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# Protective Effect of Fluvastatin Sodium (XU-62-320), a 3-Hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) Reductase Inhibitor, on Oxidative Modification of Human Low-Density Lipoprotein *In Vitro*

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**ABSTRACT.** We investigated the protective effect of fluvastatin sodium on the oxidation of low-density lipoprotein (LDL) induced *in vitro* by copper ions. The extent of lipid peroxidation was assessed by monitoring the increase of UV absorbance at 234 nm, which is the peak absorbance of a conjugated diene. Fluvastatin sodium (1–30 μM) not only prolonged the lag time of oxidation in the initiation step, but also decreased the rate of oxidation in the propagation step, both concentration dependently. Fluvastatin sodium and α-tocopherol showed an additive effect when both compounds were added before oxidation. However, when the lag time was prolonged initially by α-tocopherol, and fluvastatin sodium and α-tocopherol, were further added into the reaction mixture at the end point of the lag phase, fluvastatin sodium still showed an antioxidative effect, whereas α-tocopherol showed a pro-oxidative effect. Therefore, the antioxidative property of fluvastatin sodium differs from that of α-tocopherol. In this experiment, as neither the double bond-reduced derivative of fluvastatin sodium nor pravastatin sodium showed any protective effect, we concluded that the antioxidative effect of fluvastatin sodium is not a common property of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, but may be derived from its unique chemical structure. Since the oxidative modification of LDL plays an important role in the genesis of atherosclerosis, fluvastatin sodium may help reduce the risk of atherosclerosis, not only by reducing plasma LDL levels but also by protecting LDL from oxidative modification. BIOCHEM PHARMACOL **57**;6:697–703, 1999. © 1999 Elsevier Science Inc.

**KEY WORDS.** fluvastatin sodium; HMG-CoA reductase inhibitor; antioxidant; low-density lipoprotein; conjugated diene; lipid peroxidation

Fluvastatin sodium is the first totally synthesized HMG-CoA§ reductase inhibitor, and its chemical structure, a mevalonolactone derivative of a fluorophenyl-substituted indole (Fig. 1), differs from those of other HMG-CoA reductase inhibitors that were obtained by chemical modification of the fungal metabolites [1, 2]. The fluorophenyl indole moiety of fluvastatin sodium mimics coenzyme A in interacting with HMG-CoA reductase and the side chain mimics mevalonate, whereas the hitherto known HMG-CoA reductase inhibitors, such as pravastatin sodium, simvastatin, and lovastatin, have a hydronaphthalene ring [2]. In clinical studies, a dose of 20 or 40 mg/day of fluvastatin sodium for 6–156 weeks achieved statistically significant mean reductions of 19–31% in serum LDL-cholesterol levels and 15–21% in total cholesterol levels. Serum HDL-cholesterol levels increased by 2–10%, although these changes were not statistically significant. The ratio of LDL-cholesterol to HDL-cholesterol (an index of the risk of atherogenesis) was reduced markedly by 23–31% from the baseline [1]. Although the number of direct comparative trials with other HMG-CoA reductase inhibitors is limited, these beneficial effects of fluvastatin sodium on serum lipid profiles were similar to those of other inhibitors. Accumulated evidence supports the hypothesis that an elevated plasma level of LDL is a major risk factor of atherosclerotic disease, but little evidence has been reported that the native LDL directly promotes atherogenesis. The hypothesis that oxidative modification of LDL in the arterial wall plays an important role in atherogenesis has been presented [3]. In this hypothesis, oxidized LDL is recognized and taken up by macrophages via the scavenger receptor pathway [3]. Unlike the LDL receptor, the scavenger receptor is not regulated by intracellular cholesterol concentration, and the unregulated LDL uptake leads to foam cell formation in the early phase of atherosclerosis [4].

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§ *Abbreviations:* HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TBARS, thiobarbituric acid-reactive substances; DMTU, dimethylthiourea; AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; and BCA, bicinchoninic acid.

