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HOMOCYSTEINE INDUCES IRON-CATALYZED LIPID PEROXIDATION OF LOW-DENSITY LIPOPROTEIN THAT IS PREVENTED BY ALPHA-TOCOPHEROL

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Homocystinuria is an inborn error of methionine metabolism that is characterized by the premature development of arteriosclerosis. As one of the major factors in the pathogenesis of arteriosclerosis, modification of low-density lipoprotein (LDL) has received widespread attention by many investigators. In this study, to elucidate the relationship between elevated homocysteine levels and premature arteriosclerosis, we investigated the role of homocysteine in the iron-catalyzed oxidative modification of LDL. When LDL isolated from a healthy subject was incubated with homocysteine and ferric ion, a gradual decrease of polyunsaturated fatty acids (PUFA), formation of thiobarbituric acid-reactive substances (TBARS) and fluorescent substances, and the fragmentation of apoprotein B (apoB) were observed. The extent of oxidative modification was dependent on the concentration of homocysteine. Modification of LDL was suppressed until the remaining α-tocopherol concentration reached a critical level. When the α-tocopherol content of LDL was increased by 2.6-fold, both the formation of TBARS and the fragmentation of apoB were suppressed. These results suggest that homocysteine might promote iron-catalyzed oxidation of LDL and imply its role for the development of premature arteriosclerosis.

KEY WORDS: Homocysteine, lipid peroxidation, LDL, iron, α-tocopherol, premature arteriosclerosis.
Abbreviations: LDL, low-density lipoprotein; PUFA, polyunsaturated fatty acids; TBARS, thiobarbituric acid-reactive substances; apoB, apoprotein B; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; NTA, nitrilotriacetate; T.C., total cholesterol; MDA, malondialdehyde; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

INTRODUCTION

It is generally accepted that oxidative modification of low-density lipoprotein (LDL) leads to macrophage-derived foam cell formation. It has been reported that oxidatively modified LDL can be extracted from human arteriosclerotic lesions.[1] When LDL is incubated with copper ions, oxidative modification occurs and alters its physics-chemical properties. Addition of antioxidants prevents such modification of LDL, suggesting the possible involvement of free radical reactions in this

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process.[2,3] In our previous study, the effects of α-tocopherol, a potent chain-breaking antioxidant, on biomembrane lipid peroxidation and modification of LDL were investigated.[4,5,6]

In pediatric field, arteriosclerosis is well known to develop in homocystinuria,[7,8] caused by the defects in methionine metabolisms.[9] However, the role of homocysteine in the development of premature arteriosclerosis is not understood yet. Starkebaum *et al.* have suggested that extremely high homocysteine levels may be toxic to endothelial cells through the generation of hydrogen peroxide.[10] In the presence of transition metals, sulfhydryl groups have been reported to bring about the production of superoxide,[11] and in addition, sulfur containing amino acids may play a role in cellular modification of lipoproteins.[12] In patients with homocystinuria, the serum level of homocysteine has been reported to be as high as 200 μM unless appropriate therapy is performed,[9] and elevated homocysteine levels may induce the oxidative modification of LDL. The present study was carried out to determine whether homocysteine could promote the iron-catalyzed oxidative modification of LDL, and also to investigate the influence of endogenous α-tocopherol on such oxidative modification of LDL.

MATERIALS AND METHODS

Materials

DL-Homocysteine, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and nitrilotriacetate (NTA) were purchased from Nakalai Tesque, Inc. (Kyoto, Japan). Ferritin (horse spleen) was obtained from Sigma Chemical Co. (St Louis, MO.). Chelex 100 ion-exchange resin was purchased from Bio-Rad Laboratories (Richmond, CA). Phosphate buffer (100 mM sodium phosphate, pH 7.4) was passed through Chelex 100 ion-exchange resin to remove any contaminating transition metals. Homocysteine concentrations were determined by the sulfhydryl method of Ellman *et al.*[13]

