Protective Effect of Fluvastatin on Degradation of Apolipoprotein B by a Radical Reaction in Human Plasma

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Fluvastatin, which is a synthetic 3-hydroxy-3-methylglutaryl coenzyme (HMG-CoA) reductase inhibitor, its metabolites (M2, M3 and M4) and trolox all inhibited the decrease of apolipoprotein B-100 (apoB) and α-tocopherol in a radical reaction of human plasma initiated by Cu²⁺. The concentrations of fluvastatin, M2, M3, M4 and trolox for 50% inhibition (IC₅₀) of apoB fragmentation were 405, 8.55, 1.75, 305, and 43.4 μM, respectively. The IC₅₀ value of pravastatin, which is another HMG-CoA reductase inhibitor, was 2880 μM, showing that pravastatin is not an effective antioxidant. Although fluvastatin, its metabolites and trolox inhibited the decrease of α-tocopherol in a similar manner to that of apoB, pravastatin did not significantly inhibit the decrease of α-tocopherol. Since oxidation of low density lipoprotein (LDL) is an important step in the initiation and progression of atherosclerosis, fluvastatin may reduce the risk of atherosclerosis not only by lowering plasma cholesterol but also by protecting LDL from oxidation.

Key words: protein degradation; apolipoprotein B-100 (apoB); α-tocopherol; 3-hydroxy-3-methylglutaryl coenzyme (HMG-CoA) reductase; antioxidant

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 peroxidation4) and the resulting modification of apolipoprotein B-100 (molecular mass of 512 kDa) (apoB) by the aldehydes5,6) and hydroperoxide7) which are produced. Palinski et al.7) reported that malondialdehyde- or 4-hydroxynonenal-modified LDL could be 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.⁸) 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 they suggested that desialylated LDL increased in serum of these patients. Recently we reported that sialic acid moieties of LDL were decreased by oxidation with Cu²⁺, and that a radical reaction was one possible mechanism for the increase of desialylated LDL in serum of atherosclerotic patients.⁹)

Concerning the radical reaction of the protein moiety of LDL, in vitro oxidation of isolated LDL caused fragmentation and crosslinking of apoB.10–13) In these studies, products were analyzed by SDS-PAGE and dye-staining of the proteins. However, it is not clear whether such fragmentation also occurs in vivo, because no appropriate method is available which allows us to analyze the process in the presence of a great many other proteins. Recently, we have reported that immunoblotting is an effective method to follow apoB during the radical reaction in plasma. Besides, we have demonstrated that fragmented apoB proteins are present in normal serum and that they tend to increase on ageing.14) In addition, apoB shows unusually high reactivity, even comparable to α-tocopherol, in the radical reaction of plasma initiated by Cu²⁺ compared to other proteins such as albumin and transferrin.15,16) Based on these observations, the capacity of drugs to inhibit apoB fragmentation caused by the radical reaction in plasma can be an effective indicator in evaluating their anti-atherogenic activity. Based on this method in this study, we evaluated the antioxidative activity of widely used cholesterol-lowering drugs. At the same time we estimated the antioxidative effect of these drugs based on their interaction with α-tocopherol.

Fluvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, is widely used as a cholesterol-lowering drug.17,18) As with other HMG-CoA reductase inhibitors, fluvastatin was found to delay the progression of coronary heart disease in a lipoprotein and coronary atherosclerosis study (LCAS).18,19) It was reported that fluvastatin, but not pravastatin which is also an HMG-CoA reductase inhibitor, retards the copper ion-induced oxidation of human LDL.20) Studies evaluating the inhibitory effect of fluvastatin on plasma lipid peroxidation in vivo have also been reported in human21,22) and animals.23) To estimate the antioxidative ability of fluvastatin on LDL oxidation in vivo, it is important to evaluate the antioxidative activity of its metabolites as well as fluvastatin itself. The metabolic transformation of fluvastatin in human has been reported by Dain et al.24) Unchanged fluvastatin and desisopropylpropionic acid derivative (M4) are two major components in plasma. Hydroxyl derivatives (M2 and M3) are also generated in the metabolic pathway. Nakushima et al.25) reported the in vitro inhibitory effects of the fluvastatin metabolites on NADPH-induced lipid peroxidation of rat liver microsomes. They showed that the lipid peroxidation of liver microsomes was more strongly inhibited by M2 and M3 than by fluvastatin. In addition, Suzuki et al.26) also reported the inhibitory activity of these metabolites were stronger than that of fluvastatin on copper ion-induced LDL oxidation. In the present study, we investigated the inhibitory effect of fluvastatin and its metabolites M2, M3 and M4 on the degradation of apoB in human plasma, and compared it with that of pravastatin.

