsaturated fatty acid to generate $L^{\cdot}$ and lipid hydroperoxide (LOOH) (reaction 3).[21] Reactive aldehydes and ketones are the end products of lipid hydroperoxide.[22]

$$LH + {\cdot}OH \longrightarrow L^{\cdot} + H_2O \quad (1)$$

$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot} \quad (2)$$

$$LOO^{\cdot} + LH \longrightarrow L^{\cdot} + LOOH \quad (3)$$

Hydroxyl radicals may have generated by the high concentration of free transition metals such as copper or decomposition of $ONOO^-$, which also can enhance lipid peroxidation (Figure 1.6 and Figure 1.7).[21] Cu^{2+} can be reduced to Cu^+ in the presence of

superoxide ($O_2^{\cdot-}$) (reaction 4) or endogenous α-tocopherol (reaction 5).[23] Cu^+ is capable of catalyzing the hydroxyl radicals ($OH\cdot$) from hydrogen peroxide (H_2O_2) by Haber-Weiss reaction.[23]

$$Cu^{2+} + O_2^{\cdot-} \longrightarrow Cu^+ + O_2 \quad (4)$$

$$Cu^{2+} + \alpha\text{-TocOH} \longrightarrow Cu^+ + \alpha\text{-TocO}^{\cdot} + H^+ \quad (5)$$

$$Cu^+ + H_2O_2 \longrightarrow Cu^{2+} + OH^- + OH^{\cdot} \quad (6)$$

$ONOO^-$ is a powerful oxidant and nitrating agent, which is generated by the reaction between nitric oxide and superoxide anion (reaction 7).[24] $ONOO^-$ can decompose to hydroxyl radicals by metal independent pathway at acid pH. $ONOO^-$ react with a hydrogen radical (H^+) to generate the stable peroxynitrous acid (ONOOH).[24] The hydroxyl radicals ($\cdot OH$) and free nitrogen species (NO_2) is quickly formed from ONOOH (reaction 8).[24]

$$^{\cdot}NO + O_2^{\cdot-} \longrightarrow ONOO^- \quad (7)$$

$$ONOO^- + H^+ \longrightarrow ONOOH \longrightarrow {}^{\cdot}OH + NO_2 \quad (8)$$

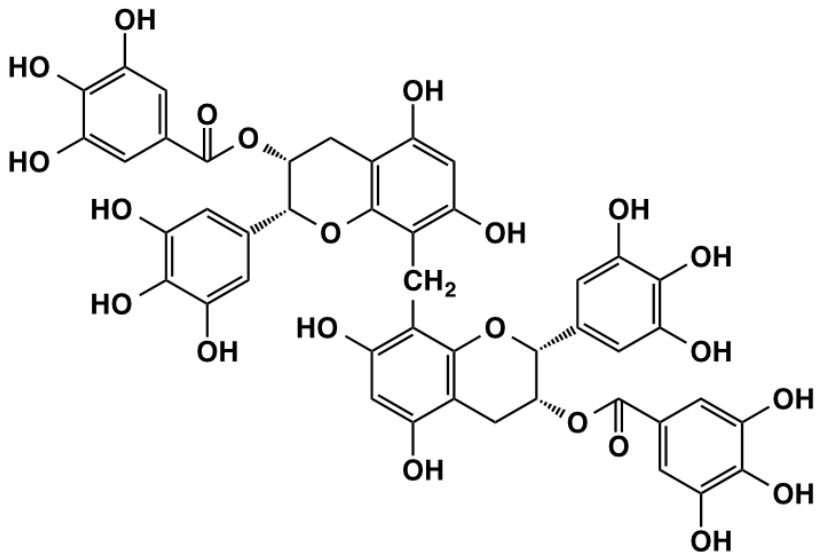
Consequent to the lipid peroxidation decomposition products such as aldehydes which can modify cysteine, lysine, histidine, and tyrosine residues of apo B-100 (Figure 1.8 and Figure 1.9).[25-27] Oxidative modification of apo B-100 may deteriorate the function of apo B-100 as a ligand to LDL receptors. In the process of atherosclerosis, oxidized LDL (oxLDL) are phagocytosed by monocyte-derived macrophages through the scavenger receptor in the intima.[28] Lipid-loaded macrophages became foam cells and accumulate to form fatty streak and fibrous plaque (Figure 1.10).[29]

In the present study, we investigated antioxidant effect of OFA on LDL oxidation by ROS and RNS *in vitro*.

Table 1.1. Contents of polyphenols in oolong tea and green tea extracts.[7)]

Components (mg/g)	**Oolong tea**	**Green tea**
Flavan-3-ol without galloyl moiety		
Gallocatechin	30	43
Epigallocatechin	6	25
Cathechin	10	5
Epicatechin	2	8
Flavan-3-ol with galloyl moiety		
Epigallocatechin gallate	14	29
Gallocatechin gallate	16	19
Epicatechin gallate	3	6
Cathechin gallate	7	5
Oolong tea polymerized polyphenols	114	-

(A)



(B)

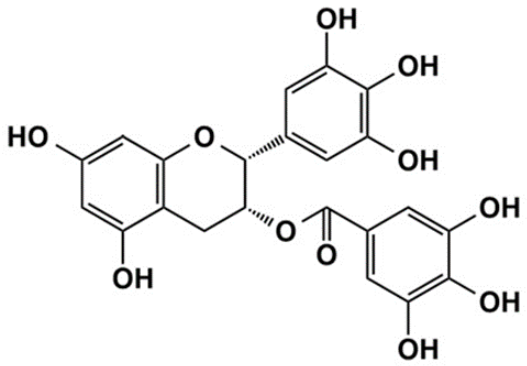


Figure 1.1. Structure of oolonghomobisflavan A (A),[46] and epigallocatechin gallate (B).[8]

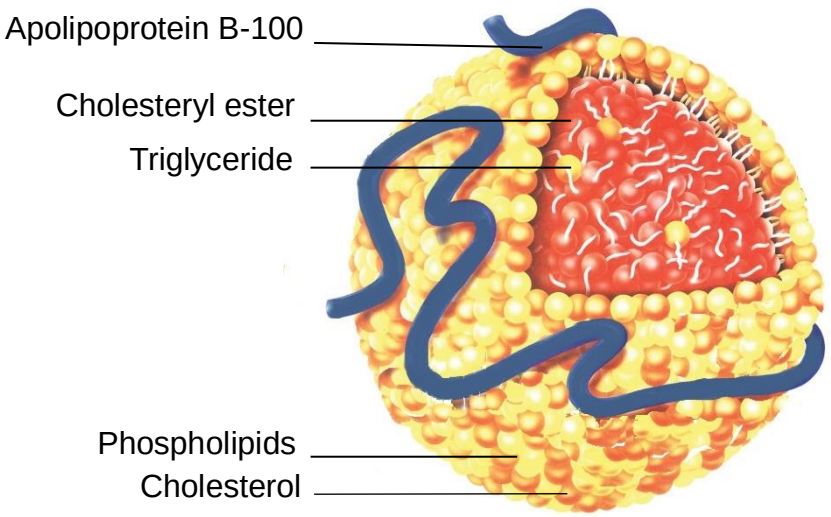


Figure 1. 2. Structure of low density lipoprotein.[11)]

Low density lipoprotein consists of apolipoprotein B-100 and cholesterol esters, triacylglycerols, and phospholipid monolayer containing unesterified cholesterol.[11)]

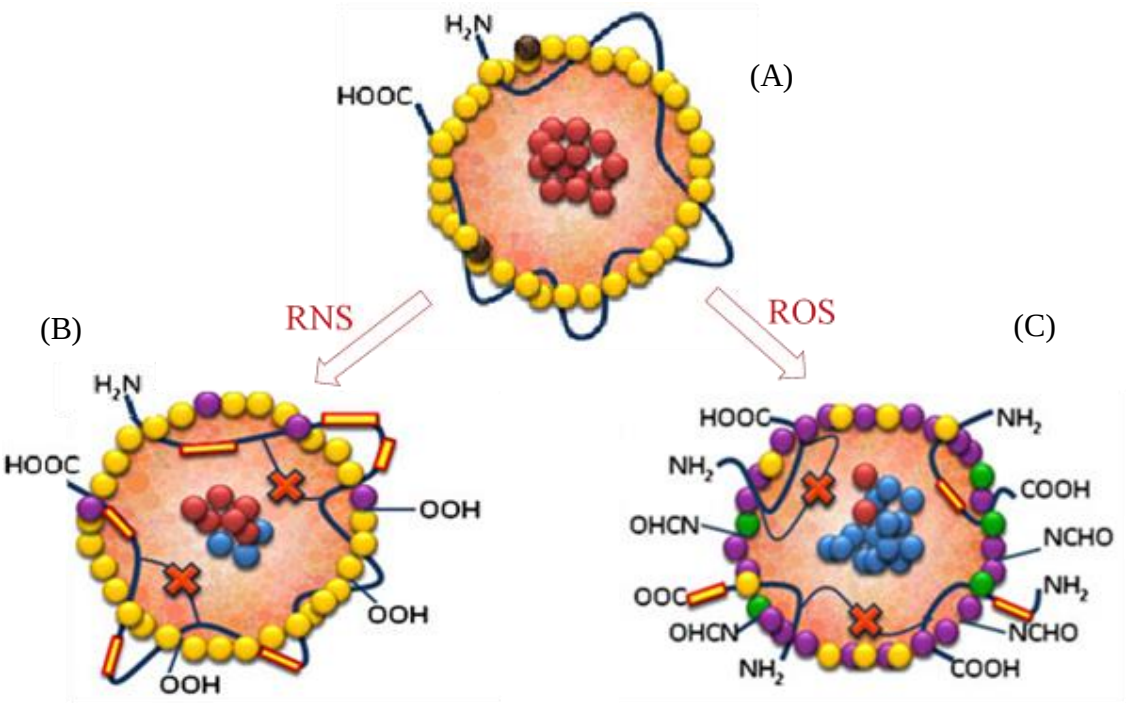


Figure 1. 3. Forms of oxidized low density lipoprotein.[14)]

● Cholesteryl ester ● Oxidized cholesteryl ester ● Antioxidants ○ Phospholipid

● Oxidized phospholipid ● Lyso phospholipid —▭— Oxidized protein

(A) Native LDL with lysine residues of apo B-100 (B) Reactive nitrogen species (RNS) induced oxidized low density lipoprotein (oxLDL) with more apo B-100 and less lipid peroxidation (C) Reactive oxygen species (ROS) induced oxLDL with extensive apo B-100 and lipid peroxidation.