Isolation and Modification of LDL

Heparinized blood samples were obtained from healthy male volunteers aged 25–35 years after an overnight fast. Plasma lipoproteins were fractionated by ultracentrifugation according to the method of Hatch and Lees.[14] The $1.006 < \text{density} < 1.063$ fraction was used as LDL in this study. The LDL fraction was then dialyzed for 24 hours at 4°C against phosphate buffer containing 5 μM EDTA. Total cholesterol (T.C.) levels in the dialyzed LDL were measured by the method of Allain.[15] To obtain α-tocopherol-loaded lipoprotein, some of the volunteers were orally administered 1.8 g of RRR-α-tocopherol daily for 7 days. LDL was then prepared from plasma samples obtained from these volunteers for use as α-tocopherol-rich LDL. Modification of LDL (0.6 mg T.C./ml) was carried out by incubation of LDL with 50 μM NTA-Fe^{3+} (50 μM ferric ion chelated by 100 μM NTA) and various concentrations of homocysteine in phosphate buffer at 37°C in 5% CO_2 and 21% O_2. The reaction was stopped by the addition of EDTA and cooling.

Analytical Procedures

The native LDL and the oxidized LDL after oxidation were analyzed for the fatty acid composition, α-tocopherol, TBARS, and fluorescenct intensity. The fatty acid composition of LDL was analyzed by GLC with a flame ionization detector (Shimadzu GC-8A, Shimadzu Corporation, Kyoto, Japan) as described previously,[16] after extraction with acetone by the one-step method of Shimasaki *et al.*[17] The α-tocopherol level in LDL was measured by HPLC with electrochemical detection, as described in our previous paper.[18] The TBARS level in LDL was determined by the method of Buege *et al.*[19] and was expressed as the equivalent of malondialdehyde (MDA) content. For analysis of fluorescent substances, LDL was diluted 50-fold in phosphate buffer, and was directly measured with a Hitachi 204 fluorescence spectrophotometer. Oxidized LDL exhibited fluorescence with an excitation maximum at 376 nm and an emission maximum at 432 nm.[20] For the observation of apoprotein B (apoB) in oxidized LDL, samples were pretreated with sample buffer containing 3% SDS and 3% 2-mercaptoethanol. Electrophoresis was performed using a linear 4–15% gradient acrylamide gel at a constant current of 4 mA for 1 hr at 4°C.

RESULTS

1. Promotion of Iron-catalyzed Modification of LDL by Homocysteine

We investigated the possibility of homocysteine to promote the iron-catalyzed oxidative modification of LDL. When LDL isolated from a healthy subject was incubated with 500 μM homocysteine and 50 μM NTA-Fe^{3+}, there was a gradual decrease of polyunsaturated fatty acids (PUFA). PUFA with higher unsaturation were oxidized faster than those with fewer unsaturated carbons (Figure 1). When LDL was incubated with 20 μM homocysteine and 50 μM NTA-Fe^{3+}, the α-tocopherol level in LDL decreased linearly with time (Figure 2-A). When the homocysteine concentration was increased, the rate of α-tocopherol consumption increased and the lag period until TBARS levels began to increase was shortened (Figure 2-B). The lag period corresponded to the depletion time of α-tocopherol in the LDL. We also examined fluorescence spectra of native LDL and LDL after varying degrees of oxidation. Oxidized LDL exhibited fluorescence with an excitation maximum at 376 nm and an emission maximum at 432 nm, and this fluorescence intensity changed with time. As shown in Figure 2-C, the fluorescence intensity increased with elevation of the homocysteine concentration. The increase of fluorescence intensity paralleled the rate of TBARS formation. The changes of apoB levels are shown in Figure 3. Silver staining of the gels showed the time-dependent loss of apoB following incubation of LDL with homocysteine. Low molecular weight fragments were detected below the apoB band, and high molecular weight proteins were found above it. The breakdown of apoB progressed more rapidly with 2 mM homocysteine than with 20 μM homocysteine. The incubation of LDL with 50 μM NTA-Fe^{3+} without homocysteine for 8 hr, did not cause the breakdown of apoB. When LDL was incubated with 2 mM homocysteine and ferritin (0.1 mg/ml), instead of NTA-Fe^{3+}, the lipid peroxide content increased with time (Figure 4). During the incubation of LDL with either ferritin or homocysteine alone for 3 hr, the lipid peroxidation did not occur (0.1 $\pm$ 0.6 nmol MDA/mg LDL T.C.).

