In Vitro Inhibitory Effects of the Optical Isomers and Metabolites of Fluvastatin on Copper Ion-Induced LDL Oxidation

Kuniharu Suzumura,* Akio Odawara, Mikiko Yasuhara, Keiko Tanaka, Hiroshi Narita, and Toshikazu Suzuki

Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd., 2-2-50 Kawagishi, Toda, Saitama 335–8505, Japan.
Received April 22, 1999; accepted June 10, 1999

Fluvastatin is a synthetic hypolipidemic drug which inhibits 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. We compared in vitro the antioxidant effects of two enantiomers (3R, 5S and 3S, 5R) of fluvastatin, which is clinically used as a racemic mixture, on copper ion-induced oxidation of human low-density lipoprotein (LDL). Although 3R,5S-enantiomer of fluvastatin has 30-fold stronger inhibitory activity on HMG-CoA reductase than its optical counterpart, the antioxidative effects of these enantiomers on copper ion-induced LDL oxidation were similar. The antioxidative effects of the metabolites of fluvastatin (M2, M3, M4 and M7) on the copper ion-induced LDL oxidation were also investigated. All the metabolites tested showed an inhibitory effect on this system. Among them, the effects of M2 and M3, which have a phenolic hydroxyl group in each indole moiety, were strong and their potencies were 30–50 times greater than that of fluvastatin. We conclude that not only 3R,5S-enantiomer of fluvastatin but also its optical counterpart and the metabolites also have a potential to show the antiatherosclerotic effect through their antioxidative activities on lipid peroxidation.

Key words fluvastatin; HMG-CoA reductase inhibitor; antioxidant; low-density lipoprotein; conjugated diene; lipid peroxidation.

The purpose of anti-hyperlipidemic therapy is to prevent the development of atherosclerosis. One of the primary factors for the pathogenesis of atherosclerosis is believed to be an elevated level of plasma low-density lipoprotein (LDL).1 Recent studies have stated that the sequence of pathogenic events involves degeneration of LDL by certain mechanisms which include both enzymic and non-enzymic modifications.2,3 Degenerated LDL is taken up by macrophages via specific scavenger receptors and leads to foam cell formation at the atherosclerotic foci which contain massive esterified cholesterol.

Oxidized lipids have recently strongly been considered a component of degenerated LDL.1 The presence of oxidized LDL was demonstrated immunohistologically in the arterial vascular walls of human and animals.4–6 Further, antioxidative agents such as probucol have been reported to inhibit the progression of atherosclerosis in Watanabe heritable hyperlipidemic (WHHL) rabbits.7,8 In human, there are relatively few reports directly demonstrating the therapeutic efficacy of antioxidants on the progression of atherosclerosis because of the difficulty of the evaluation.9 However, oxidative modification of LDL is thought to be deeply related to atherosclerosis development even in human, and inhibition of the LDL oxidation in addition to lowering plasma LDL level is viewed as an effective way to prevent progression of this disease.9

Fluvastatin (Fig. 1) is the first totally synthetic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor and its clinical efficacy as a hypolipidemic agent has been widely recognized.10,11 We previously reported in vitro that fluvastatin, but not pravastatin which is also an HMG-CoA reductase inhibitor, retards the copper ion-induced oxidation of human LDL.12 Studies evaluating the inhibitory effect of fluvastatin on plasma lipid peroxidation in vivo were also reported in human13,14 and animals.15 They showed that the fluvastatin-treatment reduces the oxidizability of plasma lipids.

Fluvastatin has two chiral carbons in its chemical structure and it is clinically used as a racemic mixture (3RS, 5SR). The hypolipidemic action of fluvastatin in vivo is thought to be predominantly caused by 3R,5S-enantiomer but not by 3S,5R-enantiomer, because the former has 30-fold stronger inhibitory activity on HMG-CoA reductase than its optical counterpart.16 Concerning the inhibitory activity on the oxidation of lipids however, the 3S,5R-enantiomer seems to have equal antioxidative ability as its optical counterpart. In this paper, we attempted to compare the antioxidative effects of each enantiomer and the racemic mixture of fluvastatin.

To estimate the antioxidative ability of fluvastatin on LDL oxidation in vivo, it is important to know the antioxidative activity of its metabolites as well as that of fluvastatin itself. The metabolic transformation of fluvastatin in human has been reported by Dain et al.17 Unchanged fluvastatin and deisopropylpropionic acid derivative (M4) are two major components in plasma. Hydroxyl derivatives (M2, M3) and deoxy derivative (M7) are also generated in the metabolic pathway. Nakashima et al.18 reported the in vitro inhibitory effects of the metabolites of fluvastatin on NADPH-induced lipid peroxidation of rat liver microsomes. They showed that the lipid peroxidation of liver was more strongly inhibited by M2 and M3 than by fluvastatin. We are interested in the antioxidative effects of these metabolites on LDL oxidation. Therefore, the inhibitory activities of major metabolites of fluvastatin (M2, M3, M4 and M7; Fig. 1) on copper ion-induced LDL oxidation were also investigated here.