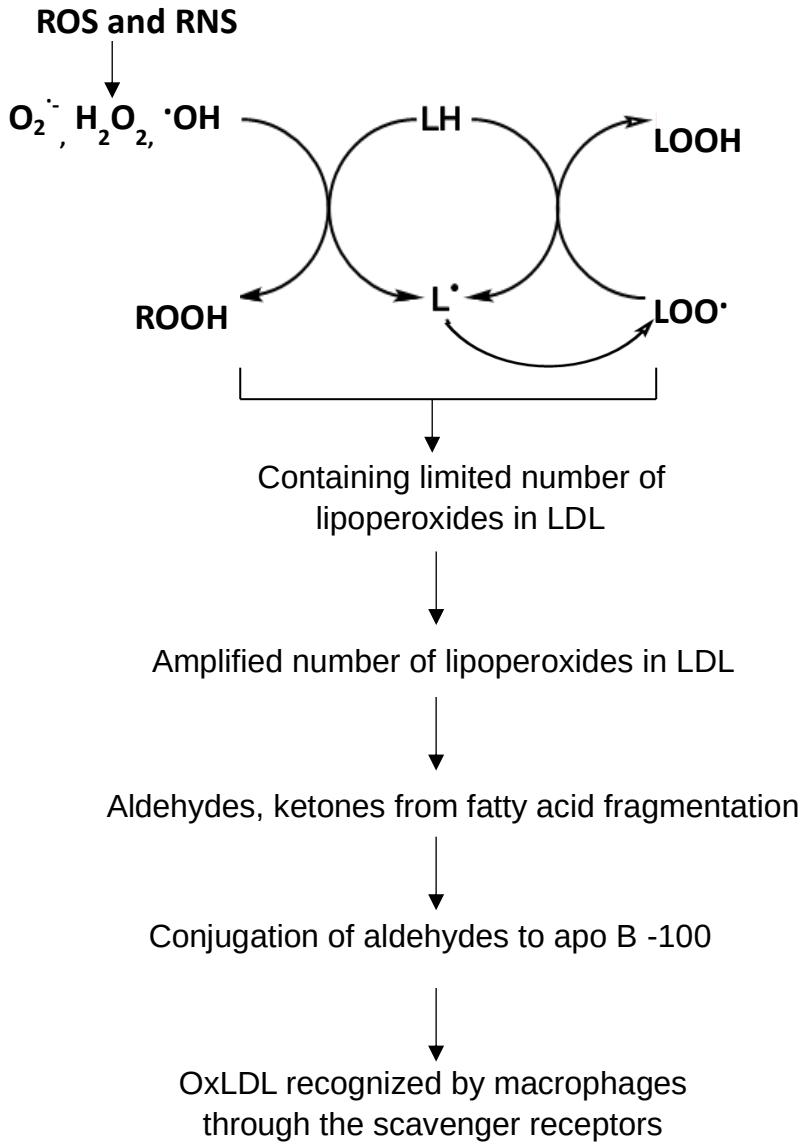


Figure 1. 4. The mechanism of LDL oxidation.

Reactive species leading to initiation of lipid peroxidation in LDL. LDL oxidation involves lipid peroxidation and modification of apolipoprotein.

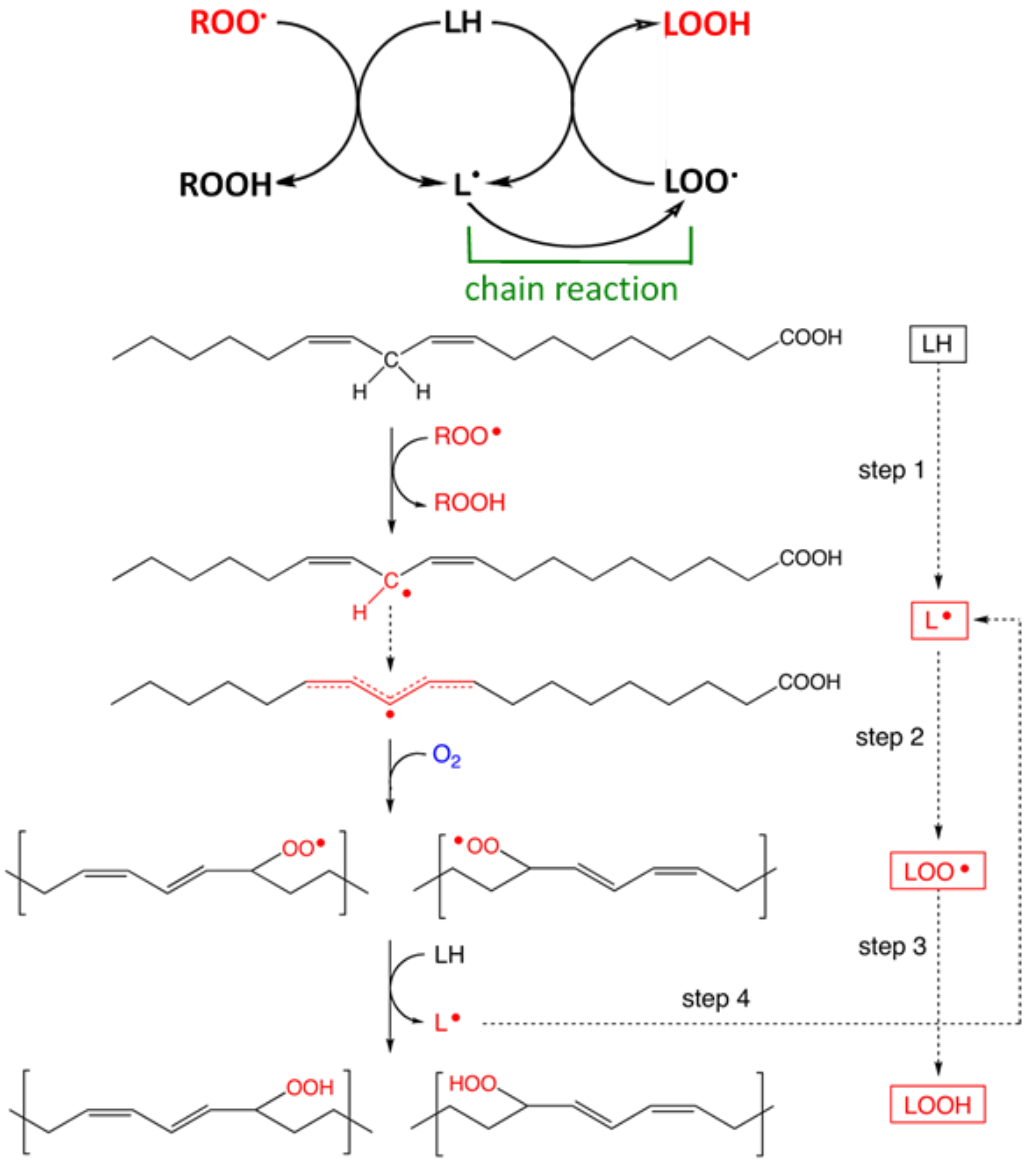


Figure 1. 5. Peroxyl radical-mediated lipid peroxidation of polyunsaturated fatty acid. Polyunsaturated fatty acid side chain (LH) containing active methylene hydrogen atom which is reactive with free radicals such as peroxyl radical (ROO·). The resulting, lipid radical (L·) adds rapidly to molecular oxygen (O_2) to generate a lipid peroxyl radical (LOO·). That itself can propagate the chain by reacting with another polyunsaturated fatty acid to generate lipid radical (L·) and lipid hydroperoxide (LOOH).

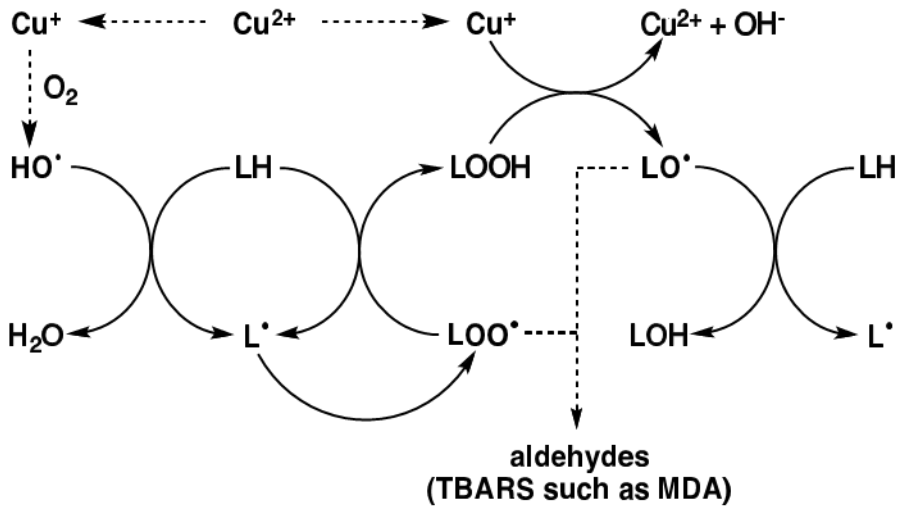


Figure 1. 6. Cu^{2+} -catalyzed lipid peroxidation.