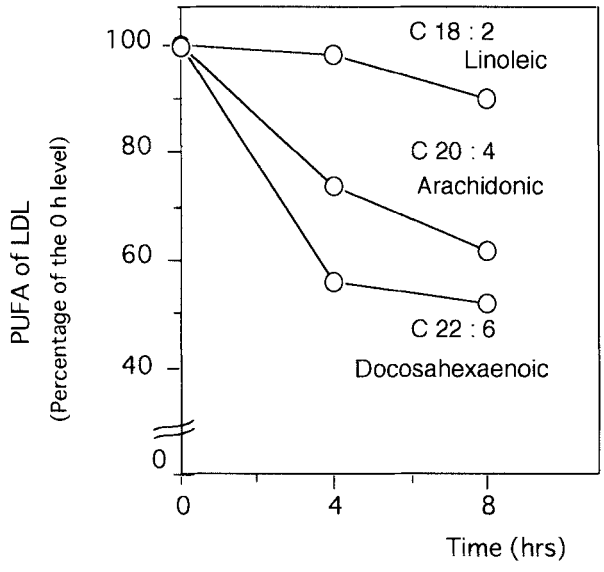

FIGURE 1 The decease of PUFA during oxidation of LDL induced by a homocysteine/iron mixture. Dialyzed LDL (total cholesterol content: 0.6 mg/ml) was incubated with 500 μM homocysteine and 50 μM NTA-Fe^{3+} at 37°C in a CO_2 incubator. Fatty acids were analyzed at the start of the reaction as well as after 4 and 8 hrs by GLC, as described in MATERIALS AND METHODS. The plots show the mean values of three experiments. Results are expressed as the percent change from native LDL.

2. *Intervention of the Modification of LDL by α-Tocopherol*

The α-tocopherol-rich LDL, which contained 2.6-fold higher than that of control LDL, was obtained from the subject after oral administration of RRR-α-tocopherol. When LDL was incubated with 1 mM homocysteine and 50 μM NTA-Fe^{3+}, the rate of α-tocopherol depletion in α-tocopherol-rich LDL closely paralleled that in the control LDL. In both reactions with α-tocopherol-rich LDL and control LDL, the production of TBARS was suppressed until the α-tocopherol content decreased to a critical level. The production of TBARS from α-tocopherol-rich LDL progressed more slowly than in the case of control LDL (Figure 5). Silver staining of the gels showed that both the formation of low molecular weight fragments and high molecular proteins in α-tocopherol-rich LDL was slower than in control LDL (Figure 6).

DISCUSSION

Homocystinuria is an inborn error of methionine metabolism, that is caused mostly by cystathionine β-synthase deficiency. Homozygous individuals are at high risk of developing premature arteriosclerosis, which can be a life-threating complication.[8,9] Recently, a few clinical studies have found that some patients with arteriosclerosis showed high plasma homocysteine level in the basal fasting state or after methionine loading.[21] The above evidences suggest that there may be a close

