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Fluvastatin Depresses the Enhanced Lipid Peroxidation in Vitamin E-deficient Hamsters

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Fluvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, has recently been reported to have the antioxidative activity *in vitro*. However, it is still unclear whether chronic treatment with this drug actually leads to amelioration of the redox status in the body. In this study, we investigated the antioxidative effect of fluvastatin *in vivo*, using a vitamin E-deficient hamster model, an *in vivo* model of enhanced oxidative stress. After pre-treatment with a vitamin E-deficient diet for 2 months, fluvastatin, pravastatin or probucol was added to the diet for 1 month. Vitamin E deficiency caused a significant increase in the levels of plasma oxidative stress markers such as 8-iso-prostaglandin F2α (8-iso-PGF2α) and hydroperoxides. Furthermore, there was a significant increase in the oxidizability of plasma lipids in the vitamin E-deficient animals, indicating that the oxidative stress was increased in the circulation. Fluvastatin markedly depressed the above oxidative stress markers in plasma, and significantly decreased the oxidizability of plasma lipids without affecting their levels. Probucol, a reference antioxidant, also showed a similar effect while pravastatin, another HMG-CoA reductase inhibitor, showed only a weak improvement. We suggest that the treatment with fluvastatin leads to a reduction of oxidative stress *in vivo*, which is mainly derived from its antioxidative property rather than its lipid-lowering activity.

Keywords: Vitamin E deficiency; Fluvastatin; 8-iso-prostaglandin F2α; Hydroperoxide; HMG-CoA reductase inhibitor; Antioxidant

INTRODUCTION

Reactive free radicals such as the superoxide anion and hydroxyl radical have been known to play an important role in the development of various diseases and oxidative tissue injuries.[1] For example, atherosclerosis, ischemia-reperfusion injury, inflammation, some types of cancer and toxic organ injuries have been reported to be closely related to free radical chain reactions.[1]

Endogenous natural antioxidants such as α-tocopherol and antioxidative enzymes such as

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superoxide dismutase play important roles in the protection of cells and tissues from oxidative tissue injury.[2] They work as a major defense system against invasive free radicals in the body. A number of studies have indicated that administration of synthetic or natural antioxidants leads to a reduction of the oxidative stress in plasma and tissues, and inhibits progression of the diseases associated with oxidative tissue injury.[2–4]

As for the patients with hyperlipidemia, the oxidative modification of low-density lipoproteins (LDL) and the oxidative injury of endothelial cells are considered to be two important factors for atherosclerosis development.[4] The presence of oxidized lipids has been reported immunohistologically in the arterial vascular walls of humans and animals.[5] Antioxidative agents such as probucol are known to reduce the LDL oxidizability and inhibit the atherosclerotic progression in hyperlipidemic animal models.[4,6] Although there are few clinical reports which directly demonstrate the therapeutic efficacy of antioxidants, inhibition of the LDL oxidation in addition to lowering the plasma LDL level is viewed as an effective way to prevent the progression of atherosclerosis.

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.[7] We have recently reported that fluvastatin has an antioxidative activity ascribable to its unique chemical structure.[8–12] Fluvastatin effectively scavenges reactive oxygen species such as superoxide anions and hydroxyl radicals,[9,10] and retards the LDL oxidation *in vitro*.[8,12] Furthermore, some major metabolites of fluvastatin also have potent antioxidative activities.[9–11] This antioxidative property of fluvastatin seems to be beneficial in treating patients with increased oxidative stress. However, in spite of these lines of evidence, it is still unclear whether the chronic treatment with this drug actually leads to amelioration of the redox status *in vivo*. Therefore, in the present study, we intended to investigate the antioxidative effect of fluvastatin *in vivo*, when the endogenous antioxidant system is depressed. Pravastatin, another HMG-CoA reductase inhibitor which lacks the chemical antioxidative property,[8,9] and probucol, a well-known antioxidant,[4,6] were also used as reference compounds.

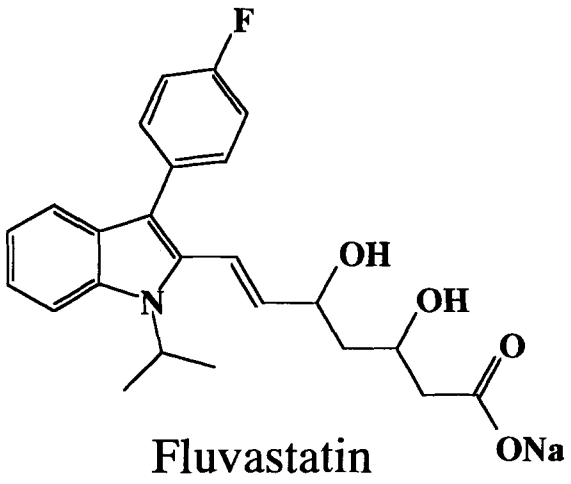

FIGURE 1 Chemical structure of fluvastatin.

