Fluvastatin, an HMG-CoA Reductase Inhibitor, Protects LDL from Oxidative Modification in Hypercholesterolemic Rabbits

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The antioxidative effect of fluvastatin sodium (fluvastatin) on low-density lipoprotein (LDL) was evaluated in vivo and in vitro. Since ex vivo measurement of the LDL oxidizability is reported to reflect the response of the atherosclerotic process, LDL isolated from rabbits fed a high cholesterol diet for 4 weeks with or without fluvastatin, pravastatin or α-tocopherol administration was oxidized by copper ions to estimate conjugated diene formation. Fluvastatin but not pravastatin significantly prolonged the lag time of LDL oxidized by copper ions ex vivo without affecting plasma cholesterol levels at a dose of 3 mg/kg after four weeks of treatment. α-Tocopherol-treated rabbits showed dramatically elongated LDL oxidation lag time at a dose of 150 mg/kg. In order to assess the mechanism, the content of α-tocopherol, a major endogenous antioxidant in LDL was measured, and we found that only LDL isolated from α-tocopherol-treated rabbits contained a significantly larger amount of α-tocopherol than that from high cholesterol control rabbits. To elucidate the mechanism further, the effect of fluvastatin on conjugated diene formation during copper-induced LDL oxidation in vitro was studied. Fluvastatin not only prolonged lag time, but also suppressed the rate of LDL oxidation, both in a dose dependent manner above 1 μM, while pravastatin showed no effect. These results suggest the direct antioxidative effect of fluvastatin on LDL oxidation in vivo. Since oxidation of LDL is an important step in the initiation and progression of atherosclerosis, fluvastatin may reduce the risk of this condition not only by lowering plasma cholesterol but also by protecting LDL from oxidation.

Key words fluvastatin; LDL; oxidation; lag time

Atherosclerosis is known to be the leading cause of cardiovascular disease. An elevated plasma level of low density lipoprotein (LDL) is a major risk factor for this disease, and several lines of mega studies suggest that cholesterol lowering therapy is effective in reducing angiographic changes and clinical event rate. 1-3) Fluvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, is widely used as a cholesterol-lowering drug. 4-5) As other HMG-CoA reductase inhibitors, fluvastatin was found to slow the progression of coronary heart disease 6) in a lipoprotein and coronary atherosclerosis study (LCAS). To our interest, fluvastatin was more effective than other cholesterol lowering drugs in patients with mildly to moderately elevated plasma cholesterol level. Thus, some mechanisms other than cholesterol lowering were thought to be involved.

Hussein et al. reported that the lag time required for the initiation of LDL oxidation was prolonged when LDL isolated from fluvastatin treated patients was incubated with copper ions. 7) Mitani et al. showed that fluvastatin (2 mg/kg per day for a period of 16 weeks) caused a reduction of the serum thiobarbituric acid reactive substances level in hypercholesterolemic rabbits. 8) These reports suggest the antioxidative effect of this inhibitor. Oxidative modification of LDL and its incorporation into macrophages are widely accepted as key processes of atherogenesis. 9) Therefore, inhibition of LDL oxidation is thought to be an effective approaches to preventing progression of this disease. Indeed, some antioxidants such as probucol and α-tocopherol are reported to prevent atherosclerosis in animal models. 10-12)

The chemical structure of fluvastatin, which is the first totally synthesized HMG-CoA reductase inhibitor, is different from those of other HMG-CoA reductase inhibitors 13, 13) (Fig. 1). Since some compounds which possess an indole ring in their chemical structure are reported to exert antioxidative activity, 14, 15) fluvastatin may exhibit an antioxidative effect based on its chemical structure. Therefore, we carried out an animal experiment to learn whether fluvastatin demonstrates an antioxidative effect and affects LDL oxidizability in vivo, using doses which would not decrease plasma cholesterol level. Further, we studied the direct antioxidative effect of fluvastatin in vitro to elucidate its mechanism of action on LDL.

MATERIALS AND METHODS

Materials Fluvastatin was donated by the Department of Pharmacology, Novartis Pharma (Ibaraki, Japan). Pravastatin sodium was extracted from Mevalotin® (Sankyo, Tokyo, Japan) at Tanabe R & D service (Saitama, Japan). All other chemicals used in this study were standard high-purity materials obtained from commercial sources.

Animals and Ethics These experiments were reviewed and approved by the Committee on Ethics of Animal Experiments, Tanabe Seiyaku Co., Ltd.