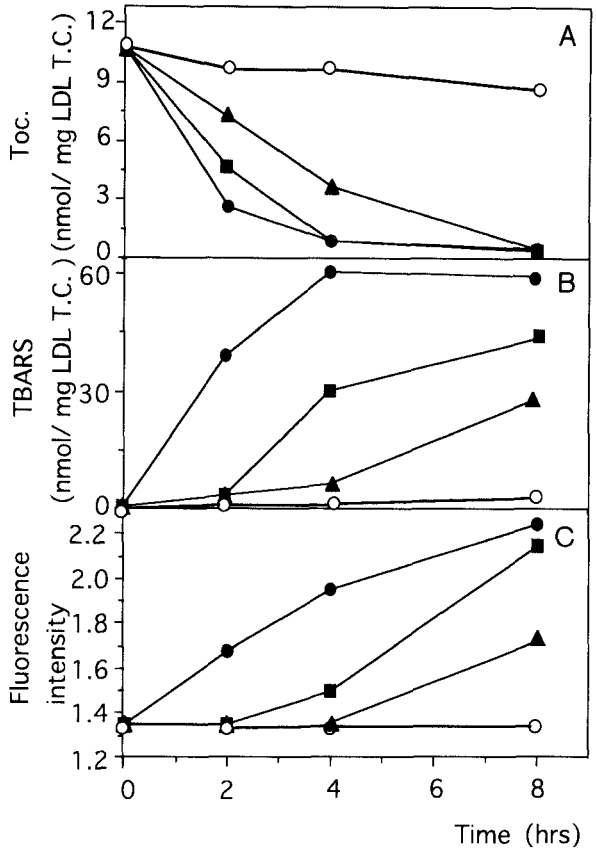

FIGURE 2 α-Tocopherol, TBARS formation and fluorescence when LDL was reacted with homocysteine and ferric ion. LDL (total cholesterol content: 0.6 mg/ml) was incubated with a given concentration of homocysteine, 2 mM (—●—), 200 μM (—■—), 20 μM (—▲—), or 0 μM (—○—) in phosphate buffer containing 50 μM NTA-Fe^{3+} (pH 7.4) at 37°C in a CO_2 incubator. Aliquots of the reaction mixture were taken to determine α-tocopherol (Toc.), TBARS, and fluorescence at the start of the reaction as well as after 2, 4, and 8 hrs. Figures 6-A, -B, and -C represent the Toc. level, the TBARS level, and the fluorescence intensity respectively.

association between high concentration of homocysteine in body fluids and development of arteriosclerosis.

Our findings indicate that the incubation of LDL with homocysteine and ferric ion resulted in the lipid peroxidation. The incubation with higher concentrations of homocysteine shortened the lag period until TBARS levels began to increase. We found that incubation of LDL with homocysteine and ferric ion resulted in the fragmentation of apoB, in addition to the formation of TBARS and fluorescent substances. These changes of LDL with oxidation were similar to those of copper-oxidized LDL, which was degraded by macrophages.[13] These findings support the hypothesis that homocysteine promote the iron-catalyzed modification of LDL,

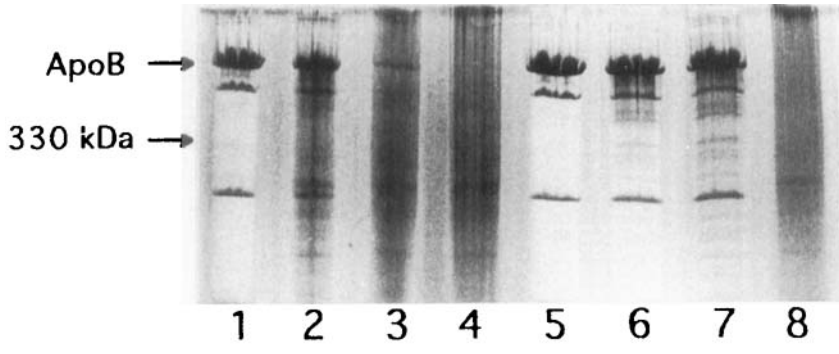

FIGURE 3 SDS-PAGE of apoB prepared from LDL oxidized with homocysteine. Native LDL (total cholesterol content: 0.6 mg/ml) was reacted with 50 μM NTA-Fe^{3+} and either 2 mM homocysteine (Lanes 1 ~ 4) or 20 μM homocysteine (Lanes 5 ~ 8). Lane 1 ~ 4 show LDL incubated with 2 mM homocysteine for 0, 2, 4, and 8 hrs, and lane 5 ~ 8 show LDL incubated with 20 μM homocysteine for 0, 2, 4, and 8 hrs, respectively.