Humans and some animals cannot synthesize vitamin E. It is well recognized that severe dietary vitamin E deficiency leads to an increase in the oxidative stress.[13,14] Because vitamin E is an important lipid-soluble antioxidant, its dietary deficiency in rats,[13] and hamsters,[15] seem to be good models to investigate the antioxidative properties of some compounds *in vivo*. We used the latter model to investigate the antioxidative efficacy of fluvastatin *in vivo* in the present study.

It is not easy to monitor the generation of free radicals directly *in vivo* because of their short lifetimes.[16] Therefore, there has been a great need for non-invasive stable biomarkers, which sensitively reflect changes of the redox status in the body. Recently, a novel series of prostaglandin F2-like compounds, especially 8-iso-PGF2α,

are emerging as a new class of selective and sensitive oxidative stress markers.[17–19] There is a substantial body of evidence which indicates the advantage of measuring 8-iso-PGF2α levels to assess the oxidative stress *in vivo* as compared with other classical methods.[16,20] It was also reported that the formation of prostaglandin F2-like compounds increased significantly in vitamin E-deficient animals.[14] Therefore, we mainly focused our attention to this parameter to investigate the antioxidative effect of drugs *in vivo*.

In this paper, we report the results of measurement of the levels of 8-iso-PGF2α in plasma and organs, as well as the levels of plasma hydroperoxides and the oxidizability of plasma lipids, and evaluation of the effect of antioxidative treatments on these redox parameters in the hamster model of vitamin E deficiency.

MATERIALS AND METHODS

Chemicals

Fluvastatin sodium salt and pravastatin sodium salt were donations from Tsukuba Research Institute, Novartis Pharma Co. Ltd (Ibaraki, Japan). Probucol was purchased from Daiichi Pharmaceutical Co. Ltd (Tokyo, Japan). Xylenol orange was obtained from Aldrich (Wisconsin, USA). 8-iso-PGF2α and [3,3,4,4,-$^2H_4$]-8-iso-PGF2α were purchased from Cayman (Michigan, USA). All other reagents used were of the highest grade commercially available.

Diets

The normal diet (AIN-93M) and vitamin E-deficient diet (based on the diet AIN-93M) were purchased from Oriental Yeast Co. Ltd (Tokyo, Japan). The composition of AIN-93M was as follows: cornstarch (46.6%), vitamin-free casein (14.0%), α-cornstarch (15.5%), sucrose (10.0%), lard (4%), cellulose (5.0%), AIN mineral mix (3.5%), AIN vitamin mix (1.0%), L-cystine (0.18%) and bitartrate choline (0.25%). In the vitamin E-deficient diet, AIN vitamin mix was replaced by vitamin E-deficient AIN vitamin mix.

Animals and Experimental Design

The experiment was undertaken by observing code of the Animal Care and Use Committee of Tanabe Seiyaku Co. Ltd. Eight-week-old male Golden Syrian hamsters were obtained from Japan SLC (Shizuoka, Japan). The animals were housed in an environmentally controlled room (22±2°C, 12 h light and dark cycles) with free access to food and water. The animals in the normal group were given the normal diet throughout the feeding period (3 months). Other animals were supplied with the vitamin E-deficient diet for the first 2 months and then divided into five groups, which received the vitamin E-deficient diet supplemented with either no drug (control), fluvastatin (0.001 and 0.003%), pravastatin (0.003%) or probucol (0.12%) for a further month. The dose of each drug, corresponding to the clinical dose, was based on the previous experiments and reports.[7,21,22] On the final day of the feeding period (around 12 a.m.), all the animals were anesthetized with ethyl ether, and heparinized blood samples and tissues were collected. The blood was immediately centrifuged (2000*g* for 5 min, 4°C) to separate the plasma as a supernatant. The liver and heart were perfused with cold saline. These samples were stored at −80°C until use.