Male Japanese white rabbits (1.9-2.3 kg) were obtained

![Chemical Structure of Fluvastatin Sodium](image-url)
from Oriental Yeast (Tokyo). These animals were acclimatized in an environmentally controlled room (temperature; 21±2°C, humidity; 55±5%, illumination time 7:00 to 19:00) with food and water available ad libitum for 1 week before the experiment.

**In Vivo Experiment** Feeding Protocol: Animals fed regular chow (LRC4: Oriental Yeast, Tokyo) were assigned to the normal control group (n=12). The other animals were fed with a 0.5% cholesterol containing diet (high cholesterol diet, HC) for 1 week and assigned to one of the 5 groups described below based on their average plasma total cholesterol level: HC control (n=11); HC with fluvastatin 1mg/kg (n=12); HC with fluvastatin 3mg/kg (n=12); HC with pravastatin 3mg/kg (n=12); HC with 0.5%, i.e. 150mg/kg, α-tocopherol (n=6). Test compounds were orally administered once each morning for 4 weeks between 9:00 to 9:30. α-Tocopherol was mixed into the high cholesterol diet. Rabbits were fed 100 g of chow per day.

Blood Collection and LDL Preparation: Two milliliters of blood was collected from the marginal ear vein using a heparinized syringe and plasma was separated by low speed centrifugation before the start of drug treatment to measure plasma lipid levels. Plasma levels of total cholesterol (TC) and triglyceride (TG) were determined by commercially available enzymatic methods (Eiken Chemical Co., Tokyo). At the end of the treatment, rabbits were anesthetized by injection of 30-mg/kg sodium pentobarbital into the marginal vein, and blood was collected from the marginal ear aorta using a heparinized syringe. LDL was isolated as the 1.019 to 1.063 density fraction by sequential ultracentrifugation (Beckman XL-90, CA, U.S.A.). Isolated LDL was stored at 4°C until use. The protein concentration of LDL was determined with the BCA protein assay reagent (Pierce, Rockford, IL, U.S.A.) and was adjusted to 50 μg/ml with phosphate buffered saline (PBS, pH 7.4).

LDL Oxidation Assays: Determination of oxidizability of LDL was done according to the previously described method by incubating 50 μg protein of LDL with 5 μM CuSO4 in 1 ml PBS for 6 h at 37°C. The production of conjugated diene was monitored at 234 nm every 5 min using a DU-650 spectrophotometer (Beckman) equipped with a 6-position automatic sample changer. Thereafter, the kinetics of conjugated diene formation was calculated to estimate the susceptibility of LDL to oxidation. The lag time was determined as the time interval between initiation and the inflection point of the slope of the absorbance curve, which corresponds to the propagation phase. The time at which optical density reaches the maximal value was determined as the maximal time.

Fluvastatin and α-Tocopherol Measurement: Portions of the plasma were stored under -20°C until use. The concentration of fluvastatin in plasma and α-tocopherol content in plasma and LDL were determined by the method reported previously using high pressure liquid chromatography (HPLC).

**In Vitro Experiment** Six male Japanese white rabbits (12 weeks) were fed HC for 2 weeks. Five milliliters of blood was drawn from the marginal ear aorta and collected in plastic tubes containing EDTA solution to give a final EDTA concentration of 0.1%. The plasma was separated and LDL was isolated as described above; it was then dialyzed against PBS (pH 7.4) to remove EDTA immediately before use. Determination of the effect of drugs on oxidizability of LDL was made by incubating 50 μg protein of LDL with 1 μM CuSO4 in 1 ml PBS for 6 h at 37°C in the presence or absence of the test compound. Test compounds were dissolved in purified water. The lag time and the maximal time were determined as described above, and the maximal rate of oxidation was expressed by the slope of the propagation phase.

**Statistics** All data were expressed as the means±S.E. Statistical comparisons of in vivo experiment data between normal diet group and HC control group in each measurement were done by Student’s t-test, and comparisons between HC control group and drug treated groups were carried out by analysis of variance (ANOVA) followed by Tukey-Kramer’s test or Mann-Whitney’s U test with Bonferroni adjustment in α level as described in the table of result of each experiment. Comparison of in vitro study was done using ANOVA followed by Dunnett’s test. For all comparisons, the probability below 5% was considered to be statistically significant.