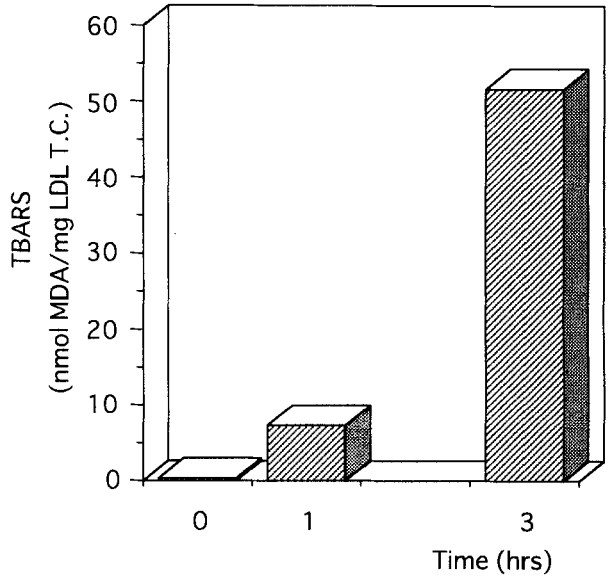

FIGURE 4 TBARS formation in LDL reacted with ferritin and homocysteine. TBARS formation was assayed in a LDL solution (total cholesterol content: 1.0 mg/ml) reacted with 1 mg/ml of ferritin and 500 μM homocysteine in phosphate buffer at 37°C in a CO_2 incubator. Values represent the mean of duplicate measurements.

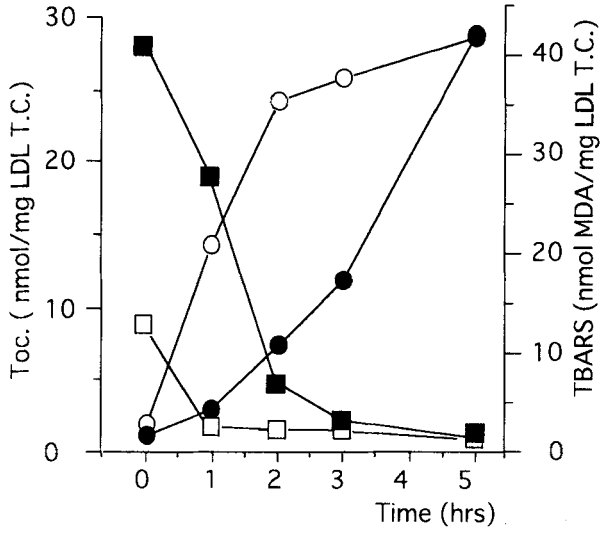

FIGURE 5 Effect of endogenous α-tocopherol on the iron-catalyzed modification of LDL in the presence of homocysteine. LDL (α-tocopherol-rich LDL and normal native LDL) was obtained from healthy human adults with or without the administration of 1.8 g of RRR-α-tocopherol daily for 7 days. Both α-tocopherol-rich LDL and control LDL (total cholesterol content: 0.6 mg/ml) were reacted with 1 mM homocysteine and 50 μM NTA-Fe^{3+} in a CO_2 incubator for 0 ~ 5 hrs. Squares show the depletion of α-tocopherol (Toc.) and circles show TBARS formation (open symbols represent native LDL and solid ones show α-tocopherol-rich LDL).

which may contribute to development of premature arteriosclerosis by endothelial cells injury and form cell formation.