MATERIALS AND METHODS

Chemicals Fluvastatin (3RS, 5SR; racemic mixture; Fig. 1) and its two enantiomers (3R, 5S and 3S, 5R) were donated by Tsukuba Research Institute, Novartis Pharma K.K. (Ibaraki, Japan). M2 (3RS, 5SR; racemic mixture), M3 (3RS, 5SR; racemic mixture), M4 and M7 (Fig. 1) were synthesized in the Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd. (Saitama, Japan). Copper sulfate was purchased from

* To whom correspondence should be addressed.
Nacalai Tesque Inc. (Kyoto, Japan). All other reagents used were of the highest grade commercially available.

**Preparation of LDL.** Human LDL in the density range of 1.019 to 1.063 was isolated from fresh EDTA-treated plasma obtained from healthy male volunteers by ultracentrifugation (XL-90 ultracentrifuge, Beckman, CA, U.S.A.) at 150,000 g and 4 °C for 22 h. The isolated LDL was stored at 4 °C (a few days), then just before use, it was dialyzed against phosphate buffered saline (PBS, pH 7.4) to remove traces of EDTA and filtered through a 0.22 μm filter (Millipore, MA, U.S.A.). The protein concentration of LDL was measured by BCA protein assay kit with bovine serum albumin as the standard.

**Oxidation of LDL.** In all experiments, 50 μg protein/ml of LDL in PBS (pH 7.4) was incubated at 37 °C in the absence or presence of various concentrations of test compounds from 5 min prior to the start of oxidation for 3 h. Oxidation was initiated by the addition of a final concentration of 5 μM copper sulfate. Test compounds were dissolved in water or ethanol, and the final concentration of ethanol was below 1% which did not show any effect on LDL oxidation. The process of LDL oxidation was followed by measuring the absorbance at 234 nm using a spectrophotometer (DU-650, Beckman) as described previously. Lag time, maximum time and maximum rate were calculated as the indexes of LDL oxidation from the record of the absorbance curve. Because LDL was taken from the blood of different volunteers in each set of experiments, the absolute values of the parameter would vary based on the difference in constituents of each native LDL used. Therefore, we prepared a control group in each set of experiment, and judged the effect of the compounds on the LDL oxidation comparing the value with each corresponding control.

**Statistical Analysis.** Data were expressed as the means±S.E.M. of 4 to 6 separate experiments. Statistical comparisons among groups were carried out by ANOVA followed by Dunnett’s test. For all comparisons, the probability below 5% was considered to be statistically significant.

**RESULTS**

**Comparison of the Inhibitory Activity of Two Enantiomers of Fluvastatin on Copper Ion-Induced LDL Oxidation.** The antioxidative activities of two enantiomers of fluvastatin were applied to the copper ion-induced LDL oxidation system. Figure 2 shows typical tracings of the experiments. Table 1 shows the lag time, max time and max rate of conjugated diene formation which were calculated from each trace. The two (10 μM) showed comparable inhibitory activity to each other and to the racemic mixture.

**Inhibitory Effects of the Metabolites of Fluvastatin on Copper Ion-Induced LDL Oxidation.** Activities of the major metabolites of fluvastatin (M2, M3, M4 and M7; Fig. 1) at the concentration of 10 μM were examined. Figure 3 shows typical tracings of the experiments. M7 showed the weakest antioxidative activity and M4 which is a main metabolite in human plasma showed almost equal activity with that of fluvastatin. Among these metabolites, M2 and

![Fluvastatin](image)

![M2](image)

![M3](image)

![M4](image)

![M7](image)

Fig. 1. Chemical Structures of Fluvastatin and Its Major Metabolites

* indicates chiral carbon.

