J. Biochem. 125, 173-176 (1999)

Facile Degradation of Apolipoprotein B by Radical Reactions and the Presence of Cleaved Proteins in Serum[1]

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Received August 3, 1998; accepted October 7, 1998

A facile cleavage of peptide bonds of apolipoprotein B (apoB) by radical reaction is reported. When human LDL was subjected to oxidative damage using Cu^{2+}, extensive degradation of apoB was observed based on immunoblotting. The degradation of apoB was inhibited by radical scavengers (β-mercaptoethanol, butylated hydroxytoluene, and probucol) and promoted by a radical initiator [2,2′-azobis(2-amidinopropane)dihydrochloride]. When human serum was treated with Cu^{2+}, a similar cleavage pattern of apoB was observed. The cleaved apoB proteins were also detected in normal serum on the basis of immunoblots. These results suggest that apoB is highly reactive toward radicals *in vitro* and *in vivo*, with reaction resulting in the cleavage of peptide bonds.

Key words: apolipoprotein B, LDL, lipid peroxidation, low density lipoprotein, protein degradation, radical reaction.

The role of oxidized low density lipoprotein (LDL) in atherogenesis has been well established (*1-3*). Although LDL is composed of lipid, sugar, and protein, studies on the oxidation of LDL have focused mainly on lipid peroxidation (*4*) and the resulting modification of apolipoprotein B-100 (molecular mass of 512 kDa) (apoB) by the aldehydes produced (*5*). Palinski *et al.* (*3*) reported that malondialdehyde- or 4-hydroxynonenal-modified LDL was detected immunochemically in the atherosclerotic lesions of Watanabe heritable hyperlipidemic rabbit aorta and in human sera by using antibodies against LDL modified with these aldehydes. As for the sugar moiety of LDL, Tertov *et al.* (*6*) showed that a part of LDL isolated from patients with coronary artery atherosclerosis bound to a Sepharose-linked *Ricinus communis* agglutinin, a lectin which interacts with galactose residues, and suggested that desialylated LDL increased in serum of these patients. Recently we reported (*7*) that sialic acid moieties of LDL decreased by oxidation with Cu^{2+}, and radical reaction was a possible mechanism for the increase of desialylated LDL in serum of atherosclerotic patients.

Concerning radical reaction of the protein moiety of LDL, *in vitro* oxidation of LDL is suggested to cause fragmentation and crosslinking of apoB (*8-17*). In these *in vitro* studies, products were analyzed by SDS-PAGE with dye-staining of proteins. However, it is not clear whether such fragmentation also occurs *in vivo*, because no appropriate method is available to analyze the process in the presence of a great many other proteins. In this paper, we used immunostaining to detect specifically proteins derived from apoB, and 4% SDS-PAGE to analyze clearly the distribution of major products with molecular masses of more than 200 kDa. This approach allowed us to investigate the oxidation of apoB in serum, which gave a characteristic pattern of fragmented proteins, and to analyze the change in these fragments with ageing.

MATERIALS AND METHODS

Reagents—Vectastain ABC kit and anti-mouse IgG (H+L) were purchased from Vector Lab. (Burlingame, CA, USA). Anti-human lipoprotein B goat IgG, molecular weight markers (cross-linked phosphorylase *b* and rabbit muscle myosin), and probucol were purchased from Sigma Chem. (St. Louis, MO, USA). Monoclonal antibody for human apolipoprotein B100 (clone:B/10) was purchased from Chemicon International (Temecula, California, USA). Polyvinylidene difluoride (PVDF) membrane filters were purchased from Millipore (Tokyo). Electrophoresis reagents were purchased from Nacalai Tesque (Kyoto). All other reagents were of analytical grade and were purchased from Wako Pure Chem. (Osaka).

LDL Isolation—Blood was taken from healthy volunteers after over-night fasting, and the serum was 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 (*18*).

Oxidation of LDL—EDTA and salt from the density

[1] This work was supported by the grant of the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; apoB, apolipoprotein B-100; BHT, butylated hydroxytoluene; LDL, low density lipoprotein; PVDF, polyvinylidene difluoride; IgG, immunoglobulin G; PBS, phosphate-buffered saline.

gradient were removed from the LDL solution with a prepacked column (Econo-Pac 10DG, Bio-Rad, Richmond, CA, USA) as described (*19*). EDTA-free LDL solution [50 μg protein/ml phosphate-buffered saline (PBS)] was transferred into a brown-colored 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 (*19*). To assess lipid peroxidation, conjugated diene formed was determined based on the absorption at 234 nm (*19*).

Electrophoresis—A 100-μl aliquot of oxidized LDL (50 μg/ml) was taken from the reaction mixture into a microtube (0.5 ml volume), and 5 μl of 4 mM EDTA and 50 μl of PBS (pH 7.4 containing 0.2 mM EDTA) were added. These samples were treated with 150 μl of 4% SDS denaturation solution as described by Laemmli (*20*). SDS gel electrophoresis on 4% polyacrylamide slab gels (1 mm thick) was performed by the method of Laemmli (*20*).