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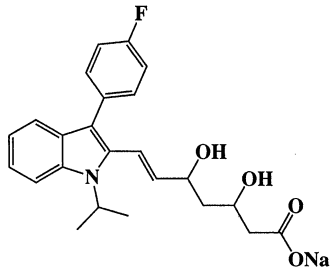
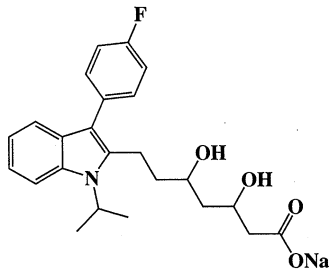



Fluvastatin sodium




Reduced derivative

**FIG. 1. Chemical structures of fluvastatin sodium and its reduced derivative.**

Immunohistochemical studies using monoclonal antibodies have demonstrated that oxidized LDL exists in atherosclerotic lesions in both experimental animal models and clinical studies [5, 6]. More recently, Sawamura *et al.* [7] reported the presence of endothelial receptors for oxidized LDL. A number of studies have indicated that synthetic antioxidants protect LDL from oxidative modification *in vitro* and reduce the development of atherosclerosis in animal models [8–10]. On the other hand, Hussein *et al.* [11] have reported that, when LDL derived from fluvastatin sodium-treated patients was incubated with copper, the lag time required for the initiation of LDL oxidation was prolonged 1.2- and 2.5-fold after periods of 12 and 24 weeks of drug therapy (40 mg/day), respectively. Moreover, Mitani *et al.* [12] reported that fluvastatin sodium (2 mg/kg per day for a period of 16 weeks) showed a reduction of the level of serum TBARS (an index of lipid peroxidation) in cholesterol-fed rabbits. However, the precise mechanism of the protective effect of fluvastatin sodium on LDL oxidation is still unknown. In the present study, we investigated the direct antioxidative effect of fluvastatin sodium against oxidative modification of LDL *in vitro*, and compared it with that of α-tocopherol.

## MATERIALS AND METHODS

### *Chemicals*

Fluvastatin sodium (XU-62-320) and pravastatin sodium were donated by the Department of Pharmacology, Sandoz Tsukuba Research Institute. The reduced derivative of fluvastatin sodium (sodium-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indol-2-yl]-3,5-dihydroxyheptanoate) was synthesized in the Lead Optimization Research Laboratory of the Tanabe Seiyaku Co., Ltd. BCA protein assay reagent was purchased from Pierce. α-Tocopherol and DMTU were purchased from Nacalai Tesque. AAPH was purchased from Wako Pure Chemical Industries, Ltd.

### *Isolation of LDL*

Human LDL in the density range of 1.019 to 1.063 g/mL was isolated from fresh EDTA-treated plasma obtained from healthy male volunteers by sequential ultracentrifugation in an XL-90 ultracentrifuge (Beckman) with a 50.2 Ti rotor at 150,000 *g* and 14° for 22 hr [13]. The isolated LDL was stored at 4° until used (for a few days). Immediately before use, LDL was dialyzed against PBS (pH 7.4) to remove all traces of EDTA and then filtered through a 0.22 μm filter (Millipore). The protein concentration of LDL was measured by the BCA protein assay with bovine serum albumin as the standard and was adjusted to 50 μg/mL with PBS (pH 7.4).

### *Oxidation of LDL*

In all experiments, 50 μg/mL of LDL was incubated at 37° in the absence or presence of various concentrations of test compounds from 5 min before the start of oxidation for 5–6 hr. Oxidation was initiated by the addition of a final concentration of 1 μM $CuSO_4$ or 300 μM AAPH. Test compounds were dissolved in a volume of ethanol to give a final concentration of 1% ethanol in the incubation medium.

