Direct free radical scavenging effects of water-soluble HMG-CoA reductase inhibitors

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3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, statins, are widely used for preventing cardiovascular and cerebrovascular diseases by controlling blood cholesterol level. Additionally, previous studies revealed the scavenging effects of statins on free radicals. We assessed direct scavenging activities of two water-soluble statins, fluvastatin and pravastatin, on multiple free radicals using electron spin resonance spectrometry with spin trapping method. We estimated reaction rate constants \( k_s \) for fluvastatin, and \( k_p \) for pravastatin. Superoxide anion was scavenged by fluvastatin and pravastatin with \( k_s \) and \( k_p \) of 4.82 M\(^{-1}\) s\(^{-1}\) and 49.0 M\(^{-1}\) s\(^{-1}\), respectively. Scavenging effects of fluvastatin and pravastatin on hydroxyl radical were comparable; both \( k_s \) and \( k_p \) were \( > 10^4 \) M\(^{-1}\) s\(^{-1}\). Fluvastatin also eliminated tert-butyl peroxyl radical with relative \( k_s \) of 2.63 to that of CYP2M6, whereas pravastatin did not affect tert-butyl peroxyl radical. Nitric oxide was scavenged by fluvastatin and pravastatin with \( k_s \) and \( k_p \) of 68.6 M\(^{-1}\) s\(^{-1}\) and 701 M\(^{-1}\) s\(^{-1}\), respectively. Both fluvastatin and pravastatin had scavenging effects on superoxide anion, hydroxyl radical and nitric oxide radical. On the other hand, tert-butyl peroxyl radical was scavenged only by fluvastatin, suggesting that fluvastatin might have more potential effect than pravastatin to prevent atherosclerosis and ischemia/reperfusion injury via inhibiting oxidation of lipids.

Key Words: water-soluble statin, electron spin resonance spectrometry, free radical, fluvastatin, pravastatin

Excessive accumulation of reactive oxygen species or free radicals increases the risk for various cardiovascular and cerebrovascular diseases.1–3 One of the major mechanisms of such pathology is atherosclerosis. Recent basic researches demonstrated that oxidized low-density-lipoprotein accelerated the formation of atherosclerosis.4–7 It was shown that metabolic abnormalities of lipids such as cholesterol and free fatty acid (FFA) correlated with the prognosis of whole body ischemia/reperfusion (I/R).4–5 These findings suggested that lipid oxidation is closely involved in various pathological conditions. Research and development of drugs to prevent the production of free radicals or oxidation of lipids have been conducted in recent years. Edaravone is the only radical scavenger that has been used in clinical practice.5–6 Although it had been confirmed that edaravone could exert anti-oxidative effect via oxidation of itself in vitro,7 the clinical usefulness of other radical scavengers has not been established yet.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, statins, are now widely used for the prevention of cardiovascular or cerebrovascular diseases via lowering serum cholesterol level. It is also known that statins have anti-oxidative effect, which encourages the usage of statins to prevent cerebrovascular and cardiovascular diseases. In fact, several clinical researches revealed such additional benefits of statins to improve patients’ outcome.8,9 Moreover, several basic researches also showed that statins could reduce hydroxyl radical in vitro, or prevent cholesterol-induced oxidative stress in laboratory animals.10–12 Fluvastatin is a compound with a fluorophenyl indole ring and an enolic acid, and pravastatin is a compound with a naphthalene frame and an enanethate (Fig. 1). Fluvastatin has double bonds in enolic acid conjugated with an indole ring that has shown to have radical scavenging activity,13 and the same structure is not involved in pravastatin. Therefore, it appears that fluvastatin could have stronger radical scavenging activity than pravastatin.