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MATERIALS AND METHODS

Materials  Fluvastatin sodium was donated by Tsukuba Research Institute, Novartis Pharma Co., Ltd. (Ibaraki, Japan). The metabolites of fluvastatin (M2, M3 and M4) were synthesized at the Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd. (Saitama, Japan). Pravastatin sodium was extracted from Mevalotin® (Sankyo, Tokyo, Japan). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid) was purchased from Aldrich (Wisconsin, U.S.A.). Vectastain ABC-PO (goat IgG) kit was obtained from Vector Lab. Inc. (Burlingame, CA, U.S.A.). Anti-human β-lipoprotein goat IgG was purchased from Sigma (St. Louis, MO, U.S.A.). Polyvinylidene difluoride (PVDF) membrane filters were purchased from Millipore (Tokyo, Japan). Polyacrylamide slab gels (3—10% gradient, 1 mm thick) were purchased from ATTO (Tokyo, Japan). All other reagents were purchased from Katayama Chemical (Osaka, Japan). Blood was taken from healthy male volunteers with heparin treatment. Plasma was separated by centrifugation at 600 g for 10 min.

Oxidation of Human Plasma  Human plasma was diluted four-fold with PBS, and then transferred into a microtube. An aliquot (100 μl) was taken for immunoblot analysis and measurement of the α-tocopherol level as the 0 h sample. Oxidation was started at 37 °C by the addition of 400 μM of Cu²⁺ at 37 °C. The oxidized plasma solutions, samples were withdrawn 4 h after the addition of Cu²⁺ and the content of apoB was measured as described in the text. Each value represents the mean±S.E. (n=4).}

was determined as described elsewhere. The conditions of HPLC and the fluorescence detector (Hitachi L-7480, Tokyo) were the same as reported previously.

Electrophoresis, Blotting, and Immunoblot Analysis  An aliquot (100 μl) taken from the reaction mixture was placed in a microtube and 10 μl of 4 mM EDTA-2Na (pH 7.4) was added. The samples were then treated with 100 μl of 4% SDS denaturation solution and SDS gel electrophoresis on 3—10% polyacrylamide slab gel (1 mm thick) was performed according to the method of Laemmli. Proteins separated on the gel were electrophoretically transferred to PVDF membrane filters as described elsewhere. Immunoblotting analysis of apoB was performed as described previously. The intensity of the stained band was measured by scanner (SHARP, JX-330M, Osaka, Japan).

Statistics  Statistical comparison among the groups was made by analysis of variance (ANOVA) followed by Schef- fe’s test. A probability below 5% was considered statistically significant.

RESULTS

Inhibitory Effect on the Degradation of ApoB in Human Plasma Induced by the Copper Ion  Human plasma subjected to a radical reaction initiated by 400 μM of Cu²⁺ at 37 °C as described previously. ApoB decreased to approximately 20% of the initial content after 4 h in the control reaction that was carried out in the absence of the inhibitors. When M3 or trolox was added, the decrease of apoB was inhibited in a dose-dependent manner (Fig. 2). Fluvastatin, and its other metabolites M2 and M4, as well as pravastatin also inhibited the fragmentation of apoB. To compare the inhibitory activity among these compounds, the concentration of each compound required for 50% inhibition (IC₅₀) of apoB fragmentation compared to the control was determined graphically at 4 h. The IC₅₀ values of fluvastatin, its metabolites M2, M3 and M4, and trolox for apoB fragmentation were 405, 8.55, 1.75, 305 and 43.4 μM, respectively (Table 1). In contrast, the IC₅₀ of pravastatin, which is another HMG-CoA reductase inhibitor, was 2880 μM. Among these compounds, M2 and M3 have higher activity as inhibitors than trolox in the radical reaction of plasma. Al-
Table 1. Relative Inhibitory Potency of Each Antioxidant on the Degradation of ApoB in Human Plasma