Measurements of Lipids and α-tocopherol

The levels of total cholesterol, triglyceride and phospholipid in plasma were measured by using commercially available enzymatic kits (Eiken Chemical Co. Ltd, Tokyo, Japan). α-Tocopherol

levels were determined by the high-performance liquid-chromatography (HPLC) method using fluorescence detection.[23]

Determination of the Oxidizability of Plasma Lipids

The oxidizability of plasma lipids was measured by the conjugated diene method.[24] Briefly, each plasma (3%) in phosphate buffered saline (pH 7.4) was incubated with $CuSO_4$ (50 μM) at 25°C. The degree of lipid peroxidation was followed by measuring the absorbance at 234 nm with a 96-well microplate reader (Molecular Devices Co., California, USA). From the record of the absorbance curve, the maximum rate of oxidation and the ending time of oxidation were calculated as the indices of the oxidizability.

Measurement of Hydroperoxides

The plasma level of hydroperoxides was determined by the FOX method.[25] Although this method is not specific to hydroperoxides, we used it as a simple method to evaluate the lipid peroxidation. Briefly, plasma (5%) was incubated with the FOX reagent at room temperature for 30 min. The absorbance was read at 560 nm after the reaction. The amount of hydroperoxides was calculated as mole equivalents of hydrogen peroxide. The standard curve was linear in the 0–10 nmol/ml range.

Measurement of 8-iso-PGF2α

A new simple assay method for 8-iso-PGF2α was developed in our laboratory, by using liquid chromatography/electrospray ionization/mass spectrometry (LC/ESI/MS).[26] The tissue homogenate (10% in phosphate buffered saline, pH 7.4) was extracted with acetonitrile/ethanol (1/1) and the resulting organic layer was evaporated under a stream of nitrogen. The residue was dissolved in 5% (v/v) ethanol (pH 3).

Tissue homogenate extract and plasma sample were spiked with [3,3,4,4-$^2H_4$]-8-iso-PGF2α as an internal standard, mixed well, and loaded onto the disk cartridge (Empore; 3M, USA), which was pre-conditioned with methanol and 1 mM HCl. The cartridge was washed with 1 mM HCl and then with heptane. The sample was eluted with ethyl acetate containing 1% (v/v) methanol, evaporated under a stream of nitrogen, and dissolved in the mobile phase of 0.1% acetic acid/acetonitrile (7/3). Extraction was performed by HPLC (model HP1100 system; Hewlett Packard, USA) with a Symmetry C8 column (3.9 × 150 mm, 5 μm; Waters, USA). Then the solution was filtered with a membrane filter (0.45 μm), and the filtrate was used for LC/ESI/MS analysis (4-Sector type MStation 700 tandem mass spectrometer; JEOL, Japan) equipped with an ESI source. The mass spectrometer was operated in the negative ion-selected ion-monitoring mode. Ions monitored were of the mass-to-charge (m/z) ratios of 353.24 and 357.26 for 8-iso-PFG2α and [3,3,4,4-$^2H_4$]-8-iso-PGF2α, respectively.

Statistics

All data were expressed as the means±S.E. of 9–10 animals. Statistical comparison among the groups was made by analysis of variance (ANOVA) followed by Dunnett's test. Probability below 5% was considered statistically significant.

RESULTS

Food Intakes and Body Weights of the Animals

There were no differences in the food intake among the groups of normal and vitamin E-deficient diet with or without drug, during the experimental period (7.5 g/day/body weight). They equally and gradually gained weight during the treatments. The approximate dose of each drug was calculated based on the

amount of food intake: fluvastatin (0.5 and 1.5 mg/kg/day), pravastatin (1.5 mg/kg/day) and probucol (60 mg/kg/day).

Profiles of Plasma Lipids

Plasma levels of lipids were determined in the normal, control, 0.001% fluvastatin, 0.003% fluvastatin, 0.003% pravastatin and 0.12% probucol groups: their mean total cholesterol levels were 147±7, 151±9, 142±6, 156±12, 151±10 and 153±10 mg/dl, respectively, their mean triglyceride levels were 267±28, 406±27, 357±36, 303±36, 379±40 and 371±50 mg/dl, respectively, and their mean phospholipid levels were 268±9, 292±11, 267±5, 267±18, 265±14 and 270±14 mg/dl, respectively. There were no significant differences in the plasma levels of total cholesterol and phospholipid between the vitamin E-deficient control and normal groups. However, the triglyceride level in the vitamin E-deficient control group was higher than that of the normal group ($p < 0.05$). All the drug treatments did not cause any statistically significant effects on the plasma lipid profiles in the vitamin E-deficient animals.