Cu^{2+} can be reduced to Cu^{+} in the presence of superoxide ($O_2^{\cdot-}$) or endogenous α-tocopherol. Cu^{+} is capable of catalyzing the hydroxyl radicals ($OH^{\cdot}$) from hydrogen peroxide (H_2O_2) by Haber-Weiss reaction. Alkoxyl radical ($LO^{\cdot}$) is generated from lipid hydroperoxide (LOOH) with Cu^{+}, resulting in generation of secondary products such as aldehydes.

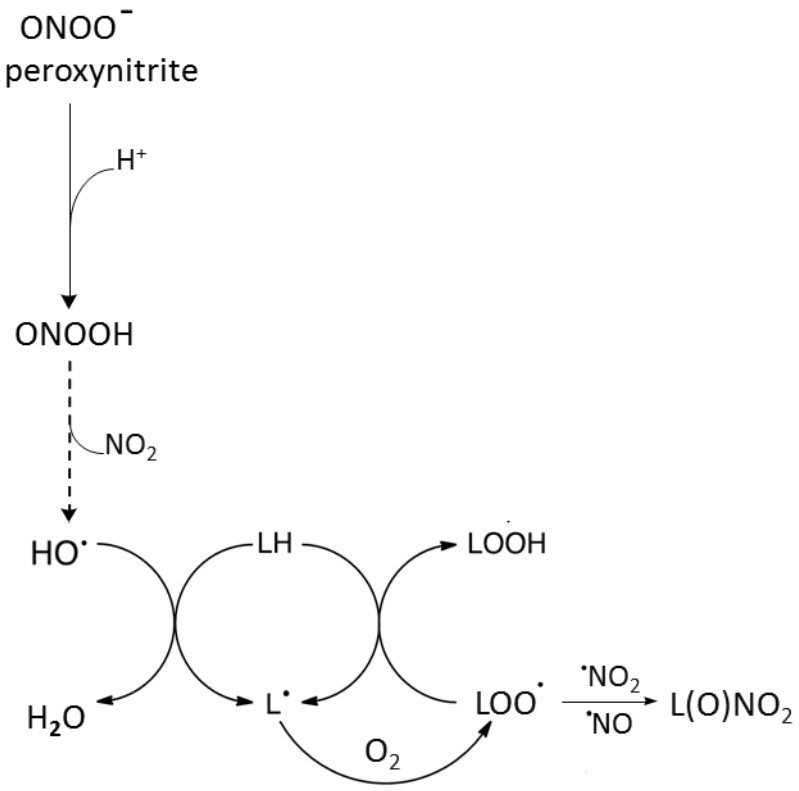


Figure 1. 7. Peroxynitrite-mediated lipid peroxidation.[24)]

Peroxynitrite ($ONOO^-$) at physiological pH to peroxynitrous acid (ONOOH) that decomposes to hydroxyl radicals (·OH) and $\cdot NO_2$. Decomposed hydroxyl radicals initiate polyunsaturated fatty acid.

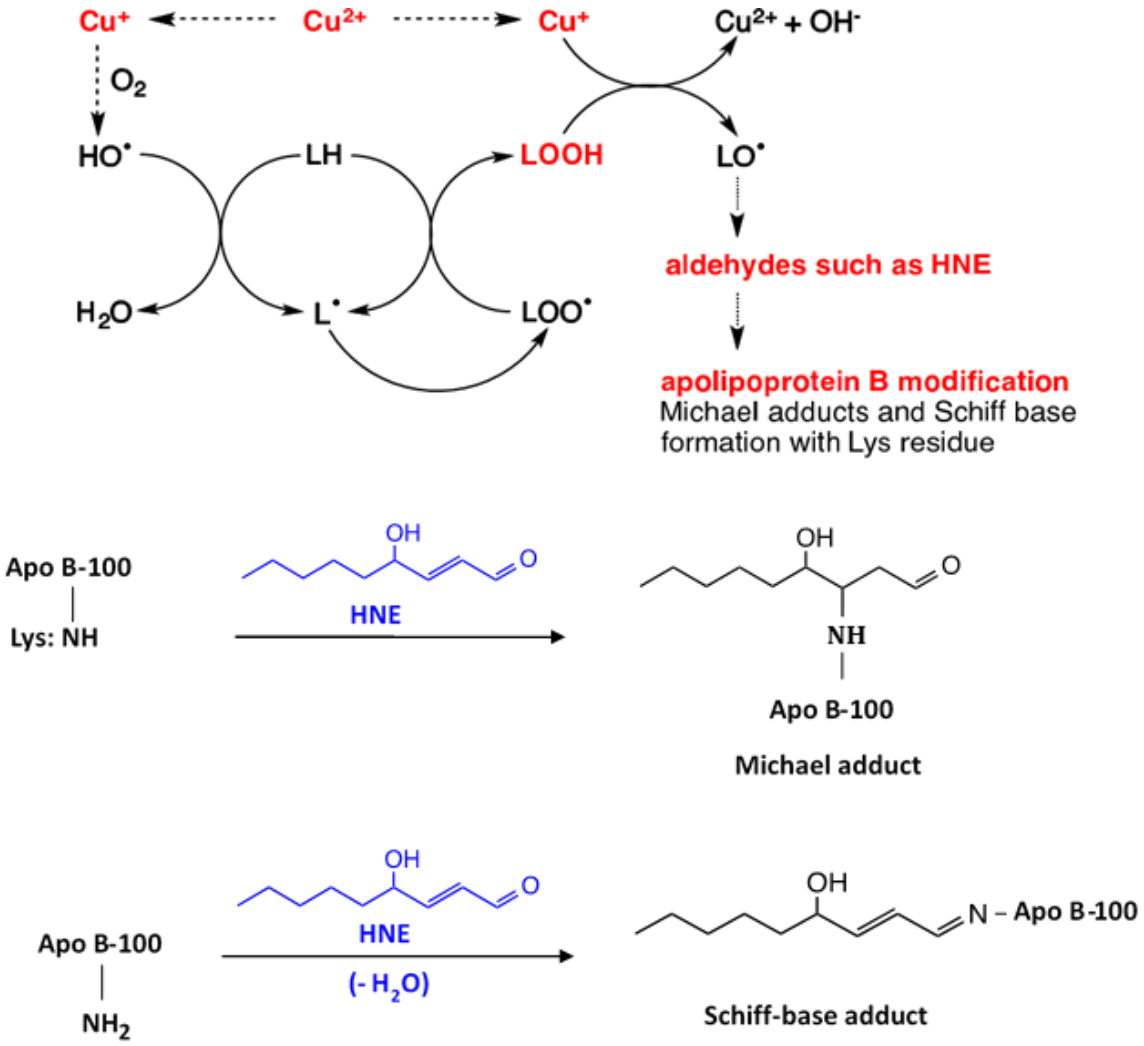


Figure 1. 8. 4-Hydroxy-2-nonenal **(**HNE) modifications for apolipoprotein B-100.[25)] Aldehydes such as HNE modify apoB-100, that is formed micheal adducts and schiff-base adducts.

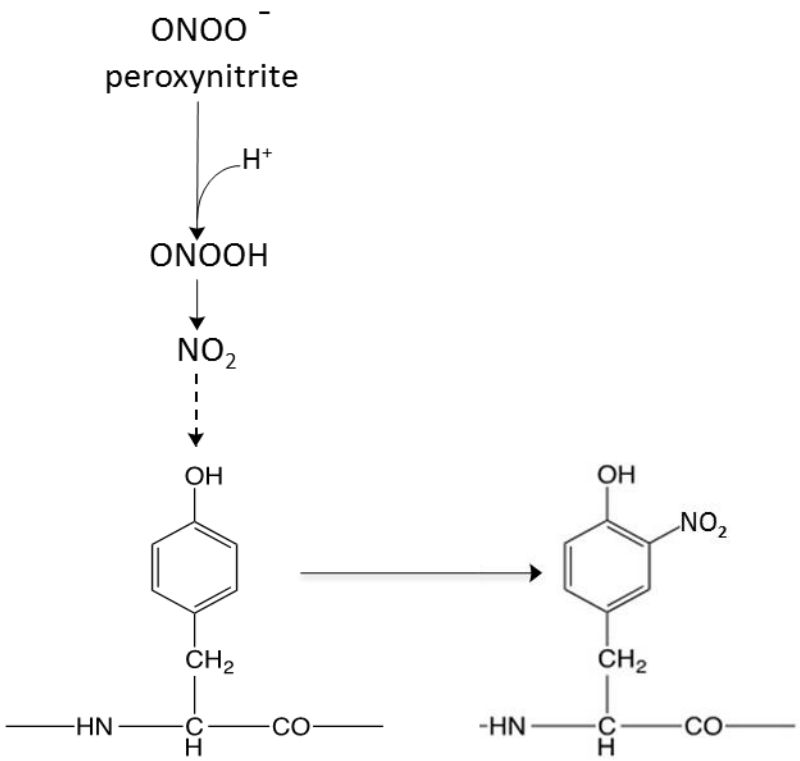
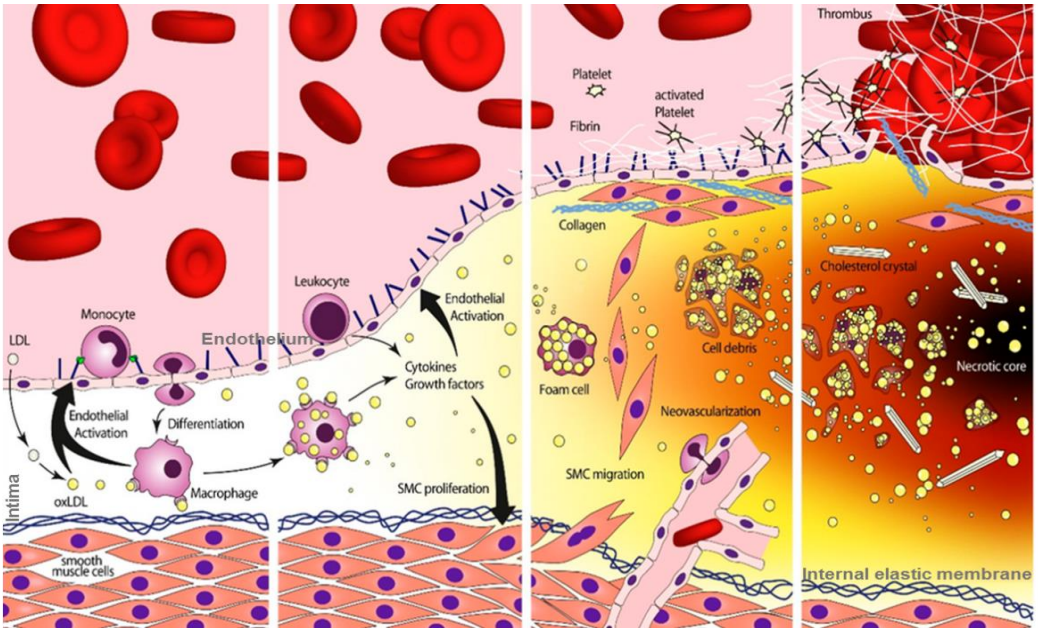