**RESULT**

**In Vivo Effects of Fluvastatin** Plasma Lipids: Table 1 shows plasma TC and TG levels of rabbits before and after the drug treatment. High cholesterol diet caused a significant increase of these levels, and neither fluvastatin, pravastatin nor α-tocopherol at the doses we used induced any significant reduction in the levels.

LDL Oxidizability: An ex vivo conjugated diene assay was performed to evaluate the effects of drug treatment on LDL oxidizability of each animal. Table 2 shows the lag time and the maximal time of conjugated diene formation of copper ion-induced LDL oxidation. Because of the low plasma cholesterol level, sufficient amount of LDL was not obtained.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Body weight (kg)</th>
<th>Plasma total cholesterol (mg/dl)</th>
<th>Plasma triglyceride (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td>4 w</td>
<td>Baseline</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td>1.95±0.05</td>
<td>3.25±0.05**</td>
<td>37.8±3.0</td>
</tr>
<tr>
<td>HC control</td>
<td></td>
<td>1.95±0.04</td>
<td>2.34±0.05**</td>
<td>481.0±131.7</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>1</td>
<td>1.93±0.04</td>
<td>2.38±0.05**</td>
<td>476.8±144.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.93±0.06</td>
<td>2.32±0.06**</td>
<td>477.8±132.8</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>3</td>
<td>1.97±0.06</td>
<td>2.46±0.05**</td>
<td>477.7±141.7</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>150</td>
<td>2.10±0.06</td>
<td>2.45±0.06**</td>
<td>483.3±129.7</td>
</tr>
</tbody>
</table>

*, p<0.05; **, p<0.01 vs. baseline (paired t-test). †, p<0.05; ††, p<0.01 vs. Normal (Tukey-Kramer's test).
Table 2. Kinetics of Oxidation of LDL Isolated from HC Fed Rabbits Treated with Fluvastatin, Pravastatin and α-Tocopherol

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Lag time (min)</th>
<th>Maximal time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC control</td>
<td>—</td>
<td>65.0±3.1</td>
<td>155.0±7.4</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>(1 mg/kg)</td>
<td>77.5±3.8</td>
<td>170.0±6.5</td>
</tr>
<tr>
<td></td>
<td>(3 mg/kg)</td>
<td>86.2±4.3*</td>
<td>180.0±5.2*</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>(3 mg/kg)</td>
<td>67.9±6.1</td>
<td>160.8±5.8</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>(150 mg/kg)</td>
<td>161.7±6.8*</td>
<td>＞240</td>
</tr>
</tbody>
</table>

LDL was isolated from rabbits of each group and adjusted to 50 μg protein/ml in PBS (pH 7.4). Oxidation was initiated by 5 μM CuSO₄ at 37°C, and the conjugated diene formation was measured by the absorbance at 234 nm. Values represent means±S.E., *p<0.05, **p<0.01 vs. HC control (Mann-Whitney’s U test with Bonferroni adjustment in α level).

Table 3. α-Tocopherol Levels in Plasma and LDL Isolated from HC Fed Rabbits Treated Fluvastatin, Pravastatin and α-Tocopherol

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Plasma (μg/ml)</th>
<th>LDL (μg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC control</td>
<td>—</td>
<td>20.89±1.71</td>
<td>2.492±0.48</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>(1 mg/kg)</td>
<td>20.29±1.64</td>
<td>3.325±0.40</td>
</tr>
<tr>
<td></td>
<td>(3 mg/kg)</td>
<td>18.78±1.78</td>
<td>3.773±0.41</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>(3 mg/kg)</td>
<td>19.51±1.60</td>
<td>3.373±0.41</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>(150 mg/kg)</td>
<td>152.53±20.55**</td>
<td>32.522±1.05**</td>
</tr>
</tbody>
</table>

LDL was isolated from rabbits of each group, and α-tocopherol content was measured using HPLC method. Values represent means±S.E. **p<0.01 vs. HC control (Tukey-Kramer’s test).