Vitamin E Levels in Plasma and the Liver

Vitamin E levels in plasma and the liver were determined in the normal, control, 0.001% fluvastatin, 0.003% fluvastatin, 0.003% pravastatin and 0.12% probucol groups: the plasma levels of α-tocopherol were 19.0±0.8, 1.7±0.1, 1.2±0.1, 1.3±0.1, 1.4±0.1 and 1.6±0.1 μg/ml, respectively, the hepatic α-tocopherol levels were 38.6±5.0, 2.0±0.3, 1.1±0.3, 1.4±0.2, 1.7±0.3 and 1.8±0.3 μg/g tissue, respectively. The animals fed the vitamin E-deficient diet showed over 90% decreases in α-tocopherol levels in both plasma and liver. All the drug treatments slightly decreased the levels of α-tocopherol in the plasma and the liver of the vitamin E-deficient animals. Although there were statistically significant decreases ($p < 0.05$) in the level of plasma α-tocopherol in both fluvastatin groups from that of the control group, the effect of fluvastatin was not dose dependent.

α-Tocopherol was by far the most abundant component of vitamin E (α,β,γ,δ) in plasma and organs. The levels of β-, γ- and δ-tocopherol in plasma and the liver were under the detection limits in all the animal groups (<0.01 μg/ml plasma, <0.01 μg/g tissue).

Oxidative Stress Markers in Plasma

Oxidizability of plasma lipids was determined by the conjugated diene method. Figure 2a shows the typical tracings of the experiments. Figure 2b,c shows the maximum rate and the ending time of oxidation, respectively, as calculated from each tracing. There was a significant increase in the plasma oxidizability of vitamin E-deficient hamsters; the maximum rate of oxidation in the control group was twice, and the ending time of oxidation was one third as compared with that in the normal. The fluvastatin treatment showed a tendency of a dose-dependent decrease in the plasma oxidizability in vitamin E-deficient hamsters, although statistical significance was not always apparent. Probucol, a reference antioxidant, also showed a similar effect on this parameter. In contrast, the treatment with pravastatin, the other HMG-CoA reductase inhibitor, did not show any significant effect.

Figure 3a,b shows the levels of hydroperoxides and 8-iso-PGF2α in plasma, respectively. Vitamin E deficiency elevated the plasma levels of these substances significantly, indicating that the oxidative stress was increased in the circulation. Both fluvastatin and probucol strongly depressed the elevated levels of these markers to the normal level, while pravastatin showed only weaker effects.

8-iso-PGF2α Levels in the Liver and Heart

The contents of 8-iso-PGF2α in the liver and heart were also determined in the normal, control, 0.001 and 0.003% fluvastatin, 0.003% pravastatin and 0.12% probucol groups: the mean levels of 8-iso-PGF2α in the liver were 4.4±0.2, 5.2±0.4, 5.2±0.3, 4.3±0.5, 4.3±0.3 and 5.5±0.3 ng/mg protein, respectively, and the mean levels of 8-iso-PGF2α in the heart were 11.5±1.4, 10.1±1.0, 10.4±0.6, 9.6±0.8, 9.8±0.9 and 9.4±0.5 ng/mg protein, respectively. In contrast to the effect on the plasma parameters, there were no significant differences between the vitamin E-deficient control and normal groups both in the liver and heart. The treatments with the drugs also caused no significant changes in the level of 8-iso-PGF2α in these organs.

DISCUSSION

Free radicals are produced continuously in the inside and outside of the cells as by-products of metabolism, even in the normal aging process.[27] Therefore, aerobic animals have developed a comprehensive set of antioxidant defense systems to protect the cells and tissues from oxidative damage.

In the present study, we investigated the effect of vitamin E deficiency on the redox parameters in the hamsters. Feeding the vitamin E-deficient diet to the animals caused a significant increase in the oxidizability of lipids (Fig. 2) and the oxidative stress markers such as 8-iso-PGF2α and hydroperoxides in plasma (Fig. 3). However, in the organs, there were no significant differences in the levels of 8-iso-PGF2α between the normal and vitamin E-deficient animals. It should be noted that the α-tocopherol level was markedly decreased in both plasma and the liver of the vitamin E-deficient animals (below 10% vs. the normal.). Although the precise reason is uncertain, it is likely that the organs are more