Fig. 1. Structure of water-soluble statins, fluvastatin (A) and pravastatin (B).
However, the answer to the question as to which of fluvastatin or pravastatin has stronger antioxidative effect has not been clarified yet. In the present study, we compared the scavenging effects of fluvastatin and pravastatin on multiple free radical species including radicals that have not been evaluated yet. For more accurate comparison of the effects of statins on each free radical, we estimated reactive rate constants based on IC\textsubscript{50} against free radicals obtained.

**Materials and Methods**

**Chemicals.** Fluvastatin (Wako, Osaka, Japan) and pravastatin (Kanto Chemical Co., Ltd., Tokyo, Japan) were dissolved in water (Milli-Q, Merck Millipore, Darmstadt, Germany) to the final concentration of 10 mM. Microwave power was set so that the signals were not saturated (9.45 GHz with 100-kHz modulation; modulation width 0.1 mT; microwave power 200 W medium-pressure mercury/xenon arc (RUVF-203SR UV illuminator, Radical Research Inc., Tokyo, Japan) with the spin trapping method. We used edaravone (Tokyo Chemical Industry, Tokyo, Japan) as a positive control for the scavenging effects on free radicals except for superoxide anion.\(^\text{[14,15]}\) Superoxide dismutase (Sigma-Aldrich, St. Louis, MO) was used as a positive control for the scavenging effects on superoxide anion. All the reagents were freshly prepared at time of use to avoid destabilization, degradation or denaturation of the chemicals due to long-term storage.\(^\text{[14,15]}\)

**Free radical measurements.** Free radicals were measured using an electron spin resonance (ESR) spectrometer (JES-RE1X, JEOL, Tokyo, Japan) with the spin trapping method.\(^\text{[16-19]}\) The ultraviolet (UV) light source was a 200 W medium-pressure mercury/xenon arc (RUVF-203SR UV illuminator, Radical Research Inc., Tokyo, Japan) with a UV-transmitting/VIS-absorbing filter (230–430 nm). Free radicals were produced in disposable ESR flat cells (Flash Point Inc., Tokyo, Japan). Instrument settings were as follows: room temperature (23°C); frequency 9.45 GHz with 100-kHz modulation; modulation width 0.1 mT; time constant 0.1 s; sweep time 1 min; center field 335.3 mT. Microwave power was set so that the signals were not saturated (1 to 10 mW). The data were analyzed by an operation software WIN-RAD ver. 1.20b (Radical Research Inc., Tokyo, Japan).

**Free radical production and spin trapping.** Each free radical was generated and trapped as previously described.\(^\text{[16-19]}\) Briefly, superoxide anion was produced by the mixture of 0.2 mM hypoxanthine dissolved with phosphate buffer (pH 7.4), 0.04 mM xanthine oxidase, and 2.5 mM CYP(MPO) as a spin-trap agent.\(^\text{[17]}\) Hydroxyl radical was produced by \textit{in situ} UV irradiation for 4 s to dilute aqueous solution of hydrogen peroxide (0.3%) in a disposable ESR flat cell and trapped by CYPMPO (2.5 mM).\(^\text{[17]}\) tert-Butyl peroxyl radical, one of alkoxyl radicals, was produced by \textit{in situ} UV irradiation for 10 s to tert-butyl hydroperoxide (160 mM) and trapped with 2.5 mM CYP(MPO).\(^\text{[18]}\) Nitric oxide radical was produced from NOC7 (250 mM) and reacted with carboxy-PTIO (25 µM) to generate carboxy-PTI, which was measured as an indicator of nitric oxide radical 60 min after the mixture.\(^\text{[19]}\) An artificial radical DPPH (15 µM), which is often used for the assessment of radical scavenging capacity of drugs,\(^\text{[20]}\) was directly observed with ESR.