Table 4. Effects of Fluvastatin and Pravastatin on the Conjugated Diene Formation in the Copper-Induced LDL Oxidation

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. (μM)</th>
<th>Lag time (min)</th>
<th>Maximal time (min)</th>
<th>Maximal rate (ΔOD×10⁻³/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>74.2±2.6</td>
<td>203.3±6.0</td>
<td>9.207±0.382</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>0.3</td>
<td>75.1±2.3</td>
<td>207.5±6.9</td>
<td>9.147±0.404</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>79.1±2.5*</td>
<td>216.7±6.4</td>
<td>8.490±0.455*</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>85.8±3.6**</td>
<td>228.3±9.0*</td>
<td>8.067±0.381*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>111.8±2.5**</td>
<td>290.0±6.9**</td>
<td>6.747±0.459**</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>10</td>
<td>76.8±3.0</td>
<td>209.0±8.5</td>
<td>9.043±0.498</td>
</tr>
</tbody>
</table>

Rabbit LDL (50 μg protein/ml) in PBS (pH 7.4) was incubated with CuSO₄ (1 μM) at 37°C. The conjugated diene formation was measured by the absorbance at 234 nm. Values represent means±S.E. of 6 experiments. *, p<0.05; **, p<0.01 vs. control (Dunnett’s test).

In Vitro Effects of Fluvastatin on Copper-Induced Oxidation of LDL

Figure 2 shows representative traces of the experiment on the kinetics of conjugated diene formation in rabbit LDL oxidized by copper ion. Fluvastatin caused a dose-dependent increase of the lag time and a reduction of the maximal rate of LDL oxidation. As shown in Table 4, the effects of fluvastatin on these parameters reached a significant level at a dose of 1 μM. The maximal time also increased significantly at a dose of 3 μM or more. Pravastatin, however, did not affect any parameters of LDL oxidation.

DISCUSSION

An animal experiment was carried out in this study to elucidate the antioxidative effect of fluvastatin on LDL in vivo. Except for some clinical studies, the animal study, in which antioxidative effect of fluvastatin is suggested, is the one by Mitani et al.20 using hypercholesterolemic rabbits. Therefore, we used cholesterol diet-fed rabbits as a model of hypercholesterolemia. Four-week fluvastatin administration prolonged the lag time for LDL oxidation in hypercholesterolemic rabbits ex vivo, suggesting suppression of LDL oxidizability.21 We measured not only LDL oxidizability, but also plasma lipid peroxide (LPO) levels using a commercial kit (Oxis International, OR, U.S.A.) and thiobarbituric acid-reactive substances (TBARS). However, we did not observe any differences in plasma LPO levels between HC control group and drug treated groups (data not shown). Plasma LPO level represents the existence of oxidation products already generated in vivo, and which are assumed to be removed immediately by the liver. On the other hand, suppression of LDL oxidizability suggests increased resistance against oxidative attack by some mechanisms. The latter method seems more sensitive in reflecting the oxidative stress status in vivo.

Generally, oxidizability of LDL is determined by the content of endogenous antioxidant, such as α-tocopherol, and lipid composition, and fatty acid composition of lipids composing the LDL.23,24 Since plasma total cholesterol and triglyceride levels were similar to each other among groups fed HC, neither HMG-CoA reductase inhibiting property nor lipid levels is likely to be a cause of the protective effect of

![Fig. 2. Effect of Fluvastatin (FV) and Pravastatin (PR) on Copper-Induced Oxidation of Rabbit LDL.](image-url)

The increase of conjugated diene formation was measured by absorbance at 234 nm. Rabbit LDL (50 μg protein/ml) in PBS was incubated with CuSO₄ (1 μM) at 37°C. Fluvastatin was added at final concentrations of 0.3, 1, 3, 10 μM and pravastatin at a final concentration of 10 μM.

from normal diet fed rabbits and no LDL oxidizability assay was performed on them. LDL isolated from fluvastatin-treated rabbits showed significantly increased resistance to oxidation compared with HC control group, as judged by both lag time and maximal time at the dose of 3 mg/kg. However, LDL isolated from pravastatin-treated rabbits exhibited the same oxidizability as that of HC control group. LDL isolated from rabbits fed the α-tocopherol containing diet, in contrast, showed an extreme increase in lag time and maximal time.

α-Tocopherol Content: Table 3 summarizes α-tocopherol levels in plasma and LDL. Neither fluvastatin nor pravastatin affected the plasma content of α-tocopherol in HC fed rabbits. α-Tocopherol content of LDL isolated from both fluvastatin- and pravastatin-treated rabbits showed a slight increase, though not statistically significant. α-Tocopherol levels in plasma and LDL of α-tocopherol treated rabbits were increased to about 7 and 13 times higher levels than those of HC control group, respectively.