Figure 1. 9. Peroxynitrite-induced nitrotrysine formation.[61)]

Peroxynitrite reacted with tyrosine residues in apo B-100 leading to the formation of nitrotyrosine.



(A) Lesion initiation (B) Fatty streak (C) Fibrous plaque (D) Thrombosis

Figure 1. 10. Atherosclerotic plaque progression.[29)]

(A) Plasma LDL is entered across the intact endothelium and undergoes oxidative modification. (B) OxLDL particles are recognized by macrophage scavenger receptors. (C) Macrophages take up oxLDL, leading to the formation of foam cells with cytokines secretion. (D) Foam cells can undergo apoptosis and release cell-debris and lipids, forming a necrotic core.

Chapter 2

Radical scavenging capacity of oolonghomobisflavan A (OFA) in solution

2.1 Objectives

The biological effects of OTPP have attracted considerable attention over the past few years, but still the mechanisms remain unclear. First, we investigated the radical scavenging capacity of OFA.

2.2 Materials and Methods

2.2.1 Materials

OFA was purchased from Nagara Science Co. (Gifu, Japan). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Wako Pure Chemical Industries (Osaka, Japan). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from EMD Chemicals, Inc. (San Diego, CA, USA). All other chemicals were of reagent grade.

2.2.2 DPPH radical scavenging assay

Radical scavenging activity of OFA in solution was determined by DPPH method (Figure 2.1).[30] Methanol solutions (400 μL) containing various concentrations of OFA (2-5 μM) were mixed with 400 μL of 200 μM DPPH in methanol. The absorbance of the reaction mixture was measured at 517 nm after incubation at room temperature for 30 min in the dark. Trolox was used as a reference antioxidant.

2.3 Results

2.3.1 DPPH radical scavenging capacity of OFA in solution

OFA and Trolox dose-dependently scavenged DPPH radicals in methanol/water solution (Figure 2.2). The half maximal inhibitory concentration (IC_{50}) of OFA and Trolox was 2.99 μM/mL and 23.7 μM/mL, respectively. The IC_{50} of OFA has a higher radical scavenging capacity as compared to Trolox.

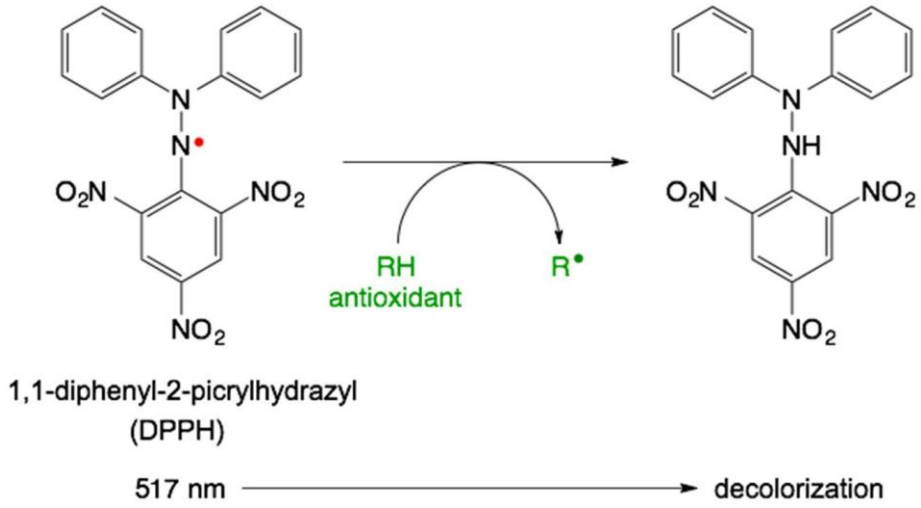


Figure 2.1. Structure of 2,2-diphenyl-1-picrylhydrazyl (DPPH).

DPPH is a stable, nitrogen-centered free radical which has an absorption maximum at 517 nm in solution. When DPPH radicals are scavenged by antioxidants, absorbance at 517 nm decreases.

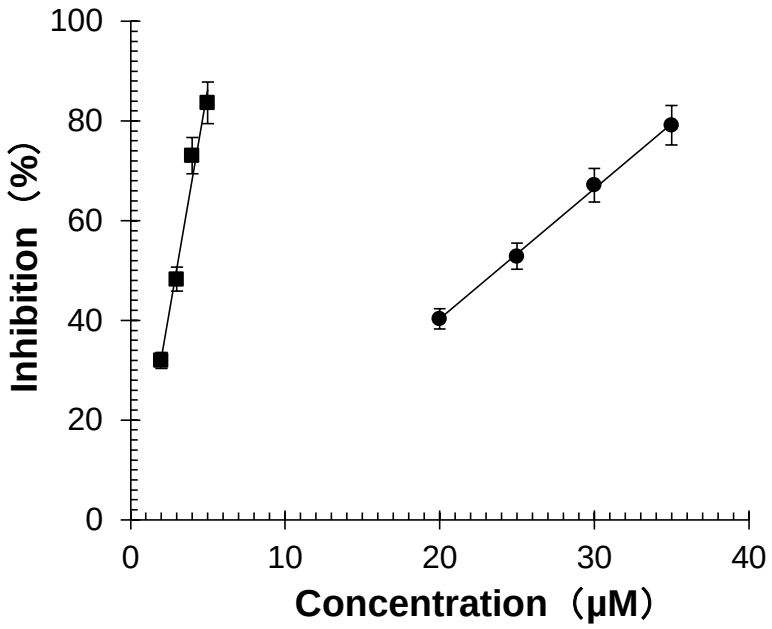


Figure 2.2. DPPH radical scavenging capacity of OFA.

DPPH was mixed with OFA and Trolox in methanol and incubated for 30 min at room temperature in the dark. The absorbance of the solutions was measured at 517 nm. Data are expressed as means ± SD (n = 3). OFA (■), Trolox (●).

Chapter 3

Effect of OFA on low-density lipoprotein (LDL) oxidation by reactive oxygen species (ROS)

3.1 Objectives

The results in Chapter 2, OFA exhibited strong radical scavenging capacity. In this chapter, we investigated the effects of OFA on lipid peroxidation by peroxyl radicals and copper *in vitro*. Also, the inhibitory effect of OFA on apo B-100 modification in LDL oxidized by copper was analyzed *in vitro*.

3.2 Materials and Methods

3.2.1 Materials

2,4-Dinitrophenylhydrazine (DNPH), 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of reagent grade.

3.2.2 Isolation and purification of LDL

Human LDL was prepared by discontinuous density gradient ultracentrifugation using KBr.[22] Human blood (200 mL) was collected from a healthy volunteer with informed consent after fasting overnight using 0.4% citric acid as an anti-coagulant, and blood plasma was separated by centrifugation at 1,200 × *g* at 4°C for 20 min. KBr solution (d = 1.019, 8 mL) was layered on 16 mL of the plasma and centrifuged at 100,000 × *g* at 16°C for 16 h. The top lipid layer was removed and density of the lower layer was adjusted to 1.063 by adding KBr. KBr solution (d = 1.063, 3.5 mL) was added to 21 mL of the lower fraction and centrifuged at 100,000 × *g* at 16°C for 20 h. The top layer including LDL fraction (d = 1.019 to 1.063) was then ultrafiltered and dialyzed against phosphate-buffered saline (PBS, pH 7.4) treated with chelating resin (Sigma-Aldrich Co., St. Louis, MO, USA) at 4°C. Protein concentration of LDL was measured by Lowry's method.[31] LDL fraction was kept at 4°C under nitrogen gas.