### *Assay Method for Measuring LDL Oxidation*

The degree of lipid peroxidation of LDL was measured by the formation of conjugated diene structures generated by the oxidative destruction of polyunsaturated fatty acids in LDL. The conjugated diene formation was measured by the increase in absorbance at 234 nm [14]. In this system, the oxidation process is divided into three stages, i.e. lag phase, propagation phase, and decomposition phase. The lag phase is an initial step of oxidation during which polyunsaturated fatty acids are protected against peroxidation by consumption of oxidizable endogenous antioxidants. In this phase, conjugated diene formation is not observed. In the following propagation step, polyunsaturated fatty acids are oxidized to lipid hydroperoxides in a chain reaction. The absorbance of conjugated diene moieties at 234 nm increases as long as the rate of diene formation exceeds its decomposition. When the rate of diene decomposition becomes greater, the absorbance begins to fall and the decomposition phase starts. Absorbance was read in a DU-650 spectrophotometer (Beckman) every 10 min. To exclude the effect of UV absorbance by the test compound itself, we calculated the final data for plotting by subtracting the absorbance of the test compound at 234 nm from

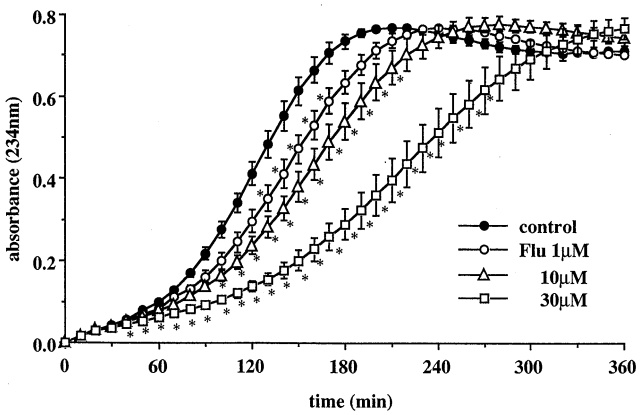



**FIG. 2. Effect of fluvastatin sodium (Flu) on copper-induced oxidation of human LDL. The increase of conjugated diene formation was measured by the absorbance at 234 nm. Human LDL (50 μg protein/mL) in PBS (pH 7.4) was incubated with $CuSO_4$ (1 μM) at 37°. Fluvastatin sodium was added at final concentrations of 0, 1, 10, and 30 μM, respectively. Values are means ± SEM. (N = 5). Key: (*) significantly different from control ($P < 0.05$).**

the raw data. The lag phase time period was determined from the intersection of two straight lines drawn along the changing optical density curve in the graph, one through the start point and tangent to the initial slowly rising curve and the other extended from the subsequent rapidly rising curve, which corresponds to the propagation step of LDL oxidation. The speed of oxidation at the propagation step was expressed by the slope of the latter line.

### *Statistics*

All data are expressed as the mean ± SEM of five experiments. Statistical comparisons among groups were carried out by ANOVA followed by Dunnett's test. For all comparisons, the probability below 5% was considered statistically significant.

## RESULTS

### *Effects of Fluvastatin Sodium on Copper-Induced Oxidation of LDL in Comparison with Other HMG-CoA Reductase Inhibitors*

The oxidation of LDL continued for 3–4 hr until the start of the decomposition phase, as shown in Fig. 2. Fluvastatin sodium at a concentration of 1–30 μM inhibited the conjugated diene formation in a concentration-dependent manner, and its effect was statistically significant even at the lowest concentration of 1 μM (Fig. 2). To investigate whether this effect is a common property among HMG-CoA reductase inhibitors or derived from the unique structure of fluvastatin, we compared fluvastatin sodium with two other HMG-CoA reductase inhibitors, i.e. the reduced derivative of fluvastatin and pravastatin sodium. The comparative experiment with the reduced derivative of fluvastatin sodium (see Fig. 1) is shown in Fig. 3. Although