Proteins separated on the gel were electrophoretically transferred to PVDF membrane filters as described (*21*).

Immunoblot Analysis—Transferred membranes were incubated with rabbit serum for blocking according to the procedure of the provider [Vectastain ABC-PO (goat IgG) kit], washed three times with TTBS [0.1 M Tris-HCl, pH 7.5, 0.9% NaCl containing 0.1% (v/v) Tween 20], then incubated with anti-human apoB goat IgG diluted 1:1,000 with TTBS for 1 h. All treatments were done according to the procedures described by the provider.

Inhibition and Initiation Experiments—In inhibition experiments, β-mercaptoethanol (final concentration 0.1 mM), butylated hydroxytoluene (BHT: final concentration 0.1 mM), or probucol (final concentration 0.1 mM) was added to 1 ml of LDL solution (50 μg/ml PBS), then 16.7 μl of $CuSO_4$ solution (0.1 mM) was added to initiate the reaction at 37°C. In an initiation experiment, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH: final concentration 0.2 mM) was used. BHT and probucol were added as a methanol solution of 10 μl. At the start and after 3 h, contents of conjugated dienes were measured, and immunoblot analysis was performed. In a control experiment, 16.7 μl of $CuSO_4$ solution (0.1 mM) or 16.7 μl of $CuSO_4$ solution (0.1 mM) and 10 μl of methanol were added to LDL solution (50 μg/ml PBS) to initiate the reaction at 37°C.

Oxidation of Serum with Cu^{2+}—To remove endogenous antioxidants, serum was dialysed against 0.02 M phosphate buffer at pH 7.4. The serum was diluted to 2 mg protein/ml with PBS, and transferred into a brown-colored tube with a Teflon-coated screw-cap. The oxidation was started at 37°C by the addition of $CuSO_4$ to a final concentration of 50 μM. An aliquot (100 μl) of oxidized serum was taken from the reaction mixture into a microtube and 5 μl of 4 mM EDTA was added to the tube. Immunoblot analysis was done as described above.

Immunoblot of Serum—Blood was taken from healthy volunteers who did not take vitamin supplements after overnight fasting, and the serum was separated. Serum was diluted 1:10 with PBS and treated with the same volume of 4% SDS denaturation solution. Five microliters of the denatured sample was applied on 4% SDS-PAGE. Immunoblot analysis was done as described above.

Protein Assay—Protein concentrations were determined by the method of Lowry *et al.* (*22*) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Degradation of LDL by Oxidative Reaction with Cu^{2+}—Human LDL prepared by ultracentrifugation was subjected to a well-studied oxidation reaction (*19*) initiated by 1.67 μM Cu^{2+} at 37°C. The time course of the reaction was followed by 4% SDS-PAGE and immunoblotting. Degradation of apoB, with a molecular mass of 512 kDa, was detected as early as 1 h after the start of reaction [lane 2, Fig. 1A], when conjugated dienes were increasing (*7, 23*), and apoB disappeared almost completely [lane 3, Fig. 1A] after 2 h, when conjugated dienes, measured by absorbance at 234 nm, reached a plateau (*7, 23*). It is well documented (*5*) that conjugated dienes develop in LDL through the oxidation of polyunsaturated fatty acids with isolated double bonds to fatty acid hydroperoxides with conjugated double bonds with a UV-absorption maximum at 234 nm. These results indicated that lipid peroxidation, fragmentation of apoB protein, and degradation of the sialic acid moiety of apoB (*7*) proceeded simultaneously.

After 3 h, apoB disappeared completely [lane 4, Fig. 1A], and in the following 3 h, extensive degradation of the produced fragments was found [lane 5, Fig. 1A]. These results demonstrate that apoB contains peptide bonds which are highly susceptible to oxidation by Cu^{2+}. Degradation and aggregation of LDL by oxidation were reported on the basis of protein staining with a dye (*8–17*). To our knowledge, this is the first report that clearly shows a product pattern using polyclonal antibodies that discriminate products derived from apoB.

The products of a similar reaction with another LDL sample were analyzed with a monoclonal antibody to apoB [Fig. 1B]. The time course of the reaction was slightly different depending on the LDL preparation, as is well known for radical reactions of LDL (*4*), but the decrease of intact apoB by oxidation was observed for all LDL preparations. Comparison of Fig. 1, A and B, showed that staining with polyclonal antibody gave much clearer fragmentation patterns. Therefore, polyclonal antibody was used for subsequent experiments.