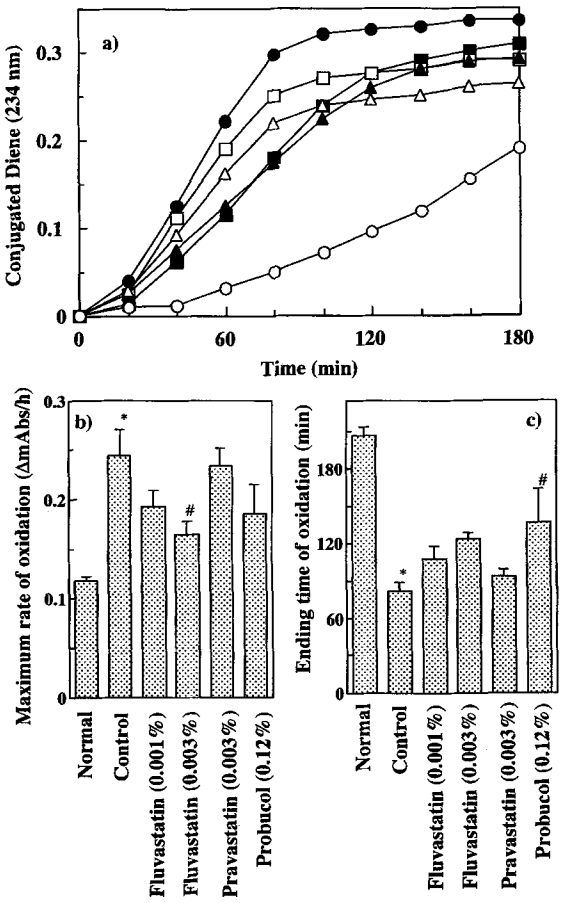

FIGURE 2 Oxidizability of plasma lipids in normal and vitamin E-deficient hamsters with or without drug-treatment. Oxidizability was determined as described in the text. (a) Typical tracings of the experiment. (○) Normal, (●) Control, (△) Fluvastatin (0.001%), (▲) Fluvastatin (0.003%), (□) Pravastatin (0.003%), (■) Probucol (0.12%). (b) The maximum rate of oxidation and (c) the ending time of oxidation were calculated from each tracing. Data are expressed as the means±S.E. ($n = 9$–10). $^{*}p < 0.05$ compared with normal; $^{\#}p < 0.05$ compared with control.

resistant against the increasing oxidative stress than plasma. Most antioxidative enzymes are located in the inside of the cells. Thus, the enzymatic oxidation and regeneration of antioxidants mainly occur in the inside of the cells, and play important roles to protect the tissues.[28,29] Furthermore, the levels of some antioxidants are extremely higher in the tissue than those in plasma. For example, the glutathione–glutathione peroxidase/reductase

system is one of the important defense systems in the cytosolic compartment. It is known that the glutathione level in tissue is about 50–100 times higher than that in plasma.[28] Therefore, this kind of distinction between the inside and outside of the tissue may be one of the causes of the difference in the resistance against the oxidative stress mentioned above.

The treatments with fluvastatin and the reference antioxidant probucol markedly depressed the plasma lipid peroxidation in vitamin E-deficient hamsters, while pravastatin treatment showed only a weak improvement (Figs. 2 and 3). Some HMG-CoA reductase inhibitors have been reported to decrease the oxidizability of plasma LDL when they are administered *in vivo*.[30–33] It is believed that there are two major mechanisms for this effect. The first one is closely related to the hypolipidemic action based on the HMG-CoA reductase inhibitory activity, e.g. promotion of the hepatic uptake of aged LDL which is more susceptible to oxidation, or modification of the lipid constituents. The second one is a direct antioxidative effect based on individual chemical structures. In this experiment, the plasma lipid profiles did not change significantly after the drug treatments. Therefore, the effect of fluvastatin on lipid metabolism seems unlikely to be the major cause of the inhibitory effect of this drug on