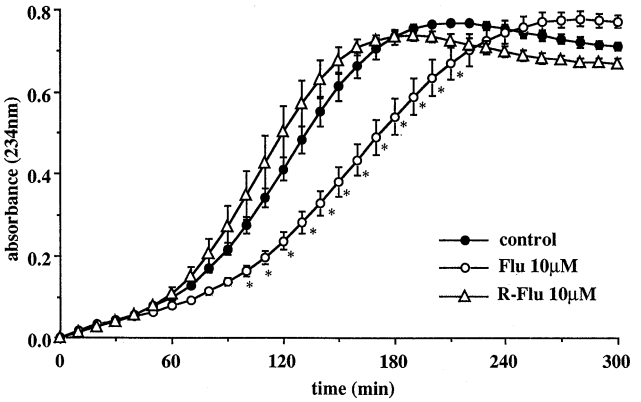



**FIG. 3. Comparative study of the effects of fluvastatin sodium (Flu) with those of its reduced derivative (R-Flu) on copper-induced LDL oxidation. Human LDL (50 μg protein/mL) in PBS (pH 7.4) was incubated with $CuSO_4$ (1 μM) at 37°. Values are means ± SEM (N = 5). Key: (*) significantly different from control ($P < 0.05$).**

fluvastatin sodium (10 μM) significantly inhibited the increase of conjugated diene formation, the reduced derivative (10 μM) had no effect. The comparative experiment with pravastatin is shown in Fig. 4. Although fluvastatin sodium showed a marked effect at 30 μM, the same concentration of pravastatin sodium demonstrated no effect.

### *Comparison of the Antioxidative Property of Fluvastatin Sodium with α-Tocopherol*

To determine the antioxidative potency of fluvastatin sodium, we performed a comparative study with α-tocopherol, an antioxidative vitamin and an important endogenous component of LDL. The results are shown in Figs. 5–8 and Table 1. Both fluvastatin sodium (10 μM) and α-tocopherol (10 μM) significantly inhibited diene formation, the latter being more potent than the former (Fig. 5). Table

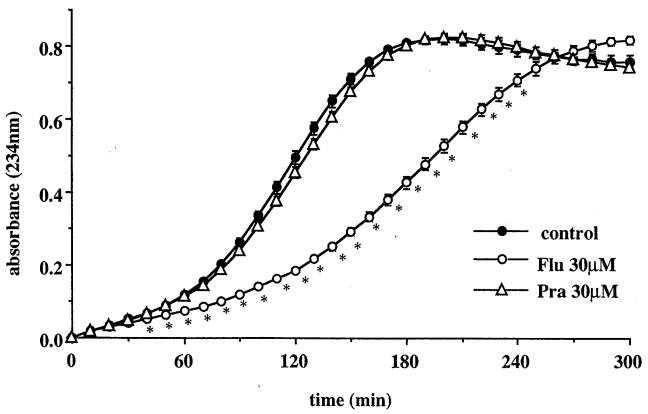



**FIG. 4. Comparative study of the effects of fluvastatin sodium (Flu) with those of pravastatin sodium (Pra) on copper-induced LDL oxidation. Human LDL (50 μg protein/mL) in PBS (pH 7.4) was incubated with $CuSO_4$ (1 μM) at 37°. Values are means ± SEM (N = 5). Key: (*) significantly different from control ($P < 0.05$).**

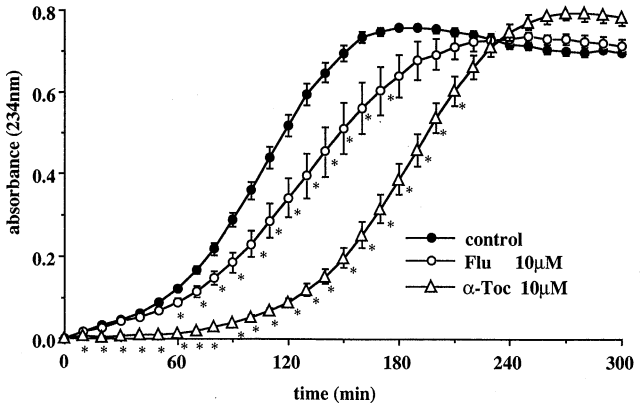



**FIG. 5. Comparative study of the effects of fluvastatin sodium (Flu) with those of α-tocopherol (α-Toc) on copper-induced LDL oxidation. Human LDL (50 μg protein/mL) in PBS (pH 7.4) was incubated with $CuSO_4$ (1 μM) at 37°. Values are means ± SEM (N = 5). Key: (*) significantly different from control ($P < 0.05$).**