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>IC_{50} (μM)</th>
</tr>
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<tbody>
<tr>
<td>Fluvastatin</td>
<td>405</td>
</tr>
<tr>
<td>M2</td>
<td>8.55</td>
</tr>
<tr>
<td>M3</td>
<td>1.75</td>
</tr>
<tr>
<td>M4</td>
<td>305</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>2880</td>
</tr>
<tr>
<td>Trolox</td>
<td>43.4</td>
</tr>
</tbody>
</table>

Human plasma diluted with PBS, was mixed with a solution of fluvastatin, its metabolites (M2, M3, and M4), pravastatin or trolox, and then, treated with 400 μM of Cu^{2+} at 37 °C. From the oxidized plasma solutions, samples were withdrawn 4 h after the addition of Cu^{2+} and the content of apoB was measured as described in the text. IC_{50} was determined graphically at 4 h for apoB.

Fig. 3. Inhibitory Effects of Fluvastatin, its Metabolites, Pravastatin and Trolox on the Decrease of α-Tocopherol in Human Plasma

Human plasma diluted with PBS, was mixed with a solution of fluvastatin (FV), its metabolites (M2, M3, and M4), pravastatin (PV) or trolox, and then, treated with 400 μM of Cu^{2+} at 37 °C. From the oxidized plasma solutions, samples were withdrawn 2 h after the addition of Cu^{2+} and the content of α-tocopherol was measured as described in the text. Each value represents the mean±S.E. (n=3). *, p<0.05; **, p<0.01; ***, p<0.001 vs. control.

though the antioxidant activity of fluvastatin was lower than that of trolox, it was approximately 7-fold higher than that of pravastatin in terms of the IC_{50} value.

Inhibitory Effect on the Decrease of α-Tocopherol in Human Plasma Induced by the Copper Ion

The antioxidative activities of these compounds to α-tocopherol were evaluated after 2 h in the radical reaction of plasma initiated by Cu^{2+} at 37 °C. As shown in Fig. 3, fluvastatin, its metabolites M2, M3 and M4, and trolox significantly inhibited the decrease of α-tocopherol at the concentrations of 1000, 10, 1, 100, and 10 μM, respectively, however, pravastatin did not significantly inhibit the decrease of α-tocopherol even at the concentration of 1000 μM.

DISCUSSION

The present study indicates that fluvastatin and its metabolites have a protective effect on apoB and α-tocopherol. In addition, these observations indicate a sequential relationship between the decrease of α-tocopherol that is caused by lipid peroxidation, and apoB fragmentation. It is worthwhile to note that M2 and M3 are more effective antioxidants than trolox, a water-soluble model of α-tocopherol in the oxidation of apoB as well as α-tocopherol. Although fluvastatin showed antioxidative activity to apoB and α-tocopherol, pravastatin showed little activity. Therefore, the antioxidative activity of fluvastatin is not considered to be a common property of HMG-CoA reductase inhibitors, but is likely to be derived from fluvastatin’s unique chemical structure. Fluvastatin is the first totally synthesized HMG-CoA reductase inhibitor. Its chemical structure, including the fluorenyl indole moiety (Fig. 1), differs from that of pravastatin. The metabolites M2 and M3 with a phenolic hydroxyl group on the indole moiety showed more potent effects than fluvastatin itself. It has been well documented that indole derivatives show some antioxidative properties, and that the phenolic hydroxyl group enhances the antioxidative property of the aromatic compounds. Hussein et al. reported that when copper ion-induced LDL oxidation was evaluated ex vivo after the administration of fluvastatin for 24 weeks to patients with hyperlipidemia, the susceptibility of LDL to oxidation was greatly decreased. Yasuhara et al. showed that the oxidizability of hypercholesteremic rabbits was decreased after the administration of fluvastatin, however it was unchanged after the administration of pravastatin even at the same dose. These results indicate that fluvastatin functions not only as a HMG-CoA reductase inhibitor but also as an effective antioxidant. Based on these studies, we suggest that fluvastatin is a more effective drug for treatment of atherosclerosis than pravastatin.

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