3.2.3 Peroxyl radical-mediated oxidation of LDL

LDL (200 μg protein/mL) was pre-incubated with OFA (0.5, 1, and 2 μM) in PBS containing 10 mM diethylene triamine pentaacetic acid (DTPA) at 37°C for 5 min. Oxidation of LDL was induced by 5 mM of AAPH, an aqueous peroxyl radical generating reagent, at 37°C. Aliquots (50 μL) of the reaction mixture were mixed with 0.9 mL of 2 mM butyl hydroxyl anisole in methanol at intervals. *n*-Hexane (1.5 mL) was added to oxLDL in methanol to extract neutral lipids. After centrifugation at 1,200 × *g* at 4°C for 5 min, the upper layer was collected and extraction with *n*-hexane from the bottom layer was repeated again. The upper layer extracts were pooled and evaporated, and the residue was dissolved in 100 μL of 2-propanol. Cholesteryl ester hydroperoxide (CE-OOH) in the sample was analyzed by reversed-phase high-performance liquid chromatography (HPLC) using an InertSustian C8 column at 40°C (150 × 4.6 mm i.d.; GL Science Inc., Tokyo, Japan) with methanol as an eluent at a flow rate of 1.0 mL/min.[32] The concentration of CE-OOH was detected at 235 nm and calculated using cholesteryl linoleate hydroperoxide (Cyaman Chemical Company, Ann Arbor, MI, USA) as an authentic standard.

3.2.4 Metal ion-catalyzed oxidation of LDL

LDL (200 μg protein/mL) was pre-incubated with OFA (0.5, 1, and 2 μM) in PBS at 37°C for 5 min. LDL oxidation was initiated by adding 5 μM $CuCl_2$ at 37°C. Aliquots (50 μL) of the reaction mixture were mixed with 450 μL of 0.2% 2-thiobarbituric acid and heated at 95°C for 60 min. After centrifugation at 20,000 × *g* at 4°C for 5 min, 2-thiobarbituric acid reactive substances (TBARS) in the supernatant were determined by reversed-phase HPLC with fluorescence detection (excitation at 515 nm, emission at 553 nm).[33] The samples were eluted with acetonitrile/water (30:70, v/v) at 1.0 mL/min on InertSustain C18 column (150 × 4.6 mm i.d.; GL Science Inc.) at 40°C. 1,1,3,3-Tetramethoxypropane was used as a standard.

3.2.5 Apolipoprotein B-100 (apo B-100) analysis in oxidized LDL

LDL (200 μg protein/mL) was oxidized by 5 μM $CuCl_2$ at 37°C for 3 h in the presence

of 2 μM OFA as described above. OxLDL was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using an acrylamide slab gel system (AnykD TGX gel; Bio-Rad Laboratories, Hercules, CA, USA) according to Laemmli's method.[34] The gel was stained with Coomassie Brilliant Blue (CBB). Proteins in the other gel were transferred to polyvinylidene difluoride membrane for Western blotting. The membrane was incubated with serum-free blocking buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA) and washed with 0.05% Tween-Tris-buffered saline (TBS). The membrane was incubated with anti-human apo B-100 monoclonal (6H12) antibody (MP Biomedicals, Santa Ana, CA, USA) for 1 h at room temperature. After washing with 0.05% Tween-TBS, the membrane was incubated with goat anti-mouse IgG-Fc conjugated with horseradish peroxidase (Bethyl Laboratories, Inc., Montgomery, TX, USA) for 1 h at room temperature. The immunoreactive substances were detected with the enhanced chemiluminescence (ECL) reagent (GE Healthcare, Little Chalfont, UK).

3.2.6 Protein carbonyl analysis of oxidized LDL

LDL (200 μg protein/mL) was oxidized by 5 μM $CuCl_2$ at 37°C for 3 h in the presence of OFA (0.5, 1, and 2 μM). Protein carbonyls in oxLDL was derivatized with DNPH and analyzed by SDS-PAGE and Western blotting as previously described.[35] Briefly, rabbit anti-dinitrophenyl antisera (Dako Cytomation, Glostrup, Denmark) and goat anti-rabbit IgG-Fc conjugated with horseradish peroxidase (Bethyl Laboratories Inc.) were used as primary and secondary antibodies, respectively.

3.2.7 Heparin-binding activity of apo B-100 in oxidized LDL

LDL (200 μg protein/mL) was oxidized by 5 μM $CuCl_2$ at 37°C for 3 h in the presence of 2 μM OFA. OxLDL (20 μg protein/100 μL) was incubated with 100 μL of heparin Sepharose 6 Fast Flow (GE Healthcare) for 3 h at 4°C. The heparin Sepharose beads were washed 10 times with 1 mL of 0.05% Tween-TBS and mixed with 100 μL of SDS-PAGE sample buffer. After heating at 95°C for 5 min, proteins in the supernatant were analyzed by SDS-PAGE and CBB staining.

3.3 Results

3.3.1 Effect of OFA on lipid peroxidation of LDL by ROS

We measured CE-OOH as the major product of lipid peroxidation of LDL induced by AAPH.[22] Figure 3.2 shows effect of OFA on peroxyl radical (AAPH)-mediated lipid peroxidation of LDL. CE-OOH concentration in the absence OFA (control) increased with time and attained 8.7 μM at 4 h. On the other hand, OFA dose-dependently suppressed CE-OOH accumulation, 2 μM of OFA exhibited strongest inhibition.

TBARS is a widely-used indicator to detect secondary oxidation products in transition metal ion-catalyzed lipid peroxidation. As shown in Figure 3.3, TBARS time-dependently accumulated up to 12.5 μM at 4 h in the absence of OFA (control). On the contrary, OFA dose-dependently inhibited TBARS formation; especially 2 μM OFA almost inhibited TBARS formation.

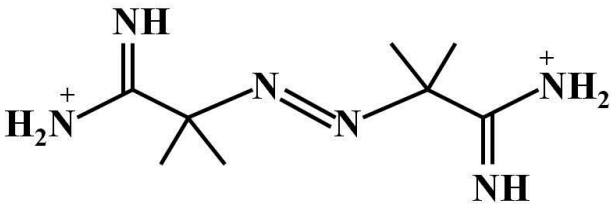
3.3.2 Effect of OFA on protein oxidation of LDL by ROS

Figure 3.4 illustrates the effect of OFA on oxidative modification of apoB-100 in LDL oxidized by Cu^{2+}, which was analyzed by SDS-PAGE with CBB staining (Figure 3.4 A) and Western blot analysis using anti-human apoB-100 antibody (Figure 3.4 B). A single band with molecular weight greater than 250 kDa was observed in native LDL (lane 1 in Figure 3.4 A). However, the band was not detected in oxLDL (lane 2 in Figure 3.4 A), whereas a weak smear band was observed. Although the single band remained in oxLDL with 2 μM OFA (lane 3 in Figure 3.4 A). As in Figure 3.4 A, apo B-100 in Figure 3.4 B was observed as a single band with molecular weight more than 250 kDa in native LDL (lane 1). The oxidative modification of apoB-100 was detected as a ladder band at 100-150 kDa (lane 2 in Figure 3.4 B). Whereas, the oxidative modification of apoB-100 in oxLDL was suppressed by 2 μM OFA (lane 3 in Figure 3.4 B).

Figure 3.5 indicates the effect of OFA on protein carbonyl formation in LDL oxidized by Cu^{2+}. Protein carbonyls that are derivertized with DNPH can be detected by western blotting using anti-dinitrophenyl antibody.[36,37] Protein carbonyls were not observed in native LDL (lane 1), while protein carbonyls were detected in oxLDL as a smear band at

high molecular weight (lane 2). OFA dose-dependently inhibited protein carbonyl formation (lane 3, 4, and 5) in oxLDL, and 2 μM OFA mostly suppressed protein carbonyl formation.

The effect of OFA on heparin-binding activity of apoB-100 in LDL oxidized by Cu^{2+} was examined by SDS-PAGE with CBB staining (Figure.3.6). A single band greater than 250 kDa was detected in native LDL (lane 1), although no protein band was observed in oxLDL by Cu^{2+} for 3 h (lane 2). A weak single band was remained (lane 3) in oxLDL with 2 μM OFA.



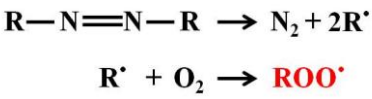


Figure 3.1. Structure of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH). AAPH is a water-soluble azo compound, which generating nitrogen and radicals ($R^{\bullet}$) undergoes thermolysis. $R^{\bullet}$ reacts with O_2 and peroxyl radical ($ROO^{\bullet}$) is formed.

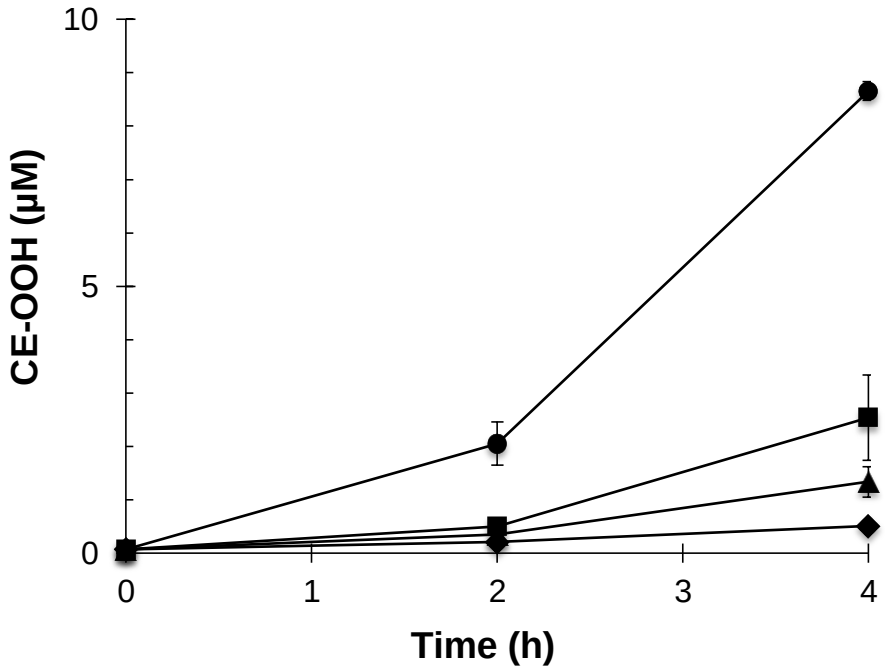


Figure 3.2. Effect of OFA on peroxyl radical-mediated lipid peroxidation of LDL. LDL (200 μg protein/ml) oxidation was initiated by 5 mM AAPH at 37°C. CE-OOH was determined by HPLC with UV detection. Data are expressed as means ± SD (n = 3). Control (●), 0.5 μM OFA (■), 1 μM OFA (▲), 2 μM OFA (◆).

