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Fluvastatin reduces modification of low-density lipoprotein in hyperlipidemic rabbit loaded with oxidative stress

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Abstract

The in vivo antioxidant effect of fluvastain, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, was investigated using Watanabe heritable hyperlipidemic (WHHL) rabbits subjected to nicotine-free cigarette smoke extracts as oxidative stress. Fluvastatin was given orally at doses of 10 and 30 mg/kg per day for 5 months. The cigarette smoke extracts were prepared by bubbling the gas phase of smoke into phosphate-buffered saline and was injected daily into the rabbit ear vein. The rabbits chronically treated with the cigarette smoke extracts showed an increase in plasma lipid peroxide levels, estimated as thiobarbituric acid-reactive substances. Oxidative modification of plasma low-density lipoprotein (LDL) was assessed by anion-exchange high-performance liquid chromatographic analysis, LDL susceptibility to oxidation, LDL incorporation into macrophages and thiobarbituric acid-reactive substances levels in LDL. Treatment with fluvastatin significantly reduced these effects induced by the cigarette smoke extracts in a dose-related manner and exerted a cholesterol-lowering effect. At the end of the experiment, the cigarette smoke extracts caused accumulation of cholesteryl ester in the thoracic aorta, while fluvastatin significantly prevented this accumulation. These results indicate that fluvastatin can exert an antioxidant effect in vivo, with a strong effect on oxidative stress such as smoking, a major risk factor of atherosclerosis.

Keywords: Fluvastatin; Antioxidant effect; Cigarette smoke extract; LDL (low-density lipoprotein); Oxidatively modified; WHHL rabbit

1. Introduction

Fluvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, has been used for the management of hypercholesterolemia. Fluvastatin has been reported to suppress atherosclerotic progression in experimental investigations with hyperlipidemic rabbits (Shiomi et al., 1998; Bandoh et al., 2000). Several trials of fluvastatin have demonstrated a beneficial effect on the progression of atherosclerosis, accompanied by a reduction in cardiac events in patients with coronary heart disease (Herd et al., 1997; Ballantyne et al., 1999; Riegger et al., 1999; Serruys et al., 1999). These effects of fluvastatin seem to be primarily due to its cholesterol-lowering effect in plasma, but there is growing evidence that fluvastatin exerts a direct anti-atherosclerotic effect on the arterial wall beyond its lipid-mediating effect (Herd et al., 1997; Bandoh et al., 1996; Shiomi et al., 1998). A number of mechanisms have been reported for its anti-atherosclerotic action, such as inhibition of vascular smooth muscle cell proliferation (Soma et al., 1993; Bandoh et al., 1996; Bellosta et al., 1998), suppression of monocyte- or leukocyte-endothelium cell adhesion (Niwa et al., 1996; Kimura et al., 1997), enhancement of vascular endothelial function (Kano et al., 1999; Bandoh et al., 2000; Kagota et al., 2000), inhibition of platelet activation (Hussein et al., 1997a) and inhibition of vascular angiotensin-converting enzyme activity (Mitani et al., 1996). These direct effects on the vascular wall seem to be in part due to its anti-oxidative action, because oxidative stress is related to vascular dysfunction and atherogenesis (Frei, 1999; Hoeschen, 1997). Fluvastatin has a characteristic antioxidant property (Mitani et al., 1996; Yamamoto et al., 1998; Obata and Yamanaka, 2000; Obata et al., 2000) and can protect low-density lipoprotein (LDL) from oxidative modification in vivo and in vitro (Suzumura et al., 1999a,b, 2000; Yasuhara et al., 2000; Hussein et al., 1997b; Leonhardt et al., 1997; Kagota et al., 2000). Since oxidized LDL has been recognized as playing

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an important role in the initiation and progression of atherosclerosis (Steinberg et al., 1989), the anti-atherogenic effect of fluvastatin must be intimately connected with its anti-oxidative action against plasma LDL in addition to its primary hypercholesterolemic effect.

The present study was designed to elucidate the effect of fluvastatin on the oxidative modification of plasma LDL in Watanabe heritable hyperlipidemic (WHHL) rabbits which were subjected to oxidative stress, i.e., injection of aqueous extracts of cigarette smoke. This is because cigarette smoke extracts are known to contain stable pro-oxidants (Yamaguchi et al., 2000a) and to oxidatively modify LDL in vitro (Yokode et al., 1988; Kagota et al., 1996) and in vivo (Yamaguchi et al., 2000b, 2001).