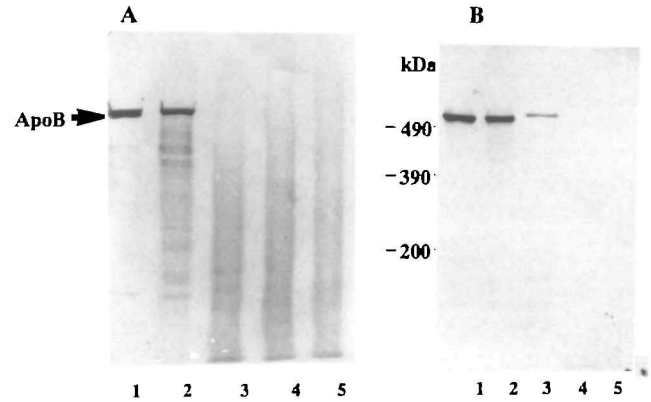

Fig. 1. **Immunoblot analysis of LDL oxidized with Cu^{2+}.** LDL (50 μg/ml) was treated with 1.67 μM Cu^{2+} at 37°C. Samples in lanes 1 to 5 were withdrawn at 0, 1, 2, 3, and 6 h, respectively. After electrophoresis, immunoblotting using anti-human apoB polyclonal (A) and monoclonal (B) antibodies was performed as described in the text.

Radical Reaction as a Mechanism of ApoB Degradation—The radical nature of the reaction was confirmed using inhibitors. ApoB was degraded almost completely by Cu^{2+} treatment for 3 h at 37°C (Fig. 2, lane 3). In the absence of Cu^{2+}, cleavage of apoB was negligible (Fig. 2, lane 2) and the blotting pattern was similar to that at the start (Fig. 2, lane 1). In the presence of β-mercaptoethanol or BHT, cleavage of apoB by Cu^{2+} was inhibited (Fig. 2, lanes 4 and 5). At the same time, these antioxidants inhibited the formation of conjugated dienes (data not shown), in agreement with previous observations (7). Probucol also inhibited the degradation of apoB by Cu^{2+} (Fig. 2, lane 6). Methanol at 10 μl/ml, which was used to dissolve BHT and probucol, did not affect the fragmentation of apoB by Cu^{2+} (data not shown). A radical initiator, AAPH, also reduced apoB content (Fig. 2, lane 7).

Noguchi *et al.* (*12*) reported that probucol did not inhibit modification of apoB based on the ratio of the relative electrophoretic mobility of oxidized LDL to the sum of lipid peroxidation products (hydroxy and hydroperoxy lipids), but that the relative electrophoretic mobility of oxidized LDL in the presence of probucol was lower than that in its absence during all the time courses studied, supporting an inhibitory effect of probucol. The present findings that a radical intiator promotes and radical scavengers inhibit the fragmentation of apoB support the view that radical reactions cause the cleavage of peptide bonds of apoB.

Degradation of ApoB in Cu^{2+}-Treated Serum—After dialysis, serum was treated with Cu^{2+} and degradation of apoB was followed by Western blotting. In the presence of 10 μM Cu^{2+}, degradation of apoB was negligible even after 5 h at 37°C. This may be due to the binding of Cu^{2+} to serum metal-binding proteins including albumin and ceruloplasmin, which inhibit the oxidation by Cu^{2+}. In the presence of 50 μM Cu^{2+}, apoB in serum was degraded progressively with reaction time (Fig. 3, lanes 4–8) and the pattern of the products was similar to that from isolated LDL (Fig. 3, lanes 1 and 2). This result suggests that similar bonds of apoB in serum are cleaved by radical reactions.

Presence of Cleaved ApoB Proteins in Serum—We next examined whether degradation of apoB occurred in blood of healthy individuals. Immunoblots of sera of seven volunteers were carried out using anti-apoB. As shown in Fig. 4, sera of females in their twenties contain lower amounts of the degradation products (lanes 3-5) than those of older volunteers in their forties (lanes 6 and 7) and seventies (lanes 8 and 9). It is worthwhile to note that the band pattern of serum is similar to that of the *in vitro* oxidation products of LDL [Fig. 4, lane 1; Fig. 1A, lane 2] and serum (Fig. 4, lane 2; Fig. 3, lanes 5-7), suggesting that radical reactions actually take place *in vivo*. Because storage of serum for one month at −80°C or at −20°C in the denaturation solution for SDS-PAGE did not cause appreciable change in the fragmentation pattern, and because apoB in dialyzed serum was resistant to oxidation with 10 μM Cu^{2+} at 37°C for 5 h (described above; dialysis largely enhanced the degradation of apoB by Cu^{2+}), it may be concluded that the presence of fragmented apoB proteins in serum and their increases with ageing are not artifacts produced after the isolation of serum. More than 20 samples from aged subjects showed a similar fragmentation pattern of LDL to those in lanes 7-9 of Fig. 4.

