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Decrease in the particle size of low-density lipoprotein (LDL) by oxidation

Atsuko Hidaka, Kana Inoue, Sahoko Kutsukake, Motoko Adachi, Yuri Kakuta and Shosuke Kojo*

Department of Food Science and Nutrition, Nara Women's University, Kita-Uoya, Nara 630-8506, Japan

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Abstract—A radical reaction of low-density lipoprotein (LDL) causes fragmentation and cross-link of apolipoprotein B-100 (apoB). LDL (50 μg/ml) was subjected to the well-studied oxidation with Cu^{2+} (1.67 μM). The concentration of α-tocopherol decreased to 10% of the initial level during the first 30 min. After this lag time, the conjugated diene content, as measured by absorption at 234 nm, started increasing and the residual apoB at 512 kDa determined by immunoblot after SDS-PAGE (sodium dodecylsulfate-polyacrylamide gel electrophoresis) was also decreased. The particle size of LDL determined by nondenaturing gradient gel electrophoresis decreased steadily during the initial 120 min, when residual native apoB was only 30% of the initial level. Plasma was also oxidized with Cu^{2+} (400 μM). Under this condition, a clear lag time was not observed and α-tocopherol content, apoB, and the LDL particle size were decreased simultaneously. Based on these experiments, we propose that an oxidation reaction is involved in the formation of small dense LDL.

1. Introduction

The role of oxidized low-density lipoprotein (LDL) in atherogenesis has been well studied.[1,2] Although LDL is composed of lipids, protein, and sugar chains, studies on the oxidation of LDL have focused mainly on lipid peroxidation[3] and the resulting modification of apolipoprotein B-100 (molecular mass of 512 kDa) (apoB) by aldehydes,[4,5] hydroperoxide,[6,7] and oxidized phosphatidylcholine[8] produced. Palinski et al.[2] reported that malondialdehyde- or 4-hydroxynonenal-modified LDL was detected immunochemically in the atherosclerotic lesions of the Watanabe heritable hyperlipidemic rabbit aorta, and in human sera using antibodies against LDL modified with these aldehydes.

The protein part of LDL, apoB, is also reactive to oxidation. Radical reaction of LDL induced by Cu^{2+} causes cleavage of peptide bonds and cross-link of apoB.[9–18] This reaction was inhibited by radical scavengers, demonstrating that the oxidation by Cu^{2+} caused radical reaction.[19] Recently, we reported that an immunoblot assay using anti-human apoB antiserum after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was an effective method to follow radical reaction of LDL in isolated solutions, as well as in plasma.[19] This approach allowed us to show that the radical reaction of serum caused by Cu^{2+} gave a characteristic pattern of fragmented apoB.[19]

Among plasma proteins, apoB is unusually reactive to radical reactions compared to albumin and transferring,[20] and its reactivity is almost the same as α-tocopherol.[20] In normal human serum, both fragmented and cross-linked apoB proteins were present and these oxidation reaction products of LDL tended to increase with age.[19] In addition, we reported[21] that the sum of fragmented and conjugated apoB proteins determined by the immunoblot assay showed significant positive correlation with IMT (intima-media thickness of the carotid artery) and LDL cholesterol and a negative correlation with high-density lipoprotein (HDL) cholesterol and

Keywords: Apolipoprotein B-100; Atherosclerosis; LDL; Oxidation; Particle size; Protein degradation; Radical reaction; Small dense LDL.
Abbreviations: apoB, apolipoprotein B-100; apoE, apolipoprotein E; HDL, high-density lipoprotein; IMT, intima-media thickness of the carotid artery; LDL, low-density lipoprotein; PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.
* Corresponding author. Tel./fax: +81 742 20 3459; e-mail: kojo@cc.nara-wu.ac.jp

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plasma vitamin C, which was an outstanding anti-oxidant in plasma.[22]

Oxidation of LDL should produce many species and normal human LDL consists of multiple subclasses.[23–25] In the present study, we investigated whether a radical reaction is a possible mechanism to generate small dense LDL because a radical reaction of LDL has been assumed as the initial event in atherogenesis,[1,2] and the role of oxidation reactions has never been examined in the formation of small dense LDL, which is associated with the incidence of atherosclerosis.[24,25]

2. Results

2.1. Oxidation of LDL

LDL (50 μg/ml) was subjected to the well-studied oxidation[27] with Cu^{2+} (1.67 μM). LDL samples from three individuals were used and the reaction profiles closely resembled each other. Therefore, a typical example is shown in Figures 1 and 2. The concentration of α-tocopherol decreased to 10% of the initial level during the first 30 min (Fig. 1). After this initiation period (often designated as a lag time), the conjugated diene content as measured by absorption at 234 nm started increasing, while the residual apoB at 512 kDa determined by immunoblot after SDS-PAGE was also decreased in a similar manner to that reported previously[19] (Fig. 1).