2. Materials and methods

2.1. Materials

Fluvastatin was provided by Tanabe Seiyaku (Osaka, Japan). The cigarettes used in this study were the Frontier Light brand (JT, Tokyo, Japan) containing 1 mg of tar and 0.1 mg of nicotine per cigarette. Cambridge filters (Borgwaldt, Germany) were used to remove 99.9% of all particles and nicotine from cigarette smoke. EDTA was from Dojindo Lab. (Kumamoto, Japan), Dulbecco's phosphate-buffered saline (PBS) was from Nissui Pharmaceutical (Tokyo, Japan) and Eagle's minimum essential medium (MEM) with balanced salt solution was from the Research Foundation for Microbial Diseases of Osaka University (Osaka, Japan). Other chemicals of analytical reagent grade were purchased from Nacalai Tesque (Kyoto, Japan) and used without further purification. DEAE-glucomannan gels for an anion-exchange high-performance liquid chromatographic (AE-HPLC) assay (Morita et al., 1990; Haginaka et al., 1995), which were kindly donated by Kurita Industries (Tokyo, Japan), were packed into a 4.6-mm I.D. × 50-mm stainless steel column. Water was purified with a Milli Q Jr. (Millipore, Tokyo, Japan) and used to prepare eluents for the HPLC method.

2.2. Preparation of cigarette smoke extract

A modification of the technique of Yokode et al. (1988) was used to obtain cigarette smoke extracts. Briefly, cigarette smoke extracts were prepared by bubbling into PBS (1 ml/three cigarettes) a stream of smoke from which tar and nicotine had been removed by gentle aspiration through a Cambridge filter; it took approximately 5 min to consume one cigarette. The cigarette smoke extract solution, with a pH of approximately 7.0, was sterilized through a 0.22-μm filter (Millipore).

2.3. Animals

Male or female WHHL rabbits (2 months old, 1.6–2.0 kg) were obtained from our colony and were bred by mating pairs of homozygous WHHL rabbits kindly supplied by Dr. T. Kita (Department of Geriatric Medicine, Faculty of Medicine, Kyoto University, Japan), originally obtained from Dr. Y. Watanabe (Institute for Experimental Animals, School of Medicine, Kobe University, Japan). As shown in Table 1, the WHHL rabbits were divided into non-treated control, control, fluvastatin-10 and fluvastatin-30 groups. The control, fluvastatin-10 and fluvastatin-30 groups received a daily intravenous injection of the cigarette smoke extracts (3 ml/rabbit per day) into the ear vein, while the non-treated control group received a daily intravenous injection of PBS (3 ml/rabbit per day) for 5 months each. The fluvastatin-10 and fluvastatin-30 groups were orally given doses of 10 and 30 mg/kg of fluvastatin daily, respectively. The concentrated granular preparations containing the respective dose of fluvastatin or the vehicle blended with standard chow (RC-4, Oriental Yeast, Tokyo, Japan) were prepared and given to individual animals deprived of food overnight. After the preparation was exhausted, standard chow was given and 5 h later the cigarette smoke extract solution or PBS was injected. The diet was given by pair-feeding and water was freely available.

2.4. Preparation of plasma and LDL samples

Blood was drawn from the ear artery of each rabbit at monthly intervals after overnight fasting. EDTA was added to the blood sample at a final concentration of 1 mg/ml for anticoagulation and prevention of autoxidation of lipoproteins. The plasma was separated by centrifugation (1500 × g for 10 min), stored at 4 °C and used to analyze the various lipids, thiobarbituric acid-reactive substances and subfractions of lipoproteins. LDL (d = 1.019–1.063 g/ml) was

Table 1
Grouping and changes in body weight

Group	Treatment	Drug	No. of rabbits	Body weight (kg)	
				Initial	Final
Non-treated control	PBS (3 ml/rabbit/day, i.v.)		5	1.79 ± 0.06	2.79 ± 0.12
Control	Cigarette smoke extracts (3 ml/rabbit/day, i.v.)		5	1.82 ± 0.07	2.74 ± 0.03
Fluvastatin-10	Cigarette smoke extracts (3 ml/rabbit/day, i.v.)	Fluvastatin (10 mg/kg/day, p.o.)	5	1.83 ± 0.04	2.86 ± 0.08
Fluvastatin-30	Cigarette smoke extracts (3 ml/rabbit/day, i.v.)	Fluvastatin (30 mg/kg/day, p.o.)	5	1.79 ± 0.04	2.63 ± 0.10

Each value represents the mean ± S.E.M. for five WHHL rabbits. PBS, phosphate-buffered saline.

isolated by ultracentrifugation (Hatch and Lees, 1968) from the plasma sample. A Beckman TL-100E ultracentrifuge was used to separate LDL with a TLA 110.3 fixed-angle rotor at 40000 × *g* for 330 min at 4 °C. The isolated LDL sample was then extensively dialyzed against PBS at 4 °C. It was used for the measurement of cholesterol and thiobarbituric acid-reactive substances, and for the experiments of LDL oxidation and cell culture.