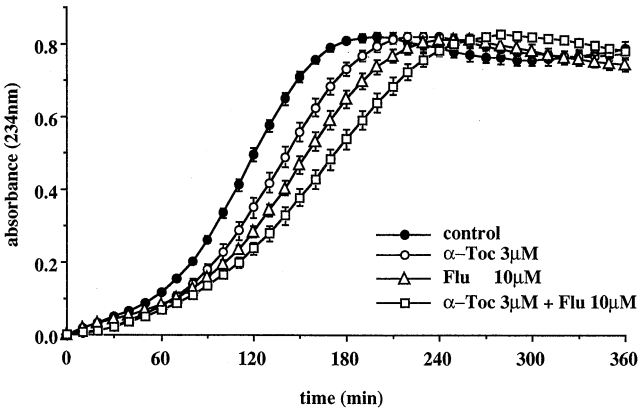



**FIG. 6. Effect of the combination of fluvastatin sodium (Flu) and α-tocopherol (α-Toc) on copper-induced LDL oxidation. Human LDL (50 μg protein/mL) in PBS (pH 7.4) was incubated with $CuSO_4$ (1 μM) at 37°. Values are means ± SEM (N = 5).**

1 shows the effects of the two compounds on the lag time of conjugated diene formation and the effects on the rate of diene formation in the propagation step. Although the prolonging effect of fluvastatin sodium on the lag phase was about three times weaker than that of α-tocopherol [fluvastatin sodium (30 μM) 203% and α-tocopherol (10 μM) 202% in Table 1], fluvastatin sodium showed a concentration-dependent reduction in the rate of diene formation in the propagation step, while α-tocopherol demonstrated no effect on it [fluvastatin sodium (30 μM) 54% and α-tocopherol (10 μM) 100% in Table 1]. Next, the interaction between fluvastatin sodium (10 μM) and α-tocopherol (3 μM) was investigated, and the results are shown in Fig. 6. The combination of both compounds showed a stronger effect than that of each individually when both compounds were added together before the start of oxidation. Methods to evaluate a synergic effect or an additive effect between drugs have been reported previously [15–17]. We used the method reported by Berenbaum [15] to determine whether the effect was additive or synergic. Figure 7 shows the lag time of LDL oxidation (percent of control average) when LDL was incubated with several combinations of both compounds. Points representing combinations with equal effects lie on the straight line joining the concentrations of fluvastatin sodium and α-tocopherol that are equally effective when used alone. Therefore, we conclude that the effect of the combination is additive. However, as shown in Fig. 8, when the test compound was added into the reaction mixture at the end point (150 min) of the lag phase, which had been prolonged by the initial addition of α-tocopherol (10 μM), additional α-tocopherol (3 and 10 μM) showed a concentration-dependent promoting effect on the conjugated diene formation (a), whereas fluvastatin sodium (10 and 30 μM) showed a concentration-dependent inhibitory effect on the conjugated diene formation (b).

**TABLE 1. Effects of the compounds on the lag time and the rate of conjugated diene formation**

| Test compounds | Lag time (% of control average) | Rate |
|---|---|---|
| Control | 100 | 100 |
| Fluvastatin sodium, 1 μM | 120 | 84 |
| 10 μM | 139 | 77 |
| 30 μM | 203 | 54 |
| α-Tocopherol, 10 μM | 202 | 100 |

Data were calculated using the mean data of Fig. 2 (fluvastatin sodium) and Fig. 5 (α-tocopherol), as described in Materials and Methods.

### *Effect of Fluvastatin Sodium on AAPH-Induced Oxidation of LDL*

To determine whether or not the inhibitory effect of fluvastatin sodium on the conjugated diene formation is