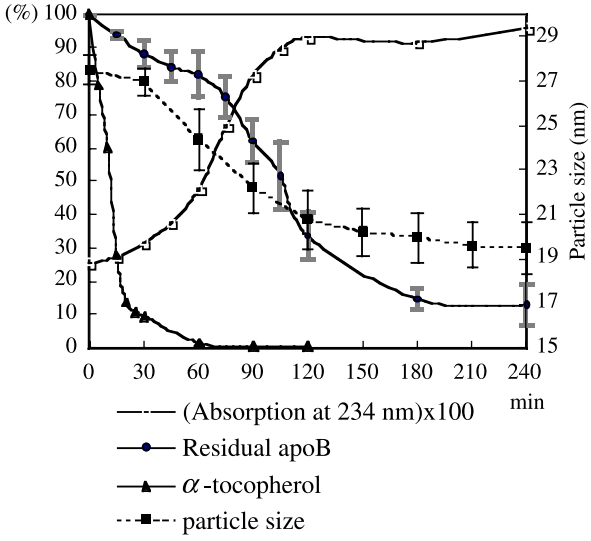

Figure 1. Reaction profile of the oxidation of LDL with Cu^{2+}. LDL (50 μg/ml) was treated with 1.67 μM Cu^{2+} at 37 °C. Samples were withdrawn at the indicated times after the addition of Cu^{2+} up to 4 h. The residual contents of α-tocopherol and apoB (% shown in the left vertical axis), and absorption at 234 nm (absorbance × 100 corresponds to the % value shown in the left vertical axis), residual apoB, and the LDL particle size (unit shown in the right vertical axis) were measured as described in the text. Each point was the mean ± SE of four independent determinations. Where no bars are shown, SE was smaller than the symbol.

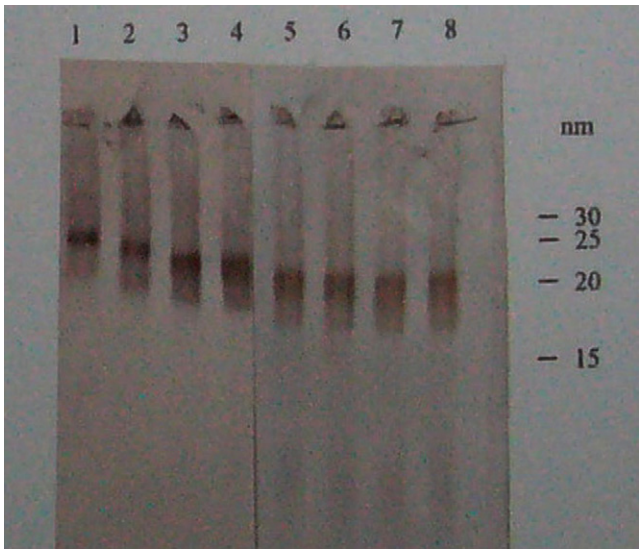

Figure 2. Change in the particle size of LDL during the oxidation of LDL. LDL (50 μg/ml) was treated with 1.67 μM Cu^{2+} at 37 °C. Samples in lanes 1–8 were withdrawn at 0, 60, 90, 120, 150, 180, 210, and 240 min, respectively. After electrophoresis, immunoblotting using anti-human apoB serum was performed and the LDL particle size was calibrated as described in the text.

The particle size of LDL was determined by nondenaturing gradient gel electrophoresis as described.[29] At the start time, LDL was detected as one broad band with the mean value of 27.5 nm. The LDL particle size as measured by the maximal, minimal, as well as the mean value decreased steadily during the initial 120 min (Figs. 1 and 2), when residual native apoB was only 30% of the initial level (Fig. 1). It was reported that LDL particles with a diameter smaller than 25.5 nm showed the strongest association with the risk of ischemic heart disease.[30] At 240 min, native apoB at 512 kDa disappeared almost totally, but the LDL particle was still detected as a broad band at approximately 19.5 nm (Fig. 2). These results demonstrate that LDL particles were retained instead of extensive cleavages in the apoB protein and that the LDL particle size was reduced by oxidation.

2.2. Oxidation of plasma

Human plasma diluted 4-fold was subjected to a radical reaction initiated by Cu^{2+} (400 μM) as previously described.[19,20] Plasma samples from three individuals were used. The reaction profiles closely resembled each other. Therefore, typical results from the same subject for LDL are shown in Figure 3. Under this condition, α-tocopherol content and the residual apoB steadily decreased in a similar manner to a previous study[20] (Fig. 3).