**Spectrometric analysis and normalization.** Intensity of the target signal was measured relative to the external standard of Mn\textsuperscript{2+} and normalized with the control signal (0 mM), and plotted against the concentration of either fluvastatin, pravastatin or edaravone to estimate IC\textsubscript{50} values of each chemical. In the case of spin trapping method, IC\textsubscript{50} values are highly dependent upon the free radical production system and the amount of spin trap agent (e.g., CYPMPO, carboxy-PTIO). Therefore, we estimated reaction rate constant (k) of each statin (k\textsubscript{fv} for fluvastatin and k\textsubscript{pv} for pravastatin) according to the following equation.\(^\text{[21]}\)

\[
k_{\text{statin}} = \frac{k_{\text{spin trap}} [\text{spin trap}]}{\text{IC}_{50}\text{-statin}}
\]

**Statistics.** IC\textsubscript{50} was estimated by sigmoid curve fitting according to Cheng-Prusoff equation. Group averages are presented as mean ± SEM. Differences between group averages were tested using a one-way ANOVA with a post-hoc test (Tukey’s HSD). Significance level was set at 0.05. All statistical analyses were carried out using a statistical software R (ver. 3.4.2).\(^\text{[22]}\)

**Results**

**Superoxide anion.** ESR spectra of superoxide anion were highly diminished by 5 mM fluvastatin and pravastatin (Fig. 2A). Fluvastatin and pravastatin scavenged superoxide anion in a dose-dependent manner with IC\textsubscript{50} of 4.98 mM and 0.49 mM, respectively (Fig. 2B). Estimated values of k\textsubscript{fv} and k\textsubscript{pv} were 4.82 M\textsuperscript{-1}s\textsuperscript{-1} and 49.0 M\textsuperscript{-1}s\textsuperscript{-1}, respectively (Table 1). Fluvastatin and pravastatin had scavenging activity on superoxide anion, when it was generated by the xanthine-xanthine oxidase system. The value of k\textsubscript{pv} was about ten-times larger than that of k\textsubscript{fv}, which suggested that the scavenging activity of pravastatin against superoxide anion was stronger than that of fluvastatin.

**Hydroxyl radical.** ESR signals of hydroxyl radical were clearly eliminated by all three compounds (Fig. 3A). Both fluvastatin and pravastatin scavenged hydroxyl radical in a dose-dependent manner with IC\textsubscript{50} of 1.19 mM and 3.04 mM, respectively (Fig. 3B). Both k\textsubscript{fv} and k\textsubscript{pv} on hydroxyl radical estimated from IC\textsubscript{50} were >10 M\textsuperscript{-1}s\textsuperscript{-1} (Table 1), which indicated that both fluvastatin and pravastatin were highly effective in scavenging hydroxyl radical.

**tert-Butyl peroxyl radical.** Fluvastatin eliminated tert-butyl peroxyl radical, but not in the case of pravastatin (Fig. 4A). Fluvastatin less than 1 mM reduced the signal in a dose-dependent manner with IC\textsubscript{50} of 0.19 mM (Fig. 4B), which gave us the value of k\textsubscript{fv} (reaction rate constant relative to that of CYP(MPO)) on tert-butyl peroxyl radical as 2.63 (Table 1). However, the signal of tert-butyl peroxyl radical in the presence of 1 mM fluvastatin increased up to 2.5 times of that in the absence of fluvastatin (Fig. 4B), suggesting that fluvastatin at high concentration might modulate the generation system of tert-butyl peroxyl radical to increase the amount of radical. Pravastatin did not affect tert-butyl peroxyl radical at all (Fig. 4A and B).

**Nitric oxide radical.** Both statins scavenged nitric oxide radical in a dose-dependent manner with IC\textsubscript{50} of 3.68 mM for fluvastatin and 0.36 mM for pravastatin (Fig. 5A and B). Estimated values of k\textsubscript{fv} and k\textsubscript{pv} on nitric oxide radical were 68.6 M\textsuperscript{-1}s\textsuperscript{-1} and 701 M\textsuperscript{-1}s\textsuperscript{-1}, respectively (Table 1). Pravastatin had ten-times stronger scavenging capacity against nitric oxide radical than fluvastatin.