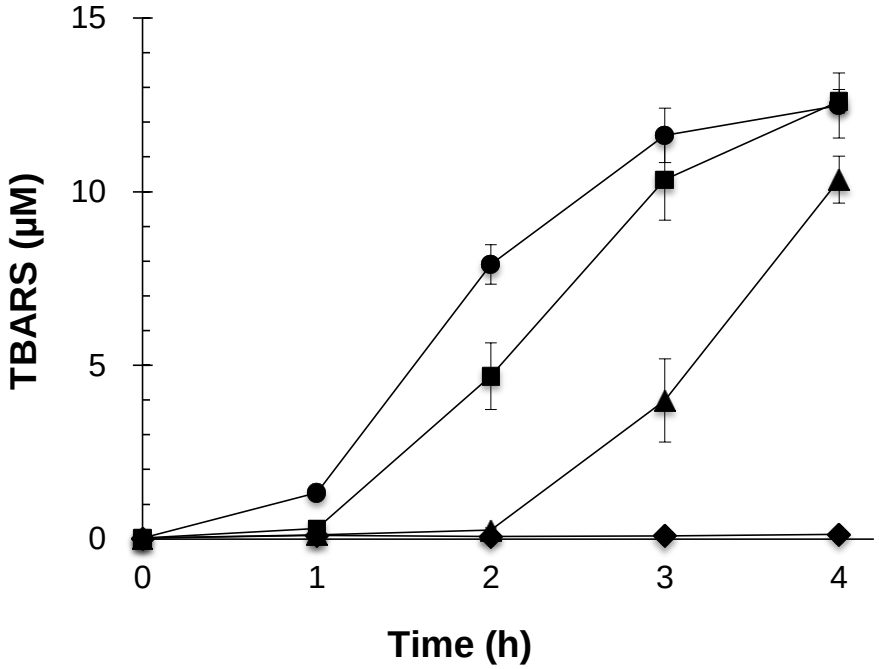


Figure 3.3. Effect of OFA on transition metal ion-catalyzed lipid peroxidation of LDL. LDL in PBS was oxidized by 5 μM Cu^{2+} at 37°C. TBARS was analyzed by HPLC with fluorescence detection. Data are expressed as means ± SD (n = 3). Control (●), 0.5 μM OFA (■), 1 μM OFA (▲), and 2 μM OFA (◆).

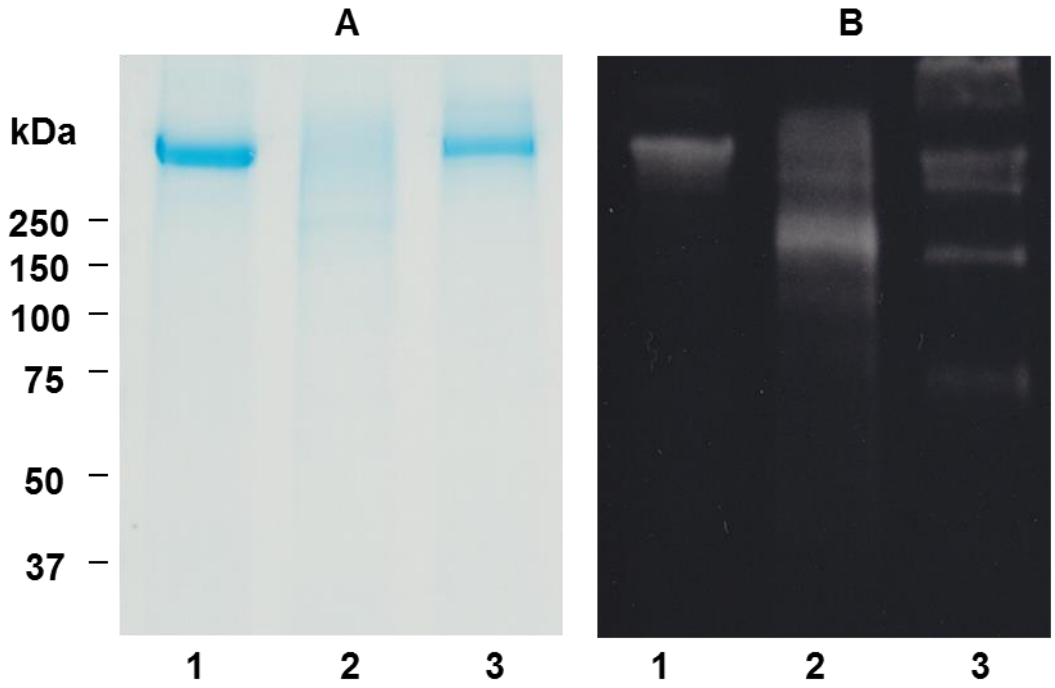


Figure 3.4. Effect of OFA on apo B-100 modification in oxidized LDL.

LDL in PBS was oxidized by Cu^{2+} at 37°C for 3 h. (A) OxLDL was analyzed by SDS-PAGE and CBB staining. (B) OxLDL was determined by Western blotting using anti-human apo B-100 antibody. Immunoreactive bands were detected with ECL reagent. Lane 1, native LDL; lane 2, LDL oxidized by Cu^{2+}; lane 3, LDL oxidized by Cu^{2+} in the presence of 2 μM OFA.

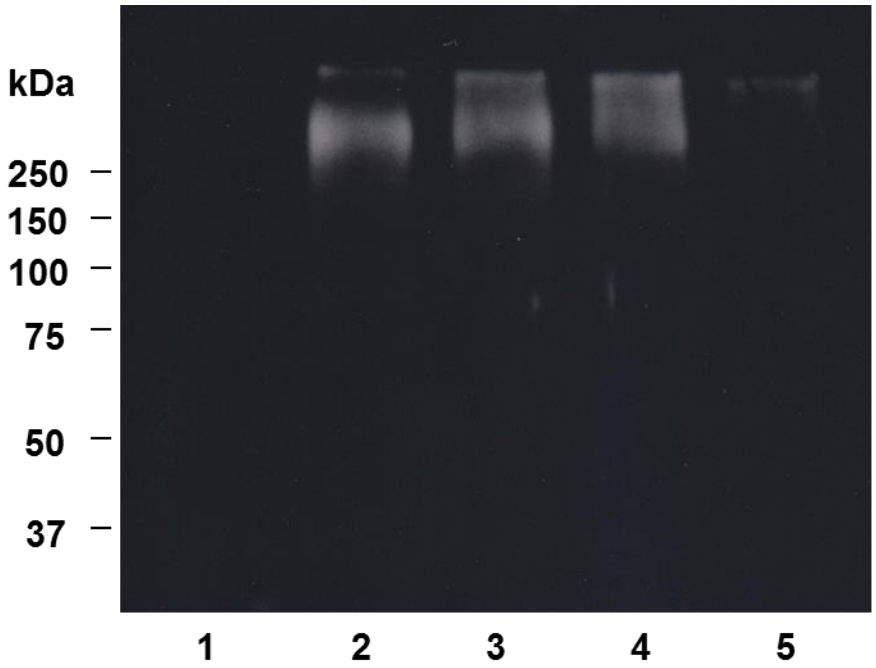


Figure 3.5. Effect of OFA on protein carbonyl formation in oxidized LDL.

LDL in PBS was oxidized by Cu^{2+} at 37°C for 3 h. OxLDL was treated with DNPH and analyzed by SDS-PAGE and Western blotting using rabbit anti-dinitrophenyl antisera as primary antibody and by ECL detection. Lane 1, native LDL; lane 2, LDL oxidized by Cu^{2+}; lanes 3, 4, and 5, LDL oxidized by Cu^{2+} in the presence of 0.5, 1, and 2 μM of OFA, respectively.