2.5. LDL oxidation

A portion of LDL (100 μg protein/ml) obtained from each group was incubated in the presence of 2 μM $CuCl_2$ for 3 h at 37 °C. At the end of the incubation, the LDL was extensively dialyzed against PBS at 4 °C, and then the amount of oxidized LDL was measured by the thiobarbituric acid-reactive substances assay. In the cell culture experiment, a portion of LDL from each group was incubated with macrophages, and the cell-mediated oxidation of LDL was also measured.

2.6. Cell culture experiments

Unstimulated mouse peritoneal macrophages were harvested from female DDY mice (30–32 g) and cultured monolayers were prepared according to a previously described method (Yamaguchi et al., 2001). The macrophage monolayers were incubated with LDL from rabbits of each group to estimate the oxidative state of the LDL and the cell-mediated LDL oxidation. Two milliliters of each LDL solution in MEM (500 μg protein/2 ml MEM/dish) was added to individual macrophage monolayers, which were then incubated in a humidified CO_2 (5%) incubator at 37 °C. After 24 h of incubation, the medium was removed and the macrophage monolayers were rinsed three times with PBS. Cells were stripped from the plastic Petri dishes, transferred to plastic tubes and collected by centrifugation. Cell lipids were extracted by addition of 1 ml of *n*-hexane/isopropanol (3:2 v/v) to each plastic tube followed by gentle shaking for 30 min. Lipid extracts were transferred to glass tubes, evaporated under nitrogen, and used for cholesterol determination. The remaining non-lipid material in the plastic tube was used for protein determination. The amount of cholesteryl ester that accumulated in the macrophages was calculated from the difference between the cholesteryl ester content of the cells cultured with and without the LDL. This value is expressed as μg of cholesteryl ester per mg of cell protein. The incubation medium removed was also used for the determination of cholesterol, thiobarbituric acid-reactive substances and protein.

2.7. Preparation of artery samples

At the end of the experiment, the rabbits of each group were anesthetized with pentobarbital sodium (30 mg/kg, i.v.) after overnight fasting and killed by exsanguination from the common iliac bifurcation by cannulation. The thoracic aorta, coronary artery and renal artery were rapidly dissected and cleared of adhering fat and connective tissue. The arteries were freeze-dried to a constant weight and lipids were extracted at 50 °C for 20 min with chloroform–methanol (2:1 v/v). After the solvent had been evaporated under nitrogen, the residue was dissolved in acetone (100 μl) containing 2.5 mg of Triton X-100 and used for cholesterol determination.

2.8. Anion-exchange high-performance liquid chromatography method

An AE-HPLC assay was used to assess the modification of LDL in the plasma of WHHL rabbits injected with the cigarette smoke extracts, according to a previously described method (Yamaguchi et al., 1998a, 2000b). Plasma LDL was separated by stepwise elution into three subfractions: LDL1, LDL2 and LDL3. The cholesterol level of each subfraction was evaluated using the enzymatic post-column reaction. Cigarette smoke extract-modified LDL, which might be anticipated in plasma, was prepared by incubation of native LDL (200 μg protein/200 μl PBS) with the cigarette smoke extracts (100 μl), as described in previous reports (Kagota et al., 1996; Yamaguchi et al., 2000b). Typical chromatograms of native LDL and cigarette smoke extract-modified LDL are shown in Fig. 1. The degree of modification of plasma LDL after the injection of cigarette smoke extracts was evaluated by the increase in the ratio of LDL3 to LDL2.

2.9. Biochemical analysis

Total and free cholesterol levels in plasma, LDL and lipid extracts were determined by a fluoro-enzymatic method as described previously (Kunitomo et al., 1984; Yamaguchi et al., 1998b). The cholesteryl ester level was calculated from

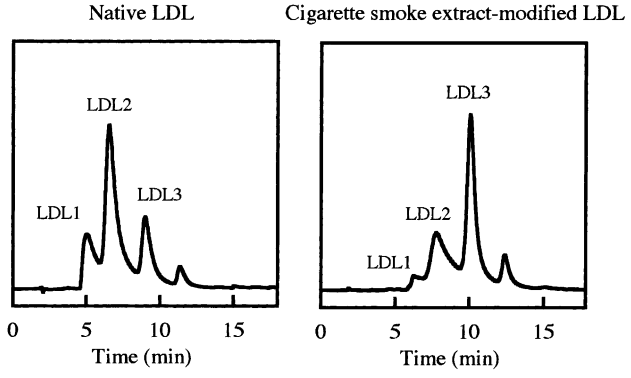

Fig. 1. Chromatograms of native LDL and cigarette smoke extract-modified LDL from anion-exchange HPLC (AE-HPLC) assay. LDL1, LDL2, and LDL3 represent subfractions separated by this method. The increase in the ratio of LDL3 to LDL2 was used as an indicator of oxidative modification of LDL.