**DPPH radical.** Representative ESR spectra of DPPH radical are shown in Fig. 5A. Pravastatin scavenged DPPH with IC\textsubscript{50} of 2.91 mM (Fig. 6B and Table 1). However, fluvastatin did not scavenge DPPH radical.

**Discussion**

We evaluated the direct scavenging effects of fluvastatin and pravastatin on multiple free radicals using ESR spectrometry with a configuration of spin trapping method. We found that both...
fluvastatin and pravastatin had potency to scavenge superoxide anion, hydroxyl radical and nitric oxide radical. On the other hand, tert-butyl peroxyl radical was eliminated only by fluvastatin. tert-Butyl peroxyl radical is an experimental reagent used for simulating alkoxyl radical or peroxyl radical, which is related to oxidation chain reaction of lipids including oxidation of LDL cholesterol as one of the major mechanisms of atherosclerosis. The present result that fluvastatin scavenged tert-butyl peroxyl radical suggested that fluvastatin might preventively affect the oxidative chain reaction of lipids, and consequently prevent atherosclerosis via inhibiting oxidation of LDL cholesterol. In fact, Miwa et al.\(^{23}\) had reported that fluvastatin reduced not only oxidative products such as malondialdehyde-modified low-density-lipoprotein (MDA-LDL) but also 3,5,7-cholestadiene in erythrocyte membrane, whereas pravastatin only reduced MDA-LDL. Previously, Nakamura et al.\(^{10}\) clarified that the allylic carbon conjugated with the indole ring of fluvastatin might contribute to the scavenging activity of fluvastatin against tert-butyl peroxyl radical. Our present experimental results were consistent with these past reports.

FFA is another source of lipid-derived alkoxyl radical or peroxyl radical in vivo. Hara et al.\(^{4}\) showed that FFA could be a

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**Table 1.** Relative reaction rate constants for fluvastatin \(k_F\) and pravastatin \(k_P\)

| Free radical                  | hfs\(^{1}\) (mT) | Statin     | IC\(_{50}\) (mM) | Reaction rate constant (M\(^{-1}\)s\(^{-1}\)) |
|------------------------------|------------------|------------|------------------|---------------------------------------------|
| Superoxide anion             | 1.04, 1.32, 5.11 | fluvastatin | 4.98             | 4.82                                        |
|                              | 1.15, 1.30, 5.24 | pravastatin | 0.49             | 49                                          |
| Hydroxyl radical             | 1.40, 1.40, 4.93 | fluvastatin | 1.19             | >10\(^{9}\)                                  |
|                              | 1.22, 1.40, 4.90 | pravastatin | 3.04             | >10\(^{9}\)                                  |
| tert-Butyl peroxyl radical   | 1.35, 1.45, 4.95 | fluvastatin | 0.19             | \(2.63 \times k_{CYPMPO}\)                  |
|                              |                  | pravastatin | no effect        | —                                           |
| Nitric oxide                 |                  | fluvastatin | 3.68             | 68.6                                        |
|                              | au1, 0.981, au2, | pravastatin | 0.36             | 701                                         |
|                              | 0.445            |             |                  |                                              |
| DPPH\(^{§}\)                |                  | fluvastatin | no effect        | —                                           |
|                              |                  | pravastatin | 2.91             | —                                           |

\(^{1}\)Hyperfine splitting constant. \(^{2}\)Value unknown. \(^{12,2}\)-diphenyl-1-picrylhydrazyl.

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Fig. 2. (A) Representative spectra of superoxide anion measured by electron spin resonance spectrometry. Black arrows indicate the external standard of Mn\(^{2+}\) and gray arrows indicate the target signals of the radical. (B) The IC\(_{50}\) curve of fluvastatin (left) and pravastatin (right) for superoxide anion. Black rhomboids in each graph indicate the relative intensity at 0 mM of each chemical as a reference. Error bars indicate SE of the mean. The number of repetition at each concentration was four.

