Superoxide Anion Scavenging Properties of Fluvastatin and Its Metabolites

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We investigated the *in vitro* superoxide anion scavenging activities of fluvastatin and its metabolites. Fluvastatin showed dose-dependent superoxide anion scavenging activity in the NADH/phenazine methosulphate (PMS)/nitroblue tetrazolium (NBT) system, and the effect was as potent as the reference antioxidant, trolox, which is a water-soluble α-tocopherol derivative. The superoxide anion scavenging activities of the major metabolites of fluvastatin (M2, M3, M4, M7) were also determined in this system. All of these metabolites showed the activity. In particular, M2 and M3, which possess a phenolic hydroxyl group at the 5 or 6-position of the indole moiety, respectively, showed 3 times stronger activities than that of fluvastatin. Further, we also determined the effects of fluvastatin, M2 and M3 on phorbol myristate acetate (PMA)-induced superoxide anion generation in human peripheral blood polymorphonuclear leukocytes (PMN). The compounds tested also showed a depressing effect on the amount of superoxide anion in this system. We suggest that fluvastatin and its metabolites have the potential to protect cells or lipids from oxidative modification mediated by superoxide anion.

An elevated plasma level of low-density lipoprotein (LDL) has been considered strongly as a major risk factor of atherosclerosis. Hypolipidemic drugs, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors such as fluvastatin, pravastatin and simvastatin are widely used as clinical drugs for patients with hyperlipidemia.[1,2] Oxidative modification of LDL has been reported to be one of the important steps for the progression of atherosclerosis.[3] Synthetic and natural antioxidants, such as probucol and α-tocopherol, inhibited the oxidative modification of LDL and reduced the development of atherosclerosis in animal models.[4,5] Antioxidants seem to have some therapeutic potential for diseases which are associated with oxidative tissue injury.

LDL oxidation which takes place in the presence of endothelial cells, macrophages, or neutrophil is considered to be mediated by the cytotoxic chain reaction of reactive free radicals.[6] One of the important steps is superoxide anion generation caused by NADPH oxidase activation at the cell membrane.[7] Therefore, a superoxide anion scavenger should have the ability to protect cells or lipids from this kind of oxidative modification.

Recently, we reported that fluvastatin, one of the HMG-CoA reductase inhibitors, and its metabolites (M2, M3, M4, M7; Fig. 1) have the ability to scavenge hydroxyl radical,[8] which is another type of reactive oxygen species. However, the superoxide anion scavenging ability of fluvastatin and its metabolites is still unknown. The scavenging potency of an antioxidant against free radicals varies significantly corresponding to the kind of radical. For example, the well-known radical scavenger, dimethylthiourea, effectively scavenges hydroxyl radical, but does not scavenge superoxide anion.[9]

In this paper, we investigated the superoxide anion scavenging activities of fluvastatin and its metabolites (M2, M3, M4, M7; Fig. 1) using both chemical and biological superoxide anion generating systems.

Materials and Methods

Chemicals Fluvastatin sodium salt was a donation from the Tsukuba Research Institute, Novartis Pharma Co., Ltd. (Ibaraki, Japan). The metabolites of fluvastatin[1,10] (M2, M3, M4, M7; Fig. 1) were synthesized at the Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd. (Saitama, Japan). Cypridina luciferin analog (CLA, 2-methyl-6-phenyl-3,7-dihydroimidazo-[1,2-*a*]pyrazin-3-one) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Ficoll-Paque was obtained from Pharmacia AB. (Uppsala, Sweden). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Aldrich (Wisconsin, U.S.A.). All other reagents used were of the highest grade commercially available.