the difference between the total and free cholesterol levels. Lipid peroxide levels in plasma, LDL and cell culture medium were estimated by assaying thiobarbituric acid-reactive substances, using the fluorometric method described by Yagi (1976). Protein levels in LDL and delipidated macrophages and culture medium were measured by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

2.10. Statistics

The results are expressed as the means ± S.E.M. Statistical analysis was performed using unpaired Student's *t*-test between the data from the non-treated control and control groups, and using Bonferroni multiple-range test between the data from the control, fluvastatin-10 and fluvastatin-30 groups. These statistical analyses were performed using the Stat View software package (SAS Institute, Cary, NC, USA). A difference was considered significant when $P < 0.05$.

3. Results

3.1. Changes in body weight

The weight gain in the fluvastatin-30 group tended to be modest compared to that in the non-treated control or control group throughout the experiment, but there were no significant differences in body weight among all groups at the end of the experiment (Table 1).

3.2. Plasma and LDL cholesterol levels

The time course of changes in plasma total cholesterol and LDL-total cholesterol levels in the four groups is shown in Fig. 2A and B, respectively. The data are expressed as a percentage of the respective starting lipid level in WHHL rabbits before the first injection (at 0 month). The initial plasma total cholesterol concentration was 919 ± 74, 920 ± 54, 921 ± 25 and 721 ± 40 mg/100 ml in the non-treated control, control, fluvastatin-10 and fluvastatin-30 groups, respectively. The initial LDL-total cholesterol concentration was 896 ± 75, 897 ± 55, 899 ± 25 and 897 ± 39 mg/100 ml, respectively. In addition, the plasma total cholesterol levels as well as LDL-total cholesterol levels in the control group were significantly increased compared to those in the non-treated control group in the early stage of the experiment, and thereafter these higher levels were maintained. Both fluvastatin-10 and fluvastatin-30 groups showed significant decreases in plasma total cholesterol and LDL-total cholesterol levels as compared to the control group and even the non-treated control group. The lowering effect in the fluvastatin-30 group was greater than that in the fluvastatin-10 group, but there was no significant difference between the two groups.

3.3. Plasma thiobarbituric acid-reactive substances level

The initial thiobarbituric acid-reactive substances level, which was used as an indicator of lipid peroxides, was 2.60 ± 0.18, 2.66 ± 0.21, 2.68 ± 0.15 and 2.68 ± 0.14 nmol/ml in the non-treated control, control, fluvastatin-10 and fluvastatin-30 groups, respectively. As shown in Fig. 2C, the thiobarbituric acid-reactive substances level in the control group gradually increased compared to that of the non-treated control group, and a significant difference was observed in a later stage (over 3 months) of the experiment. Throughout the experiment, the fluvastatin-treated groups (fluvastatin-10 and fluvastatin-30 groups) displayed a significant decrease in the thiobarbituric acid-reactive substances level compared to the control group in a dose-related manner.