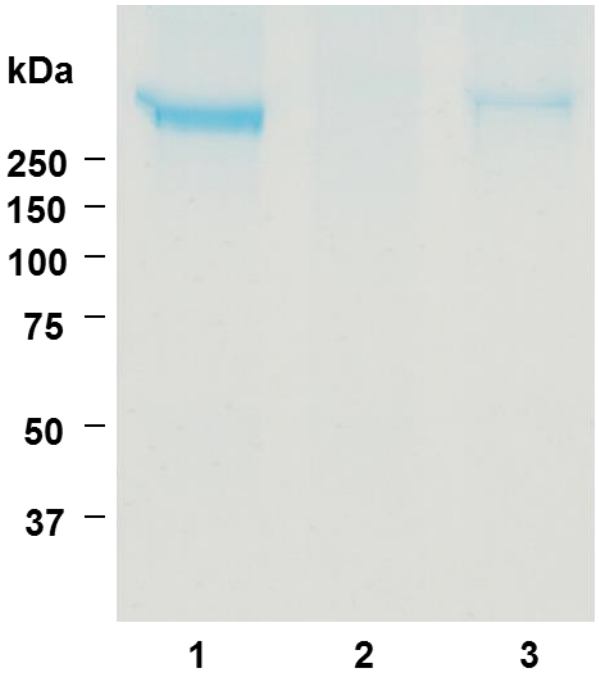


Figure 3.6. Effect of OFA on heparin-binding activity of apo B-100 in oxidized LDL. LDL in PBS was oxidized by Cu^{2+} at 37°C for 3 h. OxLDL was incubated with 100 μL heparin Sepharose at 4°C for 3 h. After washing heparin Sepharose beads with Tween-TBS, proteins bound to the beads were subjected to SDS-PAGE and CBB staining. Lane 1, native LDL; lane 2, LDL oxidized by Cu^{2+}; lane 3, LDL oxidized by Cu^{2+} in the presence of 2 μM OFA.

Chapter 4

Effect of OFA on LDL oxidation by reactive nitrogen species (RNS)

4.1 Objectives

RNS such as peroxynitrite ($ONOO^-$) is also involved in inducing oxidative stress in human body. Therefore, we evaluated the antioxidant effects of OFA on LDL oxidation induced by RNS *in vitro*; same as before both lipid peroxidation and oxidative modification of apo B-100 were analyzed as the oxidative markers.

4.2 Materials and Methods

4.2.1 Materials

3-(4-Morpholinyl) sydnonimine (SIN-1) was supplied from Dojindo Molecular Technologies Inc. (Kumamoto, Japan). All other chemicals were of reagent grade.

4.2.2 Peroxynitrite-mediated oxidation of LDL

LDL (200 μg protein/mL) was pre-incubated with OFA (2, 4, and 8 μM) in PBS containing 10 mM DTPA at 37°C for 5 min. LDL was oxidized by 1 mM SIN-1, a water-soluble peroxynitrite generator, at 37°C. Determination of CE-OOH in oxLDL was performed as previously described.

4.2.3 Peroxynitrite-mediated nitrotyrosine analysis

LDL (200 μg protein/mL) was pre-incubated with 8 μM OFA in PBS containing 10 mM DTPA at 37°C for 5 min. LDL was oxidized by 1 mM SIN-1 at 37°C. Nitrotyrosine in oxLDL was analyzed by SDS-PAGE and Western blotting using primary mouse anti-nitrotyrosine monoclonal (CC.22.8C7.3) antibody (Cayman Chemical Company) and secondary goat anti-mouse IgG-Fc conjugated with horseradish peroxidase (Bethyl Laboratories Inc.). The immune reactive substances were detected with ECL reagent.

4.3 Results

4.3.1 Effect of OFA on lipid peroxidation of LDL by RNS

Figure 4.2 shows the effect of OFA on peroxynitrite-mediated lipid peroxidation of LDL induced by SIN-1. SIN-1 generates peroxynitrite by simultaneously releasing nitric oxide and superoxide. Concentration of CE-OOH time-dependently increased in the absence of in OFA (control). OFA dose-dependently suppressed CE-OOH formation in LDL oxidized by peroxynitrite, in particular 8 μM OFA exhibited strong inhibition.

4.3.2 Effect of OFA on protein oxidation of LDL by RNS

Figure 4.3 shows the effect of OFA on oxidative modification of apoB-100 and nitrotrysine formation in LDL oxidized by SIN-1. SDS-PAGE analysis with CBB staining and Western blot analysis with primary anti-nitrotrysine antibody are exhibited in Figure 4.3 A and B, respectively. A single band with molecular weight greater than 250 kDa was observed in native LDL (lane 1 in Figure 4.3 A), although smear band was detected in oxLDL induced by SIN-1 (lane 2 in Figure 4.3 A). A weak single band was observed in oxLDL with 8 μM OFA (lane 3 in Figure 4.3 A). As in Figure 4.3 B, nitrotrysine was not detected in native LDL (lane 1 in Figure 4.3 B). Nitrotrysine was detected at greater than 250 kDa in oxLDL as a single band (lane 2 in Figure 4.3 B), while the nitrotrysine formation was suppressed by 8 μM OFA (lane 3 in Figure 4.3 B).

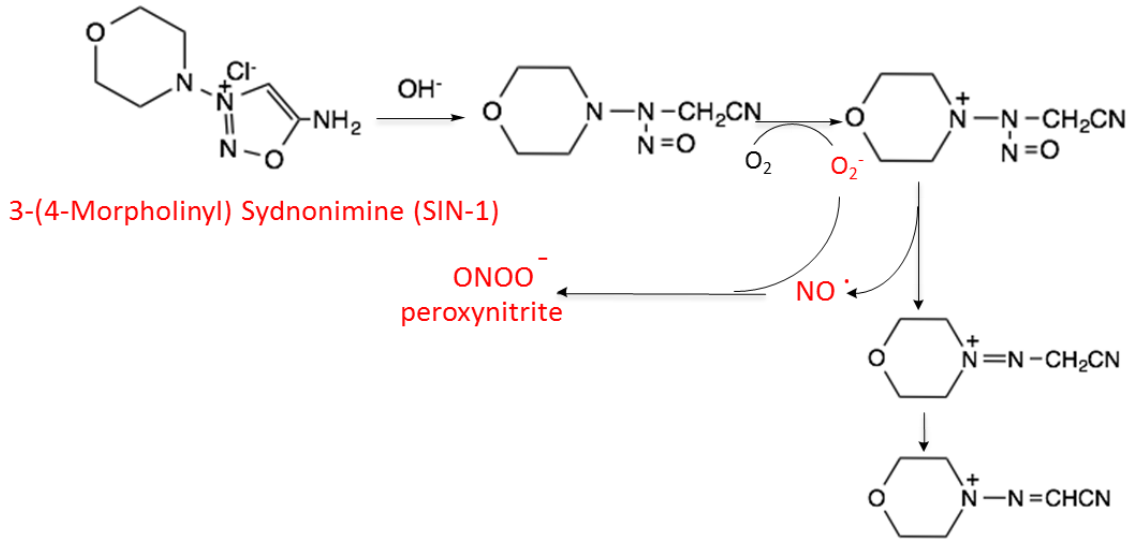


Figure 4.1. Structure of 3-(4- morpholinyl)sydnonimine (SIN-1).

SIN-1 is a metabolite of the vasodilator molsidomine and generates peroxynitrite by simultaneously releasing nitric oxide and superoxide.

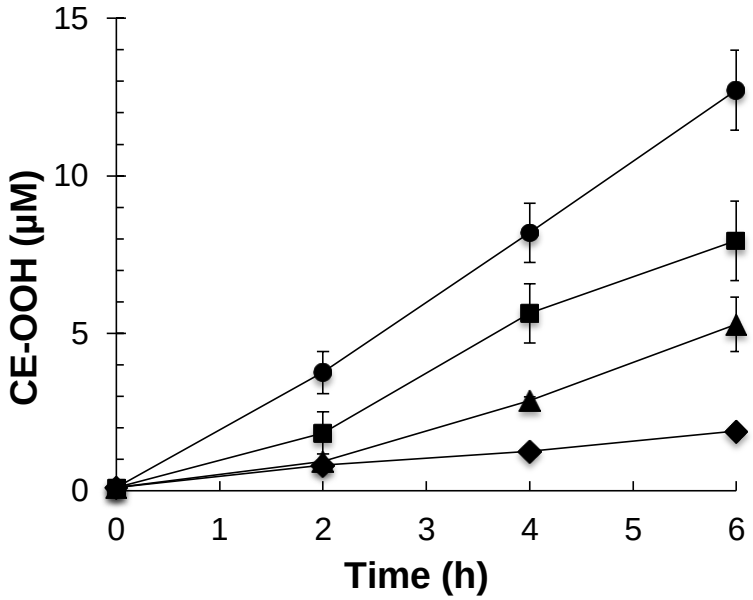


Figure 4.2. Effect of OFA on peroxynitrite-mediated lipid peroxidation of LDL.

LDL in PBS was oxidized by 1 mM SIN-1 at 37°C. CE-OOH was analyzed by HPLC with UV detection. Data are expressed as means ± SD (n = 3). Control (●), 2 μM OFA (■), 4 μM OFA (▲), and 8 μM OFA (◆).

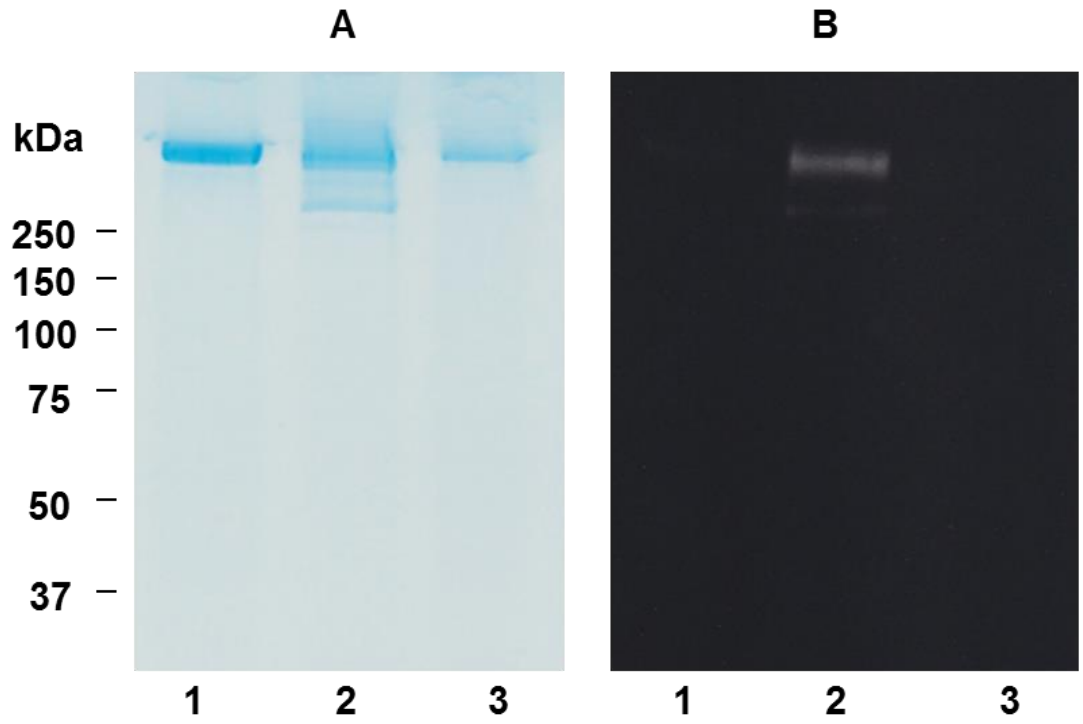


Figure 4.3. Effect of OFA on nitrotyrosine formation in oxLDL.