In lanes 4–9 of Fig. 4, bands of higher molecular weight than apoB were observed, a finding consistent with *in vitro*

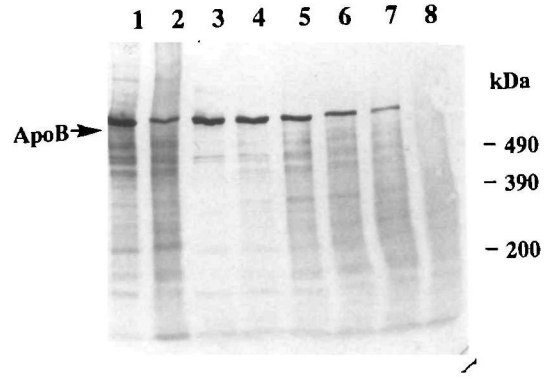

Fig. 3. **Cleavage patterns of apoB in serum treated with Cu^{2+}.** LDL (50 μg/ml) was treated with 1.67 μM Cu^{2+} at 37°C for 1 h (lane 1) and 2 h (lane 2). Dialyzed serum of a 21-year-old female at protein concentration of 2 mg/ml was treated with 50 μM Cu^{2+} at 37°C for 0 min (lane 3), 15 min (lane 4), 30 min (lane 5), 45 min (lane 6), 60 min (lane 7), and 120 min (lane 8).

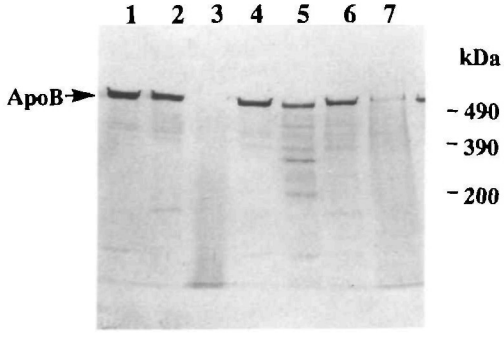

Fig. 2. **Effect of radical scavengers and initiator on the degradation of apoB.** LDL (50 μg/ml) was treated at 37°C under following conditions: lane 1, 0 h; lane 2, 0 μM Cu^{2+}, 3 h; lane 3, 1.67 μM Cu^{2+}, 3 h; lane 4, 1.67 μM Cu^{2+}, 0.1 mM mercaptoethanol, 3 h; lane 5, 1.67 μM Cu^{2+}, 0.1 mM BHT, 3 h; lane 6, 1.67 μM Cu^{2+}, 0.1 mM probucol, 3 h; lane 7, 0.2 mM AAPH, 3 h.

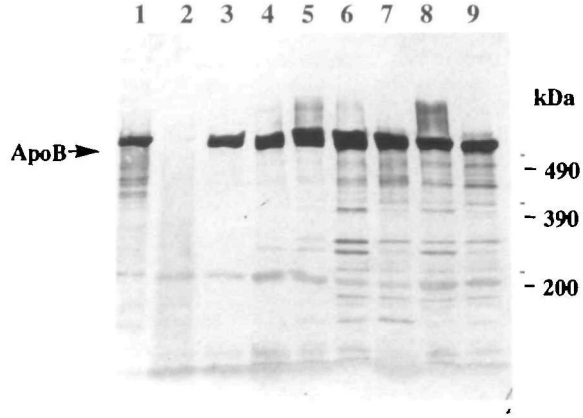

Fig. 4. **Immunoblots of human sera using anti-apoB antibody.** Lane 1, LDL treated with 1.67 μM Cu^{2+} at 37°C for 1 h; lane 2, serum of a 21-year-old female treated with 50 μM Cu^{2+} at 37°C for 1 h; lanes 3 to 9, sera of females aged 21, 22, 24, and 41, a 49-year-old male, a 74-year-old female, and a 75-year-old male, respectively.

studies (*11, 15, 16*) describing that crosslinked apoBs were formed by oxidation of apoB. Since the concentration of apoB in serum is about 1 mg/ml (*24*), which is much higher than that used in our *in vitro* reaction (50 μg/ml) where degradation predominated, intermolecular reactions leading to the formation of higher molecular weight products could take place. It is also suggested for the first time from the immunoblotting study that the aggregated products derived from apoB oxidation are present in serum.

In summary, we have demonstrated that the protein of apoB was as susceptible as the lipid and sialic acid moiety of LDL to oxidation with Cu^{2+}, giving rise to cleavage of peptide bonds. Inhibition studies confirmed that cleavage of peptide bonds in apoB was mediated by radical reactions. The pattern of fragmentation of apoB by the *in vitro* oxidation was analyzed by immunoblotting and found to be similar to the pattern obtained using serum. Similar degraded apoB proteins were detected with human serum and showed a tendency to increase with ageing, where radical reactions may play an essential role (*25*).

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