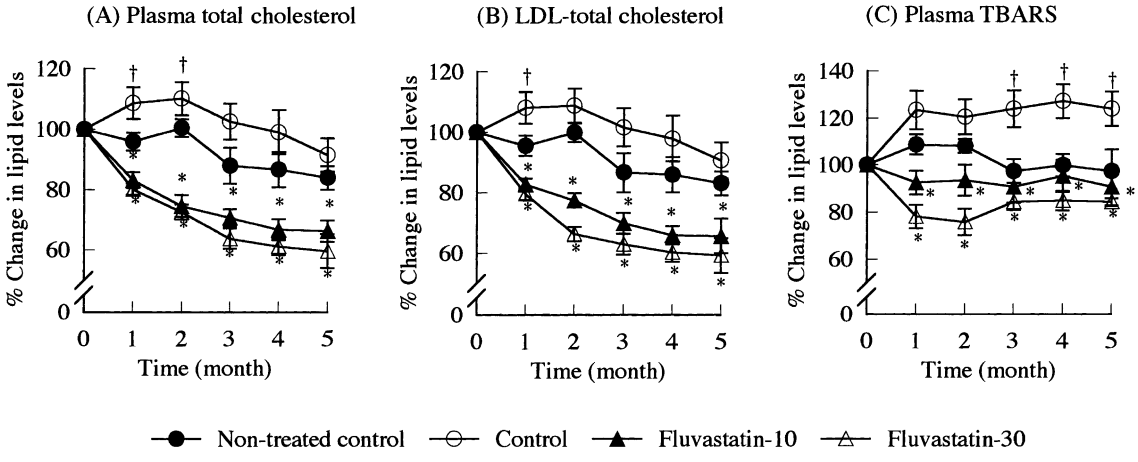

Fig. 2. Changes in relative lipid levels: plasma total cholesterol levels (A), LDL-total cholesterol levels (B) and plasma thiobarbituric acid-reactive substances (TBARS) levels (C) in WHHL rabbits treated with cigarette smoke extracts (the control group), cigarette smoke extracts plus 10 or 30 mg/kg of fluvastatin (the fluvastatin-10 or fluvastatin-30 group, respectively) for 5 months. Each point represents the mean ± S.E.M. for five rabbits. Details are given in Materials and methods. †$P < 0.05$, control group vs. non-treated control group; *$P < 0.05$, fluvastatin-10 or fluvastatin-30 group vs. control group.

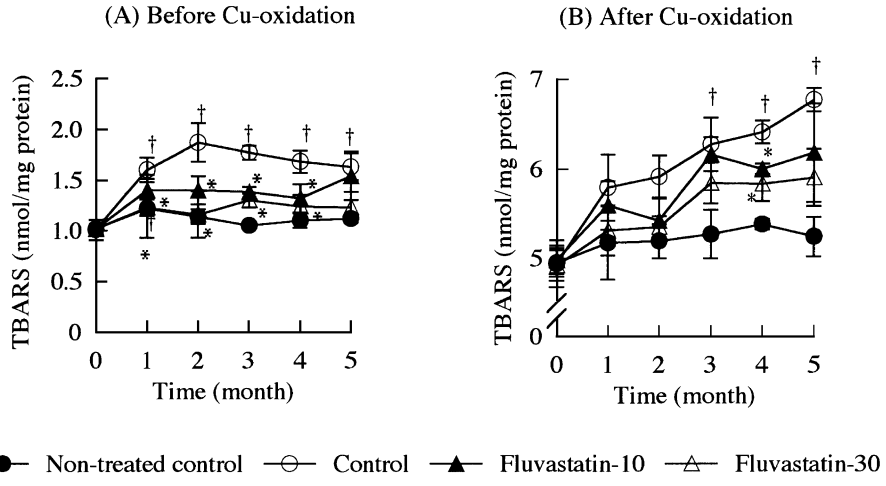

Fig. 3. Changes in thiobarbituric acid-reactive substances (TBARS) levels in LDL (A) and copper-oxidized LDL (B). Grouping is described in Fig. 2. Copper-oxidized LDL was obtained by incubation of fresh LDL from each group with 2 μM $CuCl_2$ for 3 h at 37 °C. Details are given in Materials and methods. Each point represents the mean ± S.E.M. for five rabbits. †$P<0.05$, control group vs. non-treated control group; *$P<0.05$, fluvastatin-10 or fluvastatin-30 group vs. control group.

3.4. LDL thiobarbituric acid-reactive substances level and oxidation by copper ion

Fig. 3 shows the time course changes in thiobarbituric acid-reactive substances level in LDL freshly obtained from each rabbit and in the LDL moderately oxidized by 2 μM $CuCl_2$ for 3 h at 37 °C. The thiobarbituric acid-reactive substances level in fresh LDL of the control group was significantly higher than that of the non-treated control group throughout the experiment. The fluvastatin-treated groups showed a significant decrease in the thiobarbituric acid-reactive substances level compared to the control group (Fig. 3A). The thiobarbituric acid-reactive substances level in LDL was markedly increased by copper oxidation throughout the experiment (Fig. 3B). The copper-catalyzed oxidation of LDL from the control group was the greatest among the four groups. Treatment with fluvastatin tended to inhibit the oxidation facilitated by administration of cigarette smoke extracts.

3.5. AE-HPLC assay of modified LDL

In order to assess the modification of LDL, subfractions of LDL in each group were separated by stepwise elution using the AE-HPLC method. As shown in Fig. 1, the increase in the ratio of LDL3 to LDL2 was used as an indicator of oxidative modification of LDL (Yamaguchi et al., 1998a). Fig. 4 shows the time course of changes in the relative levels of subfractions, LDL2 and LDL3. The values are expressed as a percentage of the plasma total cholesterol level. In the control group, the level of LDL2 gradually and significantly decreased while that of LDL3 significantly