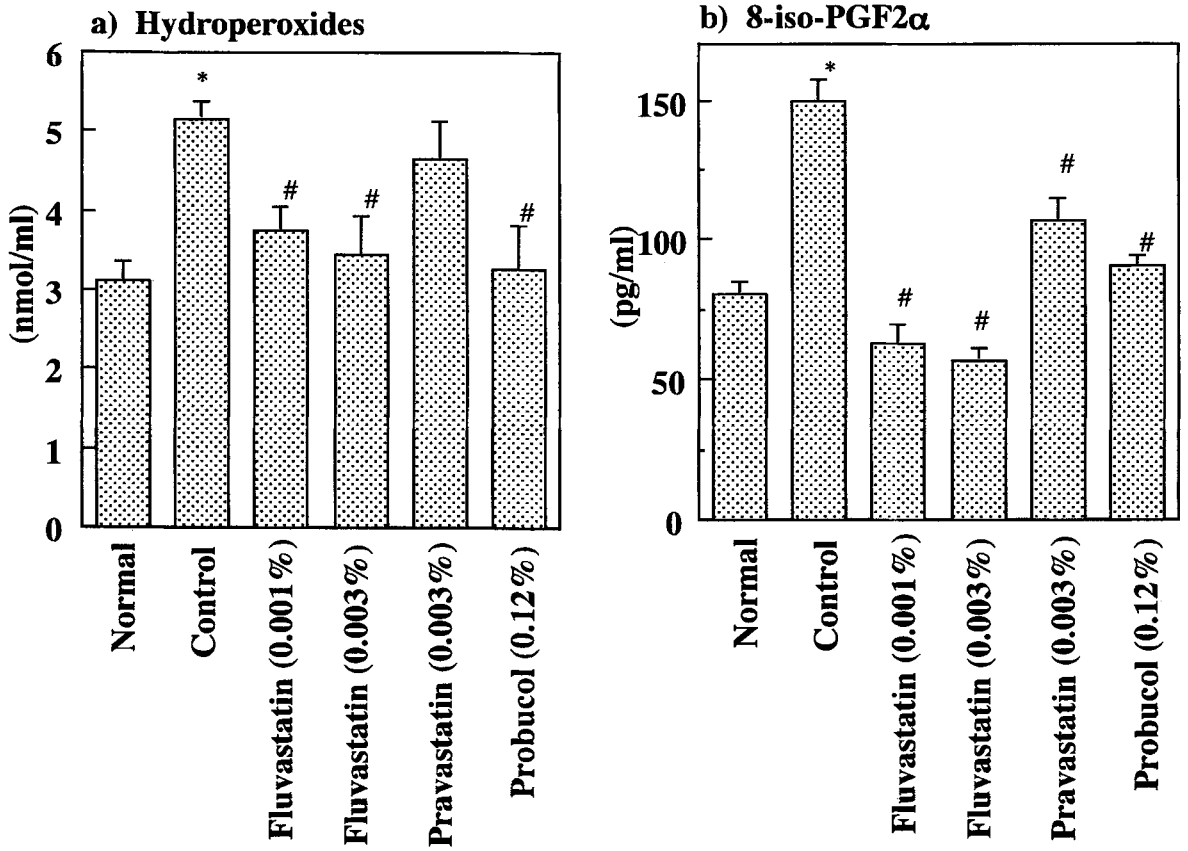

FIGURE 3 Levels of hydroperoxides and 8-iso-PGF2α in plasma. The plasma levels of (a) hydroperoxides and (b) 8-iso-PGF2α were determined as described in the text. Data are expressed as the means±S.E. ($n = 9$–10). $^{*}p < 0.05$ compared with normal; $^{\#}p < 0.05$ compared with control.

plasma lipid peroxidation. Therefore, we suggest that the inhibitory effect of fluvastatin on plasma lipid peroxidation in this study is mainly ascribable to its chemical antioxidative activity based on its unique chemical structure.[8]

In addition to this chemical antioxidant activity, a recent study also indicated that fluvastatin has a potential to improve the endogenous turnover of antioxidants, which is depressed in the rabbit model for familial hyperlipidemia.[34] Although it is not known whether similar effects are observed in other animal models and humans, such effect would also be beneficial to the endogenous protection system against free radicals.

Some studies have showed that even the clinical dose of fluvastatin delays the oxidation of plasma lipids in humans.[30,31,35] However, it is difficult to directly demonstrate the benefit from this antioxidative property when treating patients with hyperlipidemia and/or atherosclerosis. There are many clinical studies, which investigated the effectiveness of lipid-lowering therapies against coronary heart disease. Among them, the lipoprotein and coronary atherosclerosis study (LCAS) was conducted to determine whether the treatment with fluvastatin would reduce the progression of coronary atherosclerotic lesion formation in patients with CHD and moderately elevated LDL cholesterol.[36] In this study, the fluvastatin treatment significantly slowed the CHD progression. Interestingly, the angiographic benefit observed in the LCAS was as great as that in trials, which achieved a greater reduction in LDL cholesterol by other HMG-CoA reductase inhibitors. Although the precise reason is uncertain, this benefit would be, at least in part, derived from the antioxidative property of fluvastatin as described in the present paper. We speculate that the combination of the antioxidative activity with the hypolipidemic property of fluvastatin would augment its therapeutic efficacy in patients with complications of hyperlipidemia and increased risk factors for oxidative stress.

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