Superoxide Anion Scavenging Effect in the NADH/Phenazine Methosulfate (PMS)/Nitroblue Tetrazolium (NBT) System The NADH/PMS/NBT system was used to determine the superoxide anion scavenging activities of the compounds.[11,12] Briefly, generation of superoxide anion was measured in a reaction mixture containing 160 μM NADH, 40 μM NBT and 8 μM PMS in phosphate buffered saline (PBS) at pH 7.4. The reduction of NBT was followed by measuring the change in absorbance at 560 nm for 2 min, a period in which the absorbance increased linearly. Test compounds were pre-
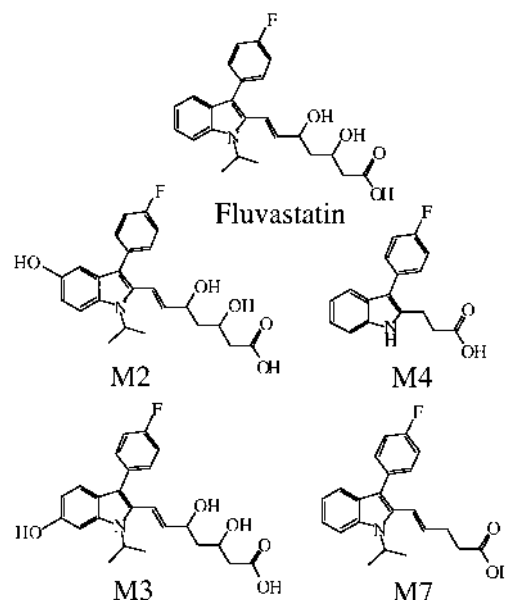

Fig. 1. Chemical Structures of Fluvastatin and Its Major Metabolites

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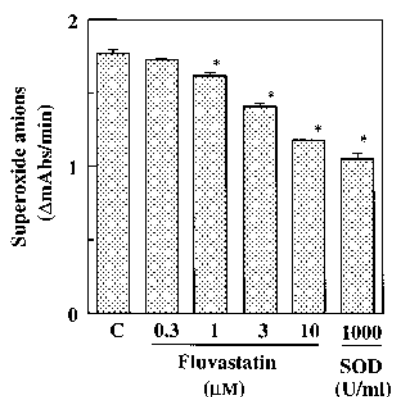* To whom correspondence should be addressed.

Fig. 2. Superoxide Anion Scavenging Effect of Fluvastatin in the NADH/PMS/NBT System

The superoxide anion scavenging activity of fluvastatin was determined as described in the text. Data are expressed as the means±S.E. ($n=4$). *: significantly different from control. $p<0.05$.
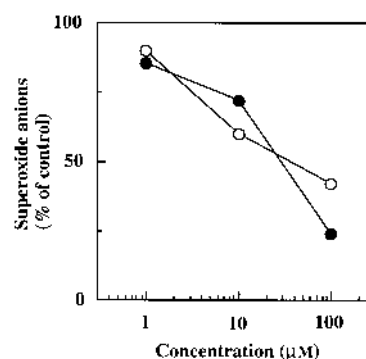

Fig. 3. Comparison of the Effects of Fluvastatin and Trolox in the NADH/PMS/NBT System

The activities of fluvastatin (○) and trolox (●) were determined as described in the text. Data are expressed as the means±S.E. ($n=4$). (Bars are smaller than symbols.)
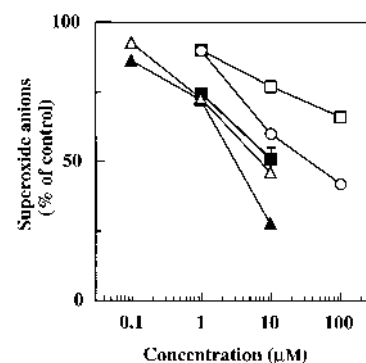

Fig. 4. Comparison of the Effects of Fluvastatin and Its Metabolites in the NADH/PMS/NBT System

The activities of fluvastatin (○), M2 (△), M3 (▲), M4 (□) and M7 (■) were determined as described in the text. Data are expressed as the means±S.E. ($n=4$).
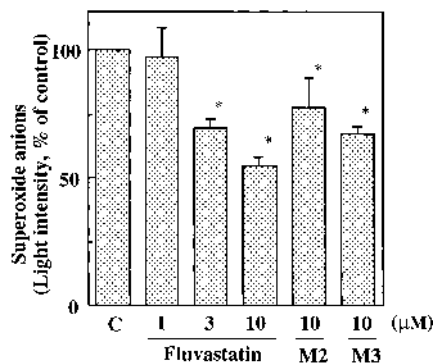

Fig. 5. The Effects of Fluvastatin, M2 and M3 on the Amount of Superoxide Anion in the PMN/CLA System

The effects of fluvastatin, M2 and M3 on the amount of superoxide anion were determined as described in the text. Data are expressed as the means±S.E. ($n=3$). *: significantly different from control. $p<0.05$.

pared in dimethylsulfoxide (DMSO) and added to the reaction mixture to give a final concentration of 0.7% DMSO. The activity of the test compound was determined in comparison with the control sample which did not contain the test compound.