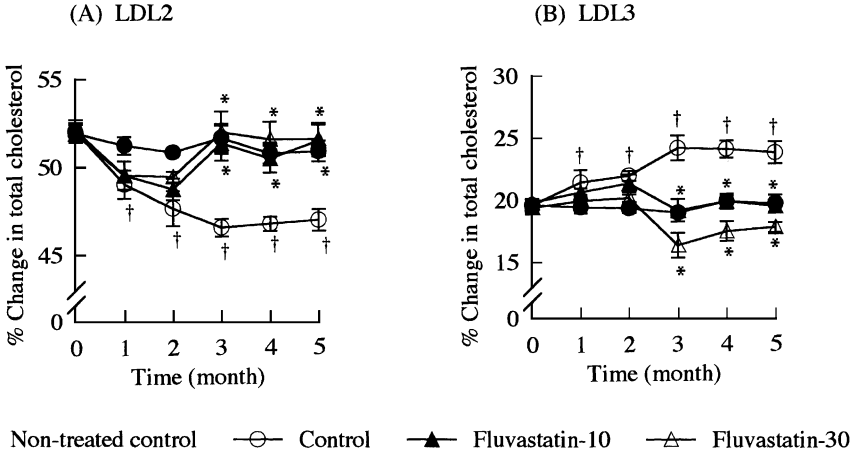

Fig. 4. Changes in total cholesterol levels in LDL2 and LDL3 subfractions of LDL. Grouping is described in Fig. 2. Each point represents the mean ± S.E.M. for five rabbits. Details are given in Materials and methods. †$P<0.05$, control group vs. non-treated control group; *$P<0.05$, fluvastatin-10 or fluvastatin-30 group vs. control group.

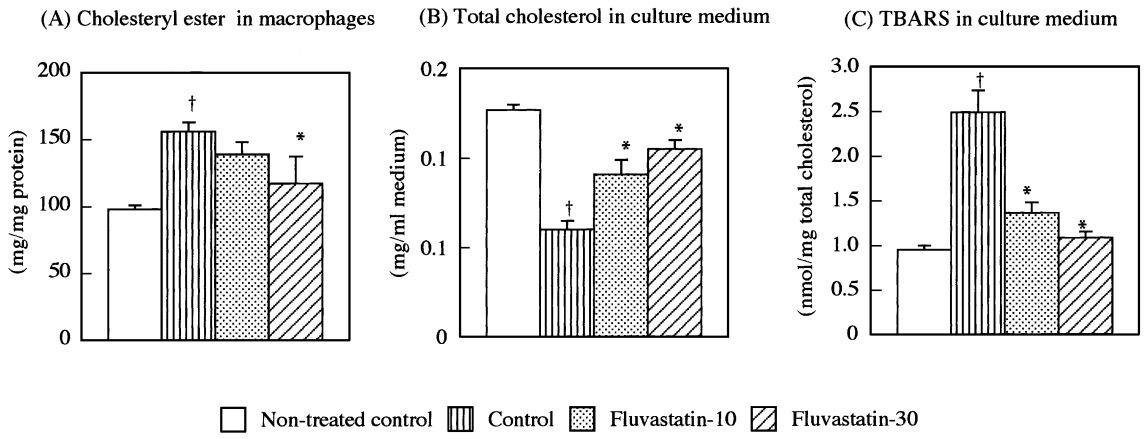

Fig. 5. Amounts of cholesteryl ester accumulated in macrophages (A), and levels of total cholesterol (B) and thiobarbituric acid-reactive substances (TBARS) (C) remaining in the medium. Mouse peritoneal macrophages were incubated with LDL from each group. Grouping is described in Fig. 2. Each point represents the mean ± S.E.M. for five rabbits. Details are given in Materials and methods. †$P<0.05$, control group vs. non-treated control group; *$P<0.05$, fluvastatin-10 or fluvastatin-30 group vs. control group.

increased as compared to the respective value in the non-treated control group throughout the experiment. These changes in LDL2 and LDL3 were significantly restored to the control levels by treatment with fluvastatin.

3.6. Cholesteryl ester accumulation in macrophages and cell-mediated LDL oxidation

At the end of the experiment, fresh LDL was isolated from each animal and incubated with mouse peritoneal macrophages for 24 h. The amounts of cholesteryl ester that accumulated in the macrophages significantly increased in the control group as compared to the non-treated control group (Fig. 5A). This increased cholesteryl ester accumulation was inhibited by treatment with fluvastatin in a dose-related manner. The total cholesterol level in the incubation medium, which corresponds to the residual LDL not incorporated into macrophages, significantly decreased in the control group compared to the non-treated control group. The total cholesterol levels in the fluvastatin-treated groups significantly increased compared to that in the control group in a dose-related manner (Fig. 5B). Furthermore, when LDL from the non-treated control group was incubated with macrophages for 24 h, the thiobarbituric acid-reactive substances level in the medium increased nearly two-fold over that without macrophages (0.50 ± 0.03 nmol/mg total cholesterol). Such cell-mediated oxidation increased further in the control group compared to the non-treated control group, but decreased significantly in the fluvastatin-treated groups compared to the control group (Fig. 5C).