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Fig. 3. (A) Representative spectra of hydroxyl radical on electron spin resonance spectrometry. Black arrows indicate the external standard of Mn$^{2+}$ and gray arrows indicate the target signals of the radical. (B) The IC$_{50}$ curve of fluvastatin (left) and pravastatin (right) for hydroxyl radical. Black rhomboids in the graphs indicate the relative intensity at 0 mM of each chemical as a reference. Error bars indicate SE of the mean. The number of repetition at each concentration was four.

Fig. 4. (A) Representative spectra of tert-butyl peroxyl radical. Black arrows indicate the external standard of Mn$^{2+}$ and gray arrows indicate the target signals of the radical. (B) The IC$_{50}$ curve of fluvastatin (left) and pravastatin (right) for tert-butyl peroxyl radical. Black rhomboids in the graphs indicate the relative intensity at 0 mM of each chemical as a reference. t-BuOO: tert-butyl peroxyl radical. Error bars indicate SE of the mean. The number of repetition at each concentration was four.
marker for oxidative stress as well as antioxidants coenzyme Q-10 in infants with asphyxia. Nagase et al.\(^5\) demonstrated that high level of plasma FFA at the onset of ischemia and following decrease in the percentage of unsaturated fatty acid over total FFA significantly correlated with poor outcomes in patients after cardiopulmonary arrest. Basic researches also showed that FFA increased oxidative stress in cultured cells and isolated organs.\(^{(24-26)}\) These previous findings indicated that FFA could be a great risk of oxidative stress in several pathophysiological states including ischemia/reperfusion injury. We showed the possibility that fluvastatin may be useful to scavenge lipid-derived free radicals. It was also speculated that fluvastatin could be useful not only for lowering cholesterol but also for preventing oxidative stress caused by FFA during I/R injury in the cases of hypertriglycerolemia for instance.

Nitric oxide radical reacts with superoxide anion and produces nitrogen dioxide or peroxinitrite that has stronger oxidative properties than nitric oxide radical itself. Both nitrogen dioxide and peroxinitrite oxidize nucleic acids, amino acids or lipids to deteriorate their functions.\(^{(27,28)}\) On the other hand, nitric oxide radical exerts vasodilator action via cyclic guanidine monophosphate (cGMP) activation.\(^{(29,30)}\) We confirmed that nitric oxide radical was significantly reduced by both fluvastatin and pravastatin, and the effect of pravastatin was more potent than that of fluvastatin. Moreover, pravastatin had stronger scavenging effects on superoxide anion than fluvastatin, which was suggested from higher value of \(k_{ov}\) than that of \(k_{ov}\). From these results, pravastatin could prevent the production of nitrogen dioxide and peroxinitrite more potently than fluvastatin via stronger scavenging activity against both superoxide anion and nitric oxide radical. However, we also have to consider that the reduction of nitric oxide radical by statins may impair vasodilation. Further experiments using cultured cells or laboratory animals are still required to conclude that the deletion of nitric oxide by statins in vivo would be beneficial or harmful.

We demonstrated that both fluvastatin and pravastatin had scavenging effects on multiple radicals, whereas tert-butyl peroxyl radical was eliminated only by fluvastatin and DPPH radical was scavenged only by pravastatin. We also evaluated the effects of fluvastatin and pravastatin on superoxide anion using the experimental system of ultraviolet irradiation to concentrated hydrogen peroxide\(^{(17)}\) and found that radical was significantly reduced only by fluvastatin but not by pravastatin (Supplemental Fig. 1*). We considered that the discrepancy was caused by the photochemical properties of the phenol group of fluvastatin.\(^{(11)}\) Furthermore, we first evaluated the scavenging effects of statins on superoxide anion in xanthine-xanthine oxidase system using 5,5-Dimethyl-1-pyrroline 1-oxide (DMPO, purchased from Dojindo, Kumamoto, Japan) as a spin trapping agent and found that both statins had less effects on the radical (Supplemental Fig. 2*). However, when CYPMPO was used as a spin trapping agent for the same experimental setting, we could clearly reveal the scavenging effects of both statins on superoxide anion (Fig. 1). DMPO is often used as a spin trapping agent to detect oxygen-centered radicals. However, the previous study revealed that CYPMPO had a larger reaction rate constant with superoxide anion than DMPO and the superoxide adduct of CYPMPO was more stable than that of DMPO.\(^{(17)}\) Thus, the authors concluded that it would be better to use CYPMPO rather than DMPO for the detection of superoxide anion in the present study.