Superoxide Anion Scavenging Effect in the Polymorphonuclear Leukocytes (PMN)/CLA System The PMN/CLA system was used as another testing method to evaluate the superoxide anion scavenging activity.[13] Superoxide anion was generated biologically by activated PMN. Briefly, PMN were isolated from the peripheral blood of healthy male volunteers by Ficoll-Pacque separation followed by hypotonic lysis of contaminating erythrocytes, and were stored at 0 °C in Hanks' balanced salt solution (HBSS) until use. The reaction mixtures contained 10^6 PMN/ml, 1 μM CLA, 100 μM diethylene-triamine-pentaacetic acid (DTPA) in a total volume of 2 ml HBSS, and were pre-incubated at 37 °C for 5 min in the presence or absence of test compounds. Superoxide anion generation by PMN was initiated by the addition of 10 nM phorbol myristate acetate (PMA). CLA is known as a highly specific indicator for superoxide anion.[13] The amount of superoxide anion which was not scavenged by the test compound was determined by the maximal light intensity in comparison with the control sample which did not contain test compound, using a luminescence reader (Aloka, Co., Ltd., Tokyo, Japan). Cell viability was assessed by trypan blue exclusion after 20-min incubation with the test compounds, and these compounds did not show cytotoxic effects under these conditions.

Statistics All data were expressed as the means±S.E. of 3 to 4 experiments. Statistical comparison among the groups was made by analysis of variance (ANOVA) followed by Fisher's PLSD test. Probability below 5% was considered statistically significant.

Results

Superoxide Anion Scavenging Effect in the NADH/PMS/NBT System The superoxide anion scavenging activities of the compounds were determined by the NADH/PMS/NBT system.[11,12] Figure 2 shows the superoxide anion scavenging activity of fluvastatin in this system. Superoxide dismutase (SOD) effectively depressed the increasing rate of absorbance, suggesting that the generation of superoxide anion actually occurred in the mixture. Fluvastatin (0.3—10 μM) showed a dose-dependent depressing effect. In order to normalize its potency, we compared its effect with that of reference compound, trolox, which is known as a water-soluble α-tocopherol derivative. As shown in Fig. 3, the potency of fluvastatin to scavenge superoxide anion was similar to that of trolox.

The superoxide anion scavenging activities of major human metabolites of fluvastatin (M2, M3, M4, M7; Fig. 1) were also determined. Figure 4 shows the superoxide anion

scavenging activities of the compounds. All of the metabolites tested showed the effect. The effect of M4 was somewhat weaker than that of fluvastatin. Among these metabolites M2 and M3, which possess a phenolic hydroxyl group at the 5 or 6-position of the indole moiety showed 3 times stronger activity than that of fluvastatin.

Superoxide Anion Scavenging Effect in the PMN/CLA System Superoxide anion scavenging activities of the compounds were also investigated in another assay system.

Human peripheral blood PMN which generates superoxide anion upon addition of PMA, were used as a biological superoxide anion generator. Figure 5 shows the effects of fluvastatin, M2 and M3 on the PMA-induced superoxide anion generation of PMN. Fluvastatin (1—10 μM) dose dependently depressed the maximal intensity of chemiluminescence, and M2 and M3 (10 μM) also showed significant effects. The effect of fluvastatin was comparable to those of M2 and M3 in this system.

Discussion

Recently, fluvastatin and its metabolites have been reported to have antioxidative activities which are related to their chemical structures.[8,14,15] In this paper, we described that the superoxide anion scavenging activities of fluvastatin and its metabolites are comparable to the reference antioxidant, trolox.

We reported previously,[8] that the fluorophenyl indole moiety is important for the manifestation of the hydroxyl radical scavenging properties of fluvastatin derivatives, and metabolic hydroxylation in the indole moiety potentiates the activity. Because all the tested metabolites showed the effects, and M2 and M3 also showed stronger effects than that of fluvastatin in the NADH/PMS/NBT system, this notion may be also applicable to the superoxide anion scavenging activities of these compounds.