LDL in PBS was oxidized by 1 mM SIN-1 at 37°C for 4 h. (A) OxLDL was analyzed by SDS-PAGE and CBB staining. (B) OxLDL was determined by Western blotting using anti-nitrotyrosine antibody and by ECL detection. Lane 1, native LDL; lane 2, LDL oxidized by SIN-1; lane 3, LDL oxidized by SIN-1 in the presence of 8 μM OFA.

Chapter 5

Discussion

The polyphenols in oolong tea are different from those in green tea and black tea.[38] Green tea leaves are rich in monomeric polyphenols known as flavan-3-ols, including (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCG).[39,40] Black tea involves lower amounts of monomeric polyphenols and higher concentration of polymeric polyphenols such as theaflavins, thearubigins.[40] The concentration of polymeric polyphenols in black tea is higher than that of oolong tea.[41] Oolong tea contains numerous kinds of polymerized-polyphenols.[38] During semi-fermentation to manufacture oolong tea, monomeric polyphenols such as catechins are converted to oligomeric flavan-3-ols by polyphenol oxidases.[38] Several studies have reported the antioxidant effects of oolong tea. Xie *et al*.[42] reported that oolong tea extract exerts higher antioxidative activity than black tea *in vitro*. Yen *et al.*[43] showed that oolong tea extract exhibits stronger radical scavenging activity than that of green and black teas. However, the active compounds in oolong tea have not been identified. The contents of polymerized polyphenols in oolong tea leaves depend on the producing procedures such as fermentation. Komatsu *et al.*[44] indicated that contents of polymerized polyphenols and EGCG are 68 mg/300 mL and 81 mg/300 mL in oolong tea prepared from 15 g of leaves, respectively. On the other hand, Toyoda-ono *et al*.[7] showed that contents of polymerized polyphenols and EGCG in oolong tea extract are 114 mg/g and 6 mg/g, respectively. Zhu *et al*.[45] have reported that oolong tea water extracts had strong free radical scavenging activity, which may be attributed to some lower molecular weight polymerized polyphenols. Hashimoto *et al*.[46] reported that dimeric polyphenols in teas are responsible for scavenging of active oxygen free radicals, which demonstrated that EGCG and ECG are not the only major antioxidant components in teas. Other minor polyphenols including monomeric and dimeric flavan-3-ols and hydrolyzed tannins also had potent anti-oxidative activity.[46] Hashimoto *et al.* [46] isolated 53 mg of OFA from 5 kg of oolong tea as a kind of polymerized polyphenol that has dimeric structure of EGCG (Figure 1.1). Several studies have shown that EGCG in green tea has many health benefits including antioxidant

effects.[47] Therefore, we made hypothesis that OFA exhibits antioxidant activity and might decrease oxidative stress in human body. As shown in Figure 2.2, OFA had very strong radical scavenging activity than that of Trolox, α-tocopherol analog.

Lipid peroxidation of polyunsaturated fatty acids moieties of phospholipids, cholesteryl esters, and triacylglycerols in LDL is mediated by radical chain reaction, resulting in the generation of various highly reactive products including lipid hydroperoxide.[48] Lipid hydroperoxide is decomposed to secondary products such as aldehydes in the presence of transition metal ions. These can react with free amino groups of apolipoprotein, generating Schiff's bases or Michael addition reactions, and making the LDL particle more electronegative.[49] We used agents such as peroxyl radicals (AAPH), coper ions for ROS generation, and peroxynitrite (SIN-1) for RNS generation to initiate LDL oxidation *in vitro*. As a major product of lipid peroxidation of LDL by AAPH, CE-OOH was measured.[50] As shown in Figure 3.2, OFA seems to be a chain-breaking antioxidant against peroxyl radical-mediated lipid peroxidation of LDL.

There are several reports indicating that tea polyphenols inhibits oxLDL catalyzed by Cu^{2+}, a transition metal ion.[51] Cu^{2+} stimulates lipid peroxidation that can decompose lipid hydroperoxides to lipid alkoxyl and peroxyl radicals.[52] During termination of lipid peroxidation secondary oxidation products such as lipofuscines, oxysterols, and aldehydes are generated.[13] TBARS is a widely used indicator of metal ion catalyzed lipid peroxidation. In Figure 3.3, OFA shows potent inhibitory effect on Cu^{2+} catalyzed LDL oxidation, due to breaking of the chain reaction by scavenging radicals.

Extensively oxLDL are characterized by a high level of oxidized lipids and more alterations of apoliportotein.[13] Aldehyde compounds such as HNE formed when LDL is oxidized by ROS, are capable of forming adducts with apolipoprotein. Several process are involved in protein oxidation of LDL, such as chemical modification of amino acid residues, cleavage of peptide chains, and cross linking of polypeptides.[53,54] The covalent binding of aldehydes to apoB-100 is leads to progressive modification of lysine residues, when LDL is oxidized by Cu^{2+}.[17] Our results indicated that OFA can protect oxidation of apo B-100 in LDL oxidized by transition metal ions.

Carbonylated protein has been used as a marker of ROS mediated protein oxidation. The decomposed products of lipid hydroperoxides generated by transition metal ions can react with the lysine, histidine, threonine, and proline side-chain of proteins to form carbonyl compounds.[55] As shown in Figure 3.5, OFA has also been shown to inhibit protein carbonyls of apoB-100 during Cu^{2+} oxidation. These results suggest that OFA protects apo B-100 from carbonylation in transition metal ion-catalyzed LDL oxidation, which may be due to preventing lipid peroxidation and its decomposition.

The interaction of heparin with apolipoprotein plays an important role in the regulation of normal physiological processes as well as LDL metabolism and cholesterol homeostasis.[56] Heparin, a sulfated polysaccharide of the family of glycosaminoglycans, has the ability to bind to the apo B (LDL) receptor.[56] Peptide analysis of apo B-100 demonstrated that positively charged amino acid residues such as lysine or arginine are responsible for specific binding to heparin.[57] Oorni *et al.* [58] suggested that reduction of the positive charge of lysine or arginine side chains by ROS results in loss of the heparin binding activity of apo B-100. The decomposed substance of lipid hydroperoxides induced by Cu^{2+} can modify of lysine residues. In our experiments, OFA showed an inhibitory effect on oxidative modification of the lysine residues of apo B-100 in LDL oxidized by transition metal ions.

RNS such as $ONOO^-$ is also involved in inducing oxidative stress in human body.[59] $ONOO^-$ is generated by the reaction between nitric oxide and superoxide anion,[59] which can efficiently induce lipid oxidation in cell membranes and lipoproteins.[16] LDL oxidized by $ONOO^-$ binds with high affinity to scavenger receptors of macrophage, leading to the accumulation of cholesteryl esters involved in the production of fatty streak and atherosclerotic lesion.[60] Therefore, we examined the effect of OFA against $ONOO^-$-mediated oxidation of LDL. Our results showed that OFA is able to inhibit CE-OOH formation in LDL oxidized by $ONOO^-$ (Figure 4.2). One of the major modification occurs during LDL oxidation induced by $ONOO^-$, which is the formation of lipid-protein.[61] Nitrotyrosine is prominently formed by radical reactions through tyrosyl radical intermediate. Nitrotrysine accumulation is useful for measuring oxidative stress. OFA is

able to prevent $ONOO^-$ -induced nitrotrysine complement of apo B-100 in oxLDL (Figure 4.3). Our data indicate that OFA prevents nitrotyrosine formation of apo B-100 as well as lipid peroxidation in LDL oxidized by $ONOO^-$.

Chapter 6

Conclusions

These results suggest that OFA, an oolong tea polymerized polyphenol, has a strong inhibitory effect on lipid peroxidation and modification of apoB-100 in LDL oxidized by ROS, such as peroxyl radicals and transition metal ions, and RNS, such as peroxynitrite. The antioxidant effects of OFA are attributable to its radical scavenging activity to reduce ROS and RNS. Oolong tea polyphenols may protect against atherosclerosis by reducing oxidative stress.

OFA is a unique polymerized polyphenol that has dimeric structure of EGCG, although information about the content of OFA in oolong tea is not enough so far. Therefore, the role of OFA in total antioxidant activity of oolong tea and comparison of antioxidant activity of OFA with EGCG would be important to elucidate. In addition, further studies using cells and animals are required to determine bioavailability of OFA including the absorption and metabolism because antioxidant activity of OFA may depend on their bioavailability *in vivo*.

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