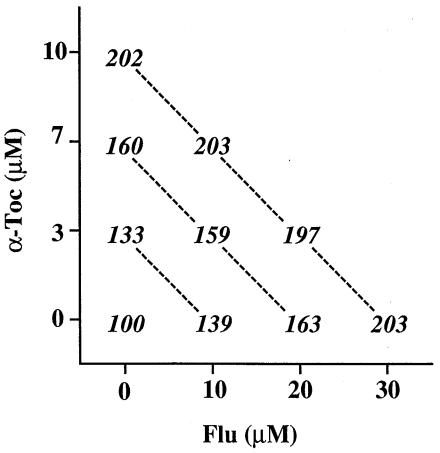



**FIG. 7. Isobologram showing the effects of combinations of fluvastatin (Flu) and α-tocopherol (α-Toc) on copper-induced LDL oxidation. Values in italics represent mean lag time of LDL oxidation (% of control average) when each combination of these compounds was added before the start of oxidation. Points representing combinations with equal effects lie on the straight line joining the concentrations of fluvastatin sodium and α-tocopherol that are equally effective when used alone.**

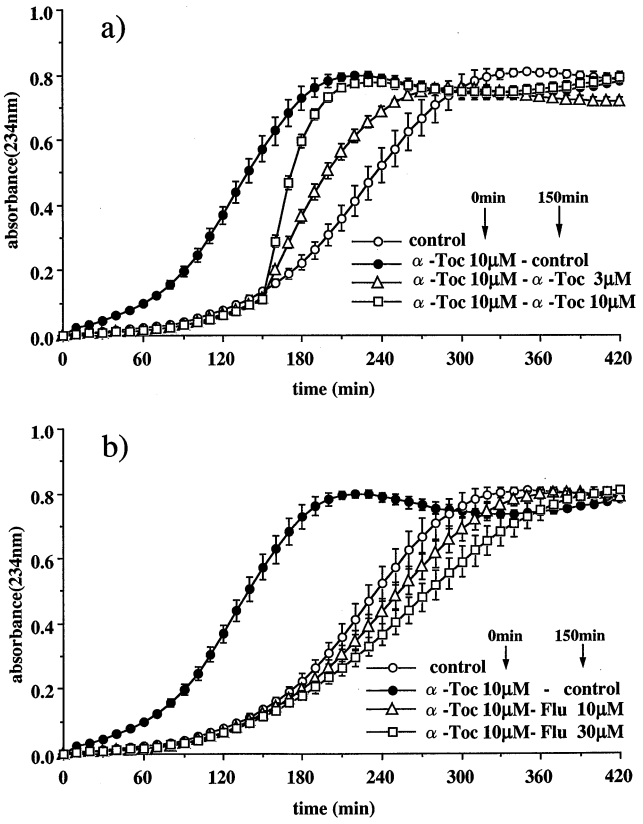



**FIG. 8. Effects of (a) α-tocopherol (α-Toc) and (b) fluvastatin sodium (Flu) on LDL oxidation when the compounds were added at the end point of the lag phase. Human LDL (50 μg protein/mL) in PBS (pH 7.4) was incubated with $CuSO_4$ (1 μM) at 37°. α-Tocopherol (10 μM) was added before the start of oxidation in all experiments except the control. At the end point of the lag phase (150 min), (a) α-tocopherol (0, 3, 10 μM) and (b) fluvastatin sodium (0, 10, 30 μM) were further added to the reaction mixture. Values are means ± SEM (N = 5).**

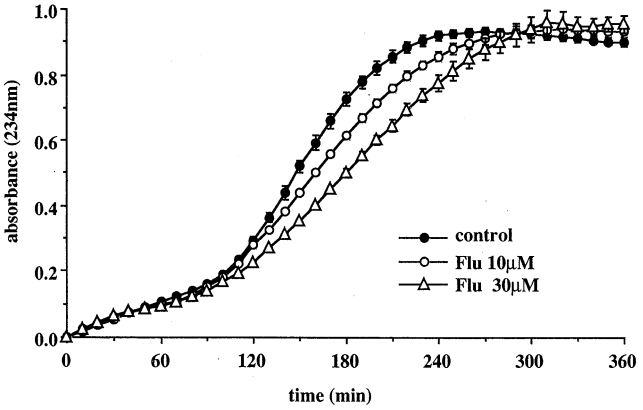



**FIG. 9. Effect of fluvastatin sodium (Flu) on AAPH-induced oxidation of human LDL. Human LDL (50 μg protein/mL) in PBS (pH 7.4) was incubated with AAPH (300 μM) at 37°. Fluvastatin sodium was added to the reaction mixture at final concentrations of 10 and 30 μM. Values are means ± SEM (N = 5).**

limited to the oxidation induced by metal ions, we performed oxidation of LDL by the non-metal and aqueous radical initiator AAPH (300 μM). As shown in Fig. 9, fluvastatin sodium also showed a concentration-dependent inhibition of the conjugated diene formation.