Fluvastatin Content in Plasma: Plasma concentration of fluvastatin 30 min after the final oral administration of 3 mg/kg was 1456±204 ng/ml, which is 3.36±0.47 μM.
fluvalastatin on LDL from oxidation. We also investigated lipid and fatty acid composition of the lipid in LDL isolated from each group, and found no significant differences among groups fed HC (data not shown). LDL oxidizability depends on the amount of oxidizable substrate, such as polyunsaturated fatty acid, available within the LDL particle. However, fluvastatin did not affect fatty acid composition. Therefore, the fatty acid composition does not seem to be an essential factor of decreased oxidizability of LDL by fluvastatin.

Secondly, we examined the content of dominant endogenous antioxidant, α-tocopherol in plasma and LDL, because Trible et al. reported that the α-tocopherol content of LDL was correlated with its resistance to oxidation. α-Tocopherol levels in plasma and LDL of the α-tocopherol-treated animals were higher than those of HC control group, and the lag time of this group was noticeably elongated. It is clear that greater resistance of LDL isolated from α-tocopherol-treated rabbits reported here against oxidation than that from the HC control group is a direct consequence of an increased concentration of α-tocopherol in LDL. However, α-tocopherol content of LDL obtained from the fluvastatin-treated group was not changed significantly, nor was that obtained from pravastatin-treated rabbits. Therefore, it is unlikely that the increased resistance of LDL isolated from fluvastatin-treated rabbits against oxidation is due to the α-tocopherol content in LDL.

We next investigated the direct action of fluvastatin on LDL oxidation. The antioxidative effect of fluvastatin on rabbit LDL oxidation induced by copper ions was tested in vitro, and we found that fluvastatin inhibited this oxidation at concentrations below the plasma concentration of fluvastatin in the in vivo experiment, which is 3.36 µM after oral administration of 3 mg/kg. Considering the clinical situation, this is a reasonable level, because maximal concentration of fluvastatin in human after oral administration of 40 mg q.d. was reported to be higher than 1 µM. Thus, it was suggested that fluvastatin actually exerts an antioxidative effect on LDL in vivo, and affects its oxidative status. In the clinical study, treatment of patients with fluvastatin resulted in a dramatic increase in the lag time of LDL oxidation after 6 months of treatment. Effect of the inhibitor on LDL oxidation observed in the clinical study was much more potent than that in the animal experiment we reported here. One reason for the difference may be the experimental period. We treated rabbits for only 4 weeks, and this may not be sufficient to exhibit the maximum antioxidative effect of fluvastatin in vivo. One other reason could be a species difference in metabolizing the drug. We reported earlier that some major human metabolites of fluvastatin have antioxidative effects on LDL oxidation, and also have superoxide radical scavenging activities.

The effects of some metabolites are more potent than those of fluvastatin, e.g. the inhibitory activity of human metabolites M2 and M3 on LDL oxidation is about 30—50 times more potent than that of fluvastatin. In human, those metabolites might exert their antioxidative property in vivo. However, the metabolism of fluvastatin in rabbits is different from that in human, and in our preliminary study the human major metabolites, which have potent antioxidative activity, were not found in the rabbit plasma (data not shown). Therefore, the antioxidative effect of fluvastatin may have been underestimated in rabbits in this study.

The precise mechanism of LDL oxidation in vivo is unclear at present, in spite of some intensive research. Recent studies suggest that NAD(P)H oxidase-dependent superoxide radical generation is involved in the oxidative process of LDL at the vessel wall. The superoxide radical scavenging activity of fluvastatin may contribute to the inhibition of LDL oxidation. In a recent clinical study, a novel action of fluvastatin of upregulating the bioavailability of nitric oxide (NO) in human was reported. Upregulation of endothelial nitric oxide synthase by HMG-CoA reductase inhibitors in vitro was also reported recently. These common actions of HMG-CoA reductase inhibitors, in addition to fluvastatin's antioxidative activity, may contribute to the suppression of LDL oxidizability. These characteristics of fluvastatin should be favorable to the cardiovascular system in addition to its cholesterol lowering effect.

In this study, we found increased resistance against LDL oxidation in rabbits treated with fluvastatin in vivo and confirmed the direct antioxidant property of fluvastatin against such oxidation induced by copper ions in vitro. This property may contribute to the beneficial effect of fluvastatin in addition to its lowering of cholesterol in the prevention of cardiovascular disease.

REFERENCES