3.7. Arterial cholesteryl ester levels

The contents of cholesteryl ester in the thoracic aorta, coronary artery and renal artery are shown in Fig. 6. In the thoracic aorta, the cholesteryl ester content in the control

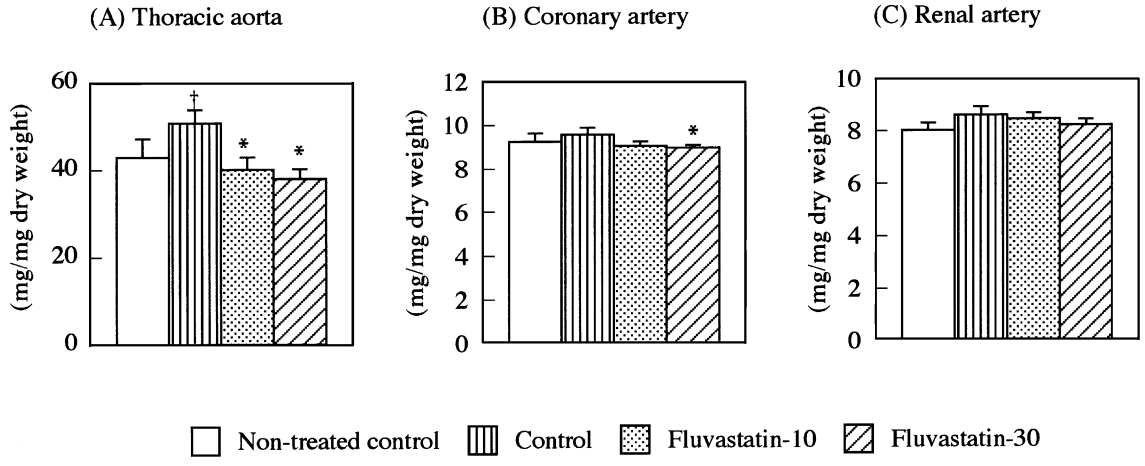

Fig. 6. Total cholesterol content of the thoracic aorta (A), coronary artery (B) and renal artery (C) of WHHL rabbits. Grouping is described in Fig. 2. Each point represents the mean ± S.E.M. for five rabbits. Details are given in Materials and methods. †$P<0.05$, control group vs. non-treated control group; *$P<0.05$, fluvastatin-10 or fluvastatin-30 group vs. control group.

group was significantly increased compared to that of the non-treated control group. The contents in the fluvastatin-10 and fluvastatin-30 groups were significantly decreased by 21.1% and 25.2%, respectively, of that of the control group. Similar but smaller changes were observed in the coronary and renal arteries.

4. Discussion

The antioxidant action of fluvastatin is unique among HMG-CoA reductase inhibitors. This property has been the subject of many studies in vitro (Kagota et al., 2000; Yamamoto et al., 1998; Obata and Yamanaka, 2000; Obata et al., 2000) and in vivo (Mitani et al., 1996; Suzumura et al., 2000). In patients and animals with hypercholesterolemia, the serum lipid peroxide level (Bandoh et al., 2000) and LDL oxidation (Yasuhara et al., 2000; Leonhardt et al., 1997; Hussein et al., 1997b) could be reduced by treatment with fluvastatin. Suzumura et al. (1999a,b) have reported that major metabolites of fluvastatin also have a similar or stronger antioxidant activity, including superoxide anion scavenging activity and inhibitory activity on LDL oxidation induced by copper ion. Therefore, fluvastatin can be expected to exhibit more potent inhibitory effects on oxidative modification of plasma LDL in vivo than in vitro.

WHHL rabbits are hypercholesterolemic (mostly due to elevated LDL) and later develop atherosclerosis. Cigarette smoke extracts contains stable radicals and pro-oxidants, one of which can gradually produce free radicals such as peroxynitrite, a strong oxidative radical (Yamaguchi et al., 2000a). Thus, WHHL rabbits injected with cigarette smoke extracts appear to be a suitable model for the investigation of the oxidative modification of LDL in vivo. Actually, the cigarette smoke extract-treated WHHL rabbit has been reported to show facilitated oxidative modification of plasma LDL and enhanced atherogenesis in the aorta (Yamaguchi et al., 2000b, 2001). Under such oxidative stress conditions, fluvastatin seems to more potently display its ability to suppress the oxidation of plasma LDL. The present study clearly demonstrated that fluvastatin can exert an antioxidant effect in addition to a lipid-lowering effect in WHHL rabbits given long-term intravenous injections of the cigarette smoke extracts as oxidative stress. This antioxidant action of fluvastatin may be considered to closely contribute to the prevention of atherogenesis together with its hypocholesterolemic action.