## DISCUSSION

It has been reported that natural antioxidants such as α-tocopherol, ascorbic acid, and ubiquinol and other synthetic antioxidants protect LDL from oxidative modification [10]. Although the exact mechanisms of the oxidative modification of LDL *in vivo* remain unclear, the oxidation of LDL in the arterial wall *in vivo* is thought to be initiated by metal ions [18], lipoxygenase [19], myeloperoxidase [20], peroxynitrate [21], and other endogenous substances. Smith *et al.* [18] reported that detectable amounts of iron and copper ions exist in human atherosclerotic lesions and that the use of transition metals to promote the peroxidation of LDL *in vitro* may be a valid model for events in the arterial wall. Moreover, O'Leary *et al.* [22] showed that an increased resistance to copper-induced oxidation was associated with a decrease of the lesion area in Watanabe heritable hyperlipidemic rabbits (WHHL). They also suggested that *ex vivo* measurement of the resistance of LDL to copper-induced oxidation is a reasonable surrogate index of the effectiveness of treatment with antioxidants [22]. Fluvastatin sodium inhibited the formation of conjugated diene structures in a concentration-dependent manner, and its effect was statistically significant at 1 μM or higher (Fig. 2). In this system, the oxidation of control LDL was finished within approximately 3 or 4 hr, but the oxidative modification of LDL *in vivo* might progress more slowly under the milder physiological conditions. According to a pharmacokinetic study of fluvastatin sodium, it was absorbed efficiently after oral administration and the mean highest plasma concentration of fluvastatin after multiple oral administrations at a dose of 40 mg qd (0.56 mg/kg) for a period of 6 days was higher than 1 μM [23]. Thus, the concentrations of fluvastatin sodium used in our study were in a reasonable range, and the present data, therefore, suggest that fluvastatin sodium might actually exert a direct antioxidative effect on LDL *in vivo*.

Since the reduced derivative of fluvastatin sodium, in which the double bond conjugated with the fluorophenyl-substituted indole ring was saturated, did not show any effect on the oxidation of LDL, the antioxidative property of fluvastatin sodium appears to be related to this conjugated double bond. A possible mechanism is that, when fluvastatin accepts or releases an electron by interaction with lipid radicals ($R^{\cdot}$) or lipid peroxy radicals ($ROO^{\cdot}$) generated in LDL, the resulting intermediate fluvastatin radical is stabilized by conjugation between the double bond and the indole ring, leading to a suppression of the production of more lipid or lipid peroxy radicals. Such an antioxidative mechanism is similar to that of β-carotene reported by Burton and Ingold [24].

Since the reduced derivative of fluvastatin sodium inhib-

its HMG-CoA reductase as effectively as fluvastatin sodium (data not shown), the antioxidative effect of fluvastatin sodium should not be a common property among HMG-CoA reductase inhibitors. This conclusion was further supported by the experiment with another HMG-CoA reductase inhibitor, pravastatin sodium, which did not show any inhibitory effect on LDL oxidation. These results indicate that the inhibitory effect of fluvastatin sodium on LDL oxidation is ascribable to its unique chemical structure.