To determine LDL particle size in plasma, blot analysis using anti-apoB serum was used. Since apoB is a component of LDL, IDL, and VLDL, LDL must be identified first. To distinguish LDL from other apoB-containing particles, an immunoblot assay using anti-apoE antiserum was performed (Fig. 4). Comparing these blots, LDL was detected as two bands indicated below

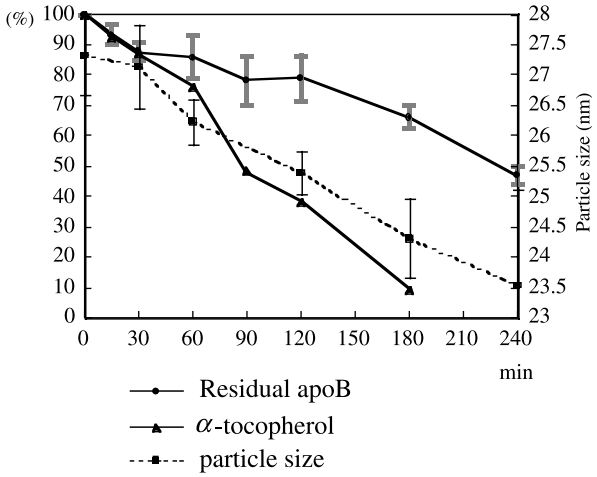

Figure 3. Reaction profile of the plasma oxidation with Cu^{2+}. Human plasma, diluted with PBS 4-fold, was treated with 400 μM of Cu^{2+} at 37 °C. From oxidized plasma solutions, samples were withdrawn at the indicated times after the addition of Cu^{2+} and the residual contents of α-tocopherol and apoB (shown in the left vertical axis), and the median LDL particle size (unit shown in the right vertical axis) were measured as described in the text. Each point was the mean ± SE. Where no bars are shown, SE was smaller than the symbol.

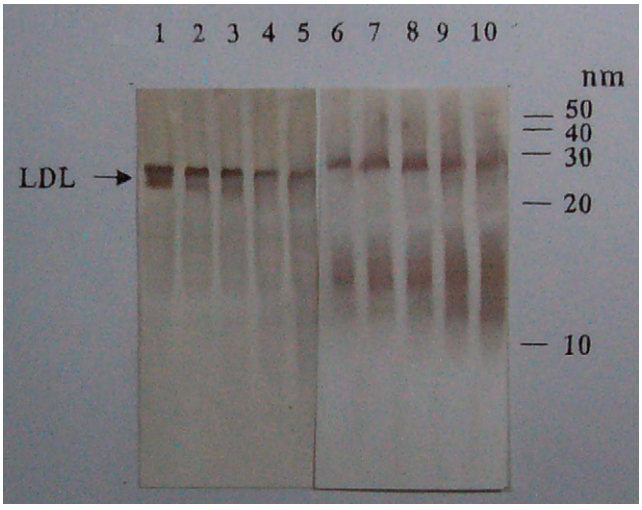

Figure 4. Change in the particle size of LDL during the plasma oxidation. Human plasma, diluted with PBS 4-fold, was treated with 400 μM of Cu^{2+} at 37 °C. From oxidized plasma solutions, samples were withdrawn at 0, 60, 120, 180, and 240 min after the addition of Cu^{2+}. After electrophoresis, immunoblotting using anti-human apoB serum was performed for lanes 1–5 (samples for 0, 60, 120, 180, and 240 min, respectively), and immunoblotting using anti-human apoE serum was performed for lanes 6–10 (samples for 0, 60, 120, 180, and 240 min, respectively). The LDL particle size was calibrated as described in the text.

apoE-containing bands (Fig. 4). Multiple bands in LDL determined with polyacrylamide gradient electrophoresis were well documented.[23–25] The median value of the particle size of these two bands was 27.3 nm. The particle size of each band became smaller and each band faded gradually as the oxidation reaction proceeded. We plotted the median value of the two bands (Fig. 4). HDL bands were also observed using anti-apoE anti-serum at about 15 nm (lanes 6–10, Fig. 4) and tended to broaden with oxidation.

The LDL particle size decreased simultaneously with vitamin E content (Figs. 3 and 4). These results indicated that a radical reaction of plasma also caused fragmentation of apoB, as well as shrinkage of LDL particle size, in a similar manner to the reaction using isolated LDL as described above.

3. Discussion

ApoB undergoes fragmentation and cross-link by oxidation reactions.[9–19] The radical nature of these reactions was confirmed on the basis of inhibition by radical scavengers, as well as promotion by a radical initiator.[19] In addition, apoB has an unusually high reactivity to radical reactions among plasma proteins and its reactivity is almost comparable to α-tocopherol,[20] a typical anti-oxidant, which is consumed during radical reactions. The extremely high reactivity of apoB to radical reaction explains why fragmented and cross-linked apoB proteins are present in normal human serum. In addition, the fragmentation and cross-link pattern of apoB positively correlated with age, IMT, and LDL cholesterol, and negatively correlated with HDL cholesterol, thus being a good index for atherosclerosis.[21]