In the present study, the antioxidant activity of fluvastatin was assessed by the lipid peroxide level (as thiobarbituric acid-reactive substances level), AE-HPLC analysis, LDL oxidation and LDL incorporation into macrophages. Fluvastatin initially inhibited the elevation of plasma thiobarbituric acid-reactive substances levels induced by injection of cigarette smoke extracts, although these levels fluctuated in parallel with the cholesterol levels with time. This result raises the possibility that lipid peroxidation could be suppressed accompanied by a concomitant decrease in the plasma cholesterol level, by treatment with fluvastatin. However, the plasma thiobarbituric acid-reactive substances levels did not correlate with the plasma cholesterol levels in the fluvastatin-untreated groups, the non-treated control and control groups ($r = 0.048$, $p > 0.9$), although there was a good correlation between them in the fluvastatin-treated groups ($r = 0.515$, $p < 0.001$). These results indicate that the inhibitory effect of fluvastatin on the elevated thiobarbituric acid-reactive substances level is independent of its cholesterol-lowering effect.

AE-HPLC analysis is also a good approach for determining oxidative modification of LDL (Yamaguchi et al., 1998a, 2000b, 2001). Oxidatively modified LDL can be characterized by changes in two separate subfractions—a decrease in LDL2 and an increase in LDL3. According to this method, treatment with cigarette smoke extracts apparently facilitated oxidative modification of plasma LDL and led to a shift in LDL2 to LDL3, and this shift could be effectively reinstated by simultaneous treatment with fluvastatin. At the same time, the thiobarbituric acid-reactive substances levels in the LDL fluctuated in parallel with the oxidative modification of LDL, as evaluated by AE-HPLC analysis, increasing with cigarette smoke extract treatment and being prevented by fluvastatin treatment.

As further documentation, treatment with fluvastatin suppressed the increased accumulation of cholesteryl ester into macrophages when incubated with LDL from WHHL rabbits treated with cigarette smoke extracts. Oxidized LDL is known to be easily incorporated by macrophages via the scavenger receptor, leading to the active synthesis of cholesteryl ester and its storage in macrophages (Steinberg et al., 1989), whereas antioxidants can prevent the formation of oxidized LDL (Aviram, 1999). Therefore, this result indicates that the LDL from fluvastatin-treated animals contains a lower concentration of oxidatively modified LDL and/or results in more difficult oxidation by macrophages than that from cigarette smoke extract-treated animals.

The ability of LDL to undergo oxidation is another index of the potential oxidative state of LDL in plasma (Scheffler et al., 1992), with supplementation with antioxidants such as vitamin E being effective for reducing LDL oxidation (Fruebis et al., 1995; Fuller et al., 2000). Enhanced oxidation of LDL seems to be associated with easy incorporation of the LDL into macrophages. In vitro oxidation of LDL is usually estimated by addition of divalent metal ions such as copper ion or by incubation with cultured cells such as endothelial cells or macrophages (Steinbrecher et al., 1987; Henriksen et al., 1981; Leake and Rankin, 1990). In the present study, we measured two types of LDL oxidation, copper ion-catalyzed and macrophage-mediated. Oxidation of LDL from the cigarette smoke extract-treated rabbits by both routes was markedly increased, and this increase was significantly inhibited by simultaneous treatment with fluvastatin. These findings suggest that fluvastatin administration can effectively prevent the formation of oxidatively modified LDL in

hypercholesterolemic WHHL rabbits treated with cigarette smoke extracts, by reducing oxidative stress.

It is noteworthy that treatment with cigarette smoke extracts significantly augmented the accumulation of cholesteryl ester in the thoracic aorta of WHHL rabbits, and that the augmentation could be markedly reduced by administration of fluvastatin. This seems to result from the formation of oxidatively modified LDL by cigarette smoke extracts and the antioxidant-combating activity of fluvastatin, respectively.

In conclusion, the present study indicates that injection of cigarette smoke extracts facilitates oxidative modification of LDL through some pro-oxidants in cigarette smoke extracts in hypercholesterolemic (LDL-rich) WHHL rabbits, and that this oxidation of LDL can be effectively prevented by fluvastatin. Our findings suggest that fluvastatin may be of potential benefit for hypercholesterolemic patients with oxidative stress such as smoking.

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