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*See online. [https://www.jstage.jst.go.jp/article/jcbn/64/1/jcbn18-48/_article](https://www.jstage.jst.go.jp/article/jcbn/64/1/jcbn18-48/_article)

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Fig. 5. (A) Representative spectra of nitric oxide radical indicated by the amount of carboxyl-PTI. Black arrows indicate the external standard of Mn\(^{2+}\) and gray arrows indicate the target signals of the radical. (B) The IC\(_{50}\) curve of fluvastatin (left) and pravastatin (right) for nitric oxide radical. Black rhomboids in each graph indicate the relative intensity at 0 mM of each chemical as a reference. NO: nitric oxide. Error bars indicate SE of the mean. The number of repetition at each concentration was four.
evaluating the antioxidant effects of chemicals, it is possible that a conclusion with only a single experimental system may lead to a misinterpretation. It would be important to evaluate the antioxidative reaction of the chemicals with multiple radicals and multiple experimental systems.

**Limitations**

The present study only evaluated two kinds of water-soluble statins, fluvastatin and pravastatin. In clinical practice, other lipophilic statins (e.g., atorvastatin, pitavastatin, simvastatin, etc.) are commonly used, and those drugs are also known as potent anti-oxidants,\(^{(32,33)}\) which were not assessed in the present study. To apply ESR spectroscopy to those drugs, it is necessary to dilute them in an organic solvent such as dimethyl sulfoxide or alcohol. However, such solvents could scavenge free radical species or react with them by themselves, thus it could be difficult to assess the effect of lipophilic statins on free radicals using ESR spectroscopy.

Another factor might be possible equilibrium in the statin solutions. In case of edaravone, it is present as unstable edaravone anion in aqueous solution, which is capable of transferring an electron to (i.e., reducing) free radicals and becomes edaravone radical.\(^{(14,15)}\) We prepared all the reagents freshly at time of use to prevent their denaturation and degradation, and mechanisms involving such equilibrium is not considered in the present discussion.

Fluvastatin and pravastatin reduced multiple free radical species in vitro. Both fluvastatin and pravastatin had scavenging effects on superoxide anion, hydroxyl radical and nitric oxide. On the other hand, tert-butyl peroxyl radical was scavenged only by fluvastatin, suggesting that fluvastatin might have more potential effect than pravastatin to prevent atherosclerosis and ischemia/reperfusion injury via inhibiting oxidation of lipids.

**Author Contributions**

Umeda R, Acquisition, analysis and interpretation of the data. Statistical analysis. Drafting of the manuscript. Takanari H, Study concept and design. Acquisition, analysis and interpretation of the data. Statistical analysis. Drafting of the manuscript. Obtained funding. Material support. Ogata K, Acquisition, analysis and interpretation of data. Technical support. Matsumoto S, Obtained funding. Material support. Kitano T, Obtained funding. Material support. Ono K, Study supervision. Tokumaru O, Study concept and design. Acquisition, analysis and interpretation of the data. Drafting of the manuscript. Critical revision of the manuscript. Obtained funding. Material and technical support.

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**Abbreviations**

- carboxy-PTIO: 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide
- cGMP: cyclic guanidine monophosphate
- CYPMPO: 5-(2,2-Dimethyl-1,3-propoxy) cyclophosphoryl-
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Conflict of Interest

No potential conflicts of interest were disclosed.