Paying attention to the radical scavenging activities of M4 and M7, the order of their potency to scavenge superoxide anion was somewhat different from their potency to scavenge hydroxyl radicals reported previously.[8] The scavenging activity of M4 was as strong as that of fluvastatin, and M7 showed the weakest scavenging activity among these metabolites, towards the hydroxyl radical. On the other hand, as shown in Fig. 4, M7 showed stronger scavenging activity than that of M4 towards the superoxide anion. Although the precise mechanism underlying this difference is uncertain, these results indicate that each partial structure of fluvastatin has a different level of contribution to the radical scavenging activity, based on the kind of radicals with which it reacts.

We attempted to investigate the direct superoxide anion scavenging activities of the compounds which were not mediated by other indirect mechanisms such as HMG-CoA reductase inhibitory activity. A chemical superoxide anion generating system (NADH/PMS/NBT) was mainly used to determine the scavenging activities of the compounds rather than the biological superoxide anion generating system (PMN/CLA), because the former is more simple than the latter for evaluation of radical scavenging activity. However, taking into consideration that hypolipidemic drugs are used for reducing the risk of atherosclerosis, and that NADPH oxidase-mediated superoxide anion generation is closely related to oxidative cell injury in the atherosclerotic region,[6] the PMN/CLA system has more physiological significance than the NADH/PMS/NBT system.

It is well-known that macrophages and neutrophils generate superoxide anion by NADPH oxidase in the cell membrane.[7] Its activation involves the assembly of low-molecular-weight guanosine triphosphate (GTP)-binding protein.[16] The post-translational modification of GTP-binding protein by long-chain isoprenoid products of mevalonic acid promotes binding of GTP to cell membranes and leads to activation of NADPH oxidase.[17] Compactin, lovastatin[18] and simvastatin[19] which are also known as HMG-CoA reductase inhibitors, have been reported to show inhibitory activities on NADPH oxidase-mediated superoxide anion generation of culture cells after incubation for a few days. The inhibitory effects of these compounds were specifically prevented by addition of exogenous mevalonic acid, suggesting that the effects on superoxide anion generation were based on inhibition of the isoprenoid synthesis pathway *via* their HMG-CoA reductase inhibitory activity. Fluvastatin may also have the ability to decrease the generation of superoxide anion by the same mechanism. However, in this experiment, the test compounds were pre-incubated with PMN for only a few minutes. Therefore, the decreased intensity of the chemiluminescence may be almost completely independent from the inhibition of HMG-CoA reductase catalyzed isoprenoid synthesis. This consideration is further supported by the observation that addition of exogenous mevalonic acid to PMN did not prevent the effect of fluvastatin in this experiment (data not shown).

Fluvastatin showed weaker activity than M2 or M3 in the NADH/PMS/NBT system. This order of antioxidative potency is almost consistent with other reports.[8,15] However, the order of the effects of these compounds in the PMN/CLA system was somewhat different. The effect of fluvastatin in this system was comparable to those of M2 and M3. Although the precise reason is uncertain, there is the possibility that fluvastatin depressed the generation of superoxide anion from PMN by direct inhibition of enzymes such as protein kinase C which play central roles in the PMA-induced production of superoxide anion in PMN.[20] However, even if we exclude the depressing effects on the generation of superoxide anion, it is clear that these compounds had the ability to scavenge superoxide anion, as shown in NADH/PMS/NBT system.

Although the exact mechanisms of the oxidative modification of LDL and oxidative cell injury in the atherosclerotic region have not been clearly demonstrated until now, reactive oxygen radicals such as superoxide anion[21] and hydroxyl radical[22] may participate in this process. Superoxide anion has been reported to be generated in endothelial cells and media[23] through multiple mechanisms mediated by NADPH oxidase,[7,24] cyclooxygenase,[25] lipoxygenase,[25] nitric oxide synthase[26] and so on. It has been reported that overexpression of human SOD inhibits the oxidation of LDL by endothelial cells, suggesting that the production of superoxide anion contributes to endothelial cell-induced oxidation of LDL.[21] It has also been reported that increased superoxide anion generation in the endothelium of atherosclerotic aortas contributes to the vascular dysfunction in hyperlipidemic subjects.[23] Further, generation of the hydroxyl radical in atherosclerotic lesions has also been suggested by Smith *et al.*[22] Taken together, these reports suggest that scavenging reactive oxygen species by antioxidants or antioxidative enzymes may be beneficial not only in the prevention of LDL oxidation, but also in inhibition of vascular dysfunction development.

We conclude that fluvastatin and its metabolites have the potential to protect cells or lipids from the oxidative modification which is mediated by reactive oxygen species.

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