**Table 1. Effects of Racemic Mixture and Each Enantiomer of Fluvastatin on Copper Ion-Induced LDL Oxidation**

<table>
<thead>
<tr>
<th>(μM)</th>
<th>Lag time (min)</th>
<th>Max time (min)</th>
<th>Max rate (ΔAbs×10³/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>58.6±5.9</td>
<td>140.0±9.1</td>
<td>5.01±0.55</td>
</tr>
<tr>
<td>Fluvastatin (3R, 5S)</td>
<td>81.8±9.7</td>
<td>190.0±10.0*</td>
<td>3.49±0.43</td>
</tr>
<tr>
<td>(3R, 5S)</td>
<td>73.8±7.8</td>
<td>190.0±10.8*</td>
<td>3.74±0.47</td>
</tr>
<tr>
<td>(3S, 5R)</td>
<td>79.8±11.6</td>
<td>195.0±15.5*</td>
<td>3.38±0.39</td>
</tr>
</tbody>
</table>

Data were expressed as the means±S.E.M. of 4 volunteers. *: p<0.05 vs. Control (Dunnett’s test).
M3, which have a phenolic hydroxyl group in their chemical structures, showed the strongest antioxidative effects and completely inhibited the conjugated diene formation at this concentration (10 μM). The antioxidative potencies of M2 and M3 at lower concentrations (0.03—1 μM) were also evaluated. Figure 4 and Table 2 show the effect of M2, and Fig. 5 and Table 3 show the effect of M3 on the LDL oxidation, respectively. At these concentrations, M2 and M3 exhibited dose-dependent inhibitory effects on the conjugated diene formation. Both metabolites caused significant prolongation of the lag time above the concentration of 0.1 μM. Their antioxidative activities were 30—50 times stronger than that of fluvastatin.

DISCUSSION

Since oxidized LDL was shown to be deeply involved in the pathogenesis of atherosclerosis, a number of antioxidants have been examined for their prophylactic efficacy against this disease. It was reported that oral administration of a synthetic antioxidant, butylated hydroxytoluene (BHT) to cholesterol-fed rabbits suppressed the plasma level of oxidized cholesterol and inhibited development of atherosclerosis in the aorta. Recognizing that BHT did not change significantly the level of plasma cholesterol, antioxidants are considered to have potential to suppress atherosclerosis development.

Fluvastatin has two chiral carbons in its chemical structure and is clinically used as a racemic mixture of two enantiomers (3R 5S, 3S 5R). Although the hypolipidemic action of fluvastatin in vivo is thought to be mainly due to the 3R,5S-enantiomer of fluvastatin, we thought that the antioxidative effect is caused by either enantiomer. The present results clearly demonstrate that both 3R,5S-enantiomer and 3S,5R-enantiomer have an equal antioxidative ability on the copper ion-induced LDL oxidation. This finding corroborates the notion that the antioxidative property of fluvastatin is independent from its HMG-CoA reductase inhibitory activity, and that 3S,5R-enantiomer of fluvastatin, which has a slight hypolipidemic activity, also has a chance to contribute to the anti-atherosclerotic effect in vivo through its antioxidative activity.

The major metabolites of fluvastatin also inhibit the cop-
per ion-induced LDL oxidation in vitro. In particular, the inhibitory potencies of M2 and M3 were 30—50 times stronger than that of fluvastatin. Both M2 and M3 possess a phenolic hydroxyl group at their indole moieties, and compounds which have phenolic hydroxyl groups generally show antioxidative properties. These compounds terminate a radical chain reaction by donating a hydrogen atom from their phenolic hydroxyl group to the peroxided lipid radical and turn themselves into phenoxyl radicals. Therefore, the biotransformation from fluvastatin to M2 or M3 may potentiate the antioxidative and anti-atherosclerotic property of fluvastatin. Because M2 and M3 were mostly excreted in the feces in large quantities and existed in a relatively low level in plasma after oral administration of fluvastatin to humans, these metabolites are considered to show antioxidative activity in liver and to have a chance to reduce the oxidizability of very low-density lipoprotein in that organ which is secreted from liver to the circulation. Although the antioxidative activity of M4 is weaker than those of M2 and M3, M4 also showed an inhibitory effect on the lipid peroxidation similar to that of fluvastatin. Taking into consideration that M4 is the main metabolite of fluvastatin in human plasma in vivo, M4 is also considered to show the antioxidative effect on the lipids in the circulation in addition to fluvastatin itself.

Fluvastatin has been reported to show antioxidative activity on the plasma lipids when administered to hyperlipidemic subjects in vivo. Hussein et al. reported that when copper ion-induced LDL oxidation was evaluated ex vivo after administration of fluvastatin for 24 weeks to patients with hyperlipidemia, the susceptibility of LDL to oxidation was greatly decreased. Although the antioxidative mechanisms of administered fluvastatin in vivo are thought to be more complex than those of in vitro experiments, the present results suggest that not only 3R,5S-enantiomer of fluvastatin but also its optical counterpart and the metabolites have a potential to contribute to the inhibitory effect of fluvastatin on the plasma lipid peroxidation in vivo.

We therefore anticipate that fluvastatin can serve as a good prophylactic agent for atherosclerosis by suppressing both the plasma level of LDL and its oxidation.

REFERENCES