In homocystinuria, the plasma homocysteine concentration is reported to be in a range of 100–200 μM unless therapeutical treatment is performed.[9] Although our reaction systems required a slightly higher concentration of homocysteine to produce rapid and profound changes of LDL, it may be assumed that similar reactions occur in subendotherial space at a slower but significant rate. However, since iron is usually completely bound to apoproteins and rarely exists in a free form *in vivo*, a question arises as to whether the reaction utilized in the above *in vitro* system can actually occur *in vivo*. Abdalla *et al.* also reported that ferritin is effective in promoting lipid peroxidation.[22] The present study also showed that oxidatively modified LDL was actually produced from native LDL by reaction together with ferritin and homocysteine. These findings suggest that the iron in ferritin could play a role in the *in vivo* modification of LDL. Therefore, the high levels of homocysteine detected in serum from homocystinuria patients may contribute to the oxidative modification of LDL and lead to premature arteriosclerosis, because ferritin exists in serum even though free iron does not.

Since our preliminary report[23] was published, Dudman *et al.*[24] published a paper wherein they did not observe an elevation of cholesterol ester hydroperoxides in the plasma of human hyperhomocysteinemia subjects compared with those from control subjects and they concluded that the thrombosis and premature arteriosclerosis in homocystinuria is not related to lipid peroxidation. One of the possible explanations may be that little oxidation takes place in the plasma due to a high concentration of

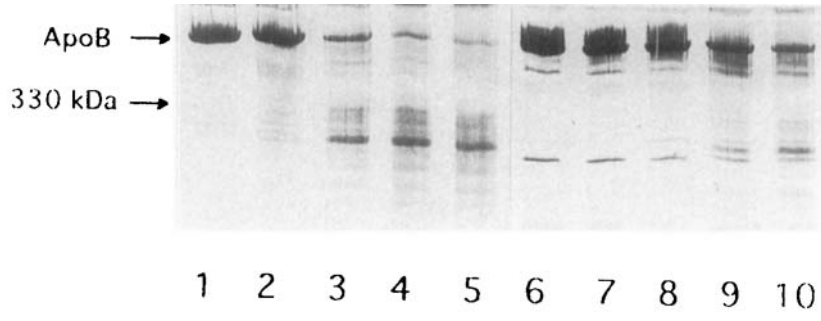

FIGURE 6 Effect of endogenous α-tocopherol on the breakdown of apoB during incubation with homocysteine and ferric ion. Samples of control LDL or α-tocopherol-rich LDL were obtained as described in the legend to Figure 5, (incubation with 1 mM homocysteine and 50 μM NTA-Fe^{3+}) and were analyzed by SDS-PAGE. Lanes 1 ~ 5 show control LDL incubated for 0, 1, 2, 3, and 5 hrs, respectively and lanes 6 ~ 10 show α-tocopherol-rich LDL incubated for 0, 1, 2, 3, and 5 hrs.

antioxidants but that the oxidation is induced in the intima by ferritin and homocysteine.

Epidemiological evidences established an inverse correlation between death caused by ischemic heart diseases and plasma antioxidant levels.[25] α-Tocopherol is known to be one of the most potent lipophilic antioxidants in the lipoproteins and biomembranes.[17,26] We found that the oxidative modification of LDL by homocysteine and NTA-Fe^{3+} was suppressed until α-tocopherol was consumed in LDL, suggesting that endogenous α-tocopherol can prevent the oxidation of LDL. When the α-tocopherol content of LDL was increased, the resistance of LDL to homocysteine-iron mediated modification was enhanced.

In conclusion, the metal-catalyzed oxidation of LDL induced by homocysteine may be involved in the development of premature arteriosclerosis in patients with homocystinuria. In these patients, it may be beneficial to prevent arteriosclerosis by maintaining higher levels of α-tocopherol in the body.

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