To estimate the relative antioxidative potency of fluvastatin sodium, we chose a well-known antioxidative vitamin, α-tocopherol, for comparison and measured the lag time of conjugated diene formation in the initiation step and the rate of diene formation in the propagation step. Although the potency of α-tocopherol was about three times stronger than that of fluvastatin sodium, the latter showed a distinguishing feature in the antioxidative property (Table 1). In general, added phenolic antioxidants, such as α-tocopherol, are gradually consumed along with endogenous antioxidants contained in the LDL preparation after the start of oxidation, and thus extend the lag phase of oxidation. When almost all these antioxidants are consumed, the propagation step starts [25]. These antioxidants would effectively extend the lag time of oxidation, but, once consumed, would not reduce the rate of oxidation expressed as the slope of the tangent in the propagation step. On the other hand, fluvastatin sodium showed both an extension of the lag time in the initiation step and a reduction in the rate of oxidation in the propagation step. As fluvastatin sodium does not have a phenolic hydroxyl group, the antioxidative property of this compound is unique and may be due to its other structural features (Fig. 1). Fluvastatin sodium may be more stable than endogenous phenolic antioxidants such as α-tocopherol in LDL. Fluvastatin sodium may be partly consumed with other endogenous antioxidants in the lag phase, but part of it may remain even after the endogenous antioxidants are exhausted. Fluvastatin sodium extended the lag phase of oxidation in the initial step, though this effect was weaker than that of α-tocopherol, and further, even after the endogenous antioxidants were exhausted, the remaining fluvastatin may have reduced the rate of oxidation in the propagation step.

Fluvastatin sodium also showed the additive effect in combination with α-tocopherol. This finding indicates that fluvastatin sodium is effective under physiological conditions in the presence of endogenous antioxidants such as α-tocopherol. When test compounds were added into the reaction mixture at the end point of the lag phase, which was prolonged by the initial addition of α-tocopherol, fluvastatin sodium still showed an inhibitory effect on conjugated diene formation. In contrast, α-tocopherol showed a promoting effect under the same conditions. It has been reported that α-tocopherol shows a pro-oxidative effect under a certain set of conditions [26]. Kontush *et al.* [27] have reported that, under normal conditions where endogenous co-antioxidants exist in LDL, added α-tocopherol acts as an antioxidant. Its major mechanism is as follows: inactivation of one radical ($R^{\cdot}$) by one molecule of α-tocopherol and subsequent scavenging of a second radical by the α-tocopheroxyl radical. The overall result is inactivation of two radicals per one molecule of α-tocopherol. However, when co-antioxidants such as ascorbate and ubiquinone, which convert the α-tocopheroxyl radical to α-tocopherol, do not exist in LDL, α-tocopherol acts as a pro-oxidant. The chain propagation is initiated by the α-tocopheroxyl radical, which exists for a certain period of time, and polyunsaturated fatty acids in LDL are directly oxidized by the radical [27]. In our experiment, as the endogenous co-antioxidants in LDL may be exhausted in the lag period [25], the above mechanism may explain the promoting effect of α-tocopherol on the conjugated diene formation observed in Fig. 8a. It is not known whether this pro-oxidative phenomenon of α-tocopherol can occur *in vivo*. It is postulated that α-tocopherol acts as an antioxidant under normal conditions, but when the co-antioxidants are exhausted by some chronic oxidative stress, α-tocopherol could act as a pro-oxidant in the arterial wall, which is isolated from the co-antioxidants of plasma. It may be one of the reasons why the studies on the effect of α-tocopherol against atherosclerosis in animal models have given inconsistent results [10, 28, 29]. Fluvastatin sodium may show the protective effect on LDL oxidation even under these conditions.

Fluvastatin sodium also showed a protective effect on the LDL oxidation that was induced by the non-metal radical initiator AAPH. Chelation of fluvastatin sodium with copper ion was examined by the spectrophotometric procedure [30, 31]. Fluvastatin sodium did not cause any spectral shift or absorbance change by the addition of $CuSO_4$ (data not shown). Considering these results, the inhibitory activity of fluvastatin sodium on oxidative modification of LDL would not be derived from its chelating action with copper ion but from its direct antioxidative effect.

In conclusion, the present data suggest that fluvastatin sodium has a protective effect on oxidative modification of LDL; this property of fluvastatin sodium is not a common property among HMG-CoA reductase inhibitors, but is a direct antioxidative effect ascribable to its unique chemical structure. We conclude that fluvastatin sodium may serve as an ideal drug for reducing the risk factor of atherosclerosis, not only by reducing plasma LDL levels but also by protecting LDL from oxidative modification.

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