The present study demonstrated that typical oxidation of LDL with Cu^{2+} caused a decrease in α-tocopherol in the lag phase, followed by an increase in the absorption at 234 nm[4] and simultaneous reductions in apoB, with a molecular mass of 512 kDa and particle size of LDL (Fig. 1). It is worth indicating that LDL particles were retained instead of extensive nicks in the apoB protein, although the LDL particle size became smaller and the band caused broadening. This is the first report showing that a radical reaction of LDL caused a decrease in the LDL particle size, although lipid hydrolysis of LDL with phospholipase A_2 caused shrinkage of LDL particles.[31]

The decrease in the LDL particle size was also observed in the oxidation of plasma (Fig. 3). In this case, a clear induction period was not observed and α-tocopherol, and apoB at 512 kDa, decreased simultaneously[19,20] while concurrently the LDL particle size was reduced. Blot analysis utilizing anti-apoE anti-serum indicated a broadening of HDL at about 15 nm (lanes 6–10, Fig. 4) and this observation suggested that HDL was also affected by oxidation.

A significant association of small dense LDL with a risk of coronary artery disease is well documented[32–34] and small dense LDL is suggested to be atherogenic.[25] In addition, the lag time in the oxidation of LDL by Cu^{2+} showed a positive correlation with LDL-peak particle diameter[35] and the level of modification by malondialdehyde was higher in small dense LDL than that in normal LDL.[29] These studies indicate that the oxidation level of small dense LDL is higher than that of

normal LDL. Taking these observations and the present study together, we propose that an oxidation reaction is involved in the formation of small dense LDL.

4. Experimental

4.1. Materials

The Vectastain ABC-PO (goat IgG) kit was from Vector Lab. Inc. (Burlingame, CA, USA). Anti-human apoB goat IgG anti-serum was purchased from Sigma Chem. Co. (St. Louis, MO, USA). Anti-human apolipoprotein E (apoE) goat anti-serum was purchased from Chemicon International Inc. (Temecula, CA, USA). Polyvinylidene difluoride (PVDF) membrane filters were purchased from Millipore (Tokyo, Japan). Electrophoresis reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan). All other reagents were of analytical grade and were purchased from Wako Pure Chem. Co. (Osaka, Japan).

Blood was taken from healthy volunteers after overnight fasting, and the serum and plasma were separated. After addition of EDTA to a final concentration of 0.1% at pH 7.4, LDL was prepared by ultracentrifugation according to Hatch and Lees.[26]

4.2. Oxidation of LDL

EDTA and salt from the density gradient were removed from the LDL solution with a prepacked column (Econo-Pac 10DG, Bio-Rad, Richmond, CA, USA) as described.[27] EDTA-free LDL solution [50 μg protein/ml phosphate-buffered saline (PBS)] was transferred into a brown tube with a Teflon-coated screw cap, and the oxidation was started at 37 °C by the addition of $CuSO_4$ to a final concentration of 1.67 μM.[27] At indicated times, 280 μl of an aliquot (200 μl for vitamin E, 20 μl for particle size, and 60 μl for residual apoB) was taken out for measurements. To assess lipid peroxidation, conjugated diene based on the absorption at 234 nm was also determined as described.[27]

4.3. Oxidation of human plasma

Human plasma was diluted with PBS 4-fold, and then transferred into a glass vial with a Teflon-covered screw cap. Oxidation was started at 37 °C by the addition of 40 mM aqueous $CuSO_4$ to a final concentration of 400 μM.[19,20] At indicated times, 170 μl of an aliquot (100 μl for vitamin E, 30 μl for particle size, and 40 μl for residual apoB) was taken to make immunoblot analysis of apoB as described previously.[19,20] Determinations of LDL particle size, residual apoB, and measurement of α-tocopherol were performed as described below.

4.4. Electrophoresis, blotting, and immunoblot analysis

For SDS-PAGE, 15 μl of the sample was applied to SDS gel electrophoresis on 4% polyacrylamide slab gels (1 mm thick) as described.[19,28] Nondenaturing gradient gel electrophoresis was performed as described.[29] In this study, a polyacrylamide gradient of 2–10% was used. Proteins separated on the gel were electrophoretically transferred to PVDF membrane filters and immunoblotting analyses of apoB and apoE were performed as previously described.[19]

LDL particle size was determined as described.[29] A calibration curve of particle size was constructed based on the migration distances of markers with known diameters: bovine serum albumin (7.1 nm), ferritin (12.2 nm), and thyroglobulin (17.0 nm). The diameter of LDL was determined by extrapolation of the calibration curve using blotted PVDF membranes.

4.5. Determination of vitamin E

The concentration of α-tocopherol was determined by HPLC.[20,36] The conditions for HPLC and fluorescence detection (Shimadzu RF-535, Kyoto, Japan) were reported previously.[37]

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