EPR studies on hydroxyl radical-scavenging activities of pravastatin and fluvastatin

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Vandjelovic, N., Zhu, H., Misra, H.P., Zimmerman, R.P., Jia, Z. , Li, Y. (2012). EPR studies on hydroxyl radical-scavenging activities of pravastatin and fluvastatin. *Molecular and Cellular Biochemistry, 364(1-2)*, 71-77. doi: 10.1007/s11010-011-1206-6

The final publication is available at Springer via http://dx.doi.org/10.1007/s11010-011-1206-6

Abstract:

Statins are known clinically by their cholesterol reduction properties through the inhibition of HMG-CoA reductase. There is mounting evidence suggesting a protective role of statins in certain types of cancer, cardiac, and vascular disease through a mechanism that extends beyond their lipid lowering ability. The root mechanism of damage likely involves the inflammatory cascade, specifically compounds known as reactive oxygen species such as the hydroxyl radical. However, direct evidence for the hydroxyl-scavenging capacity of pravastatin and fluvastatin, two forms of statins being widely used to lower LDL cholesterol, is still lacking in literature. In this study, electron paramagnetic resonance spectroscopy in combination with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO)-spin-trapping technique was utilized to determine the abilities of pravastatin and fluvastatin in scavenging hydroxyl radical generated from Fe(II) with H_2O_2 system. In addition, we examined the effects of pravastatin and fluvastatin on oxidative-induced φX-174 RF I plasmid DNA damage. We have demonstrated here for the first time that pravastatin and fluvastatin at physiologically relevant concentrations significantly decreased formation of DMPO-OH adduct indicating that both compounds could directly scavenge hydroxyl radicals. However, pravastatin and fluvastatin were not able to directly protect against oxidative DNA plasmid damage. The hydroxyl radical sequestering ability of pravastatin and fluvastatin reported in this study may contribute to their beneficial use in certain types of cancer and in cardiovascular disease.

Keywords: Statins | Hydroxyl radical | DNA strand breaks | EPR

Article:

Introduction

Statins are a group of pharmacological agents that inhibit 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the rate limiting step of the cholesterol synthesis cascade. Since their introduction, statins have become very popular and are prescribed worldwide due to their efficacy in the treatment of lipid disorders and their generally safe side effect profile. In addition to their cholesterol reduction properties, recent studies have demonstrated statins can protect against various pathological conditions with a mechanism that likely extends beyond their lipid lowering abilities [*1*].

Evidence suggest statins downregulate vascular inflammation and promote cardioprotection [*2*]. Bloom et al. [*3*] demonstrated a decreased number of ventricular arrhythmias requiring defibrillation in patients on a statin with a mechanism related to decreased derivatives of reactive oxygen species (ROS). In addition, treatment with a statin before cardiac ablation improved freedom from paroxysmal and persistent atrial fibrillation [*4*]. The use of statins in patients with early aortic valve disease reduced inflammatory biomarkers [*5*]. The protective effects of statins have also been found in certain types of cancer. The favorable effects of statin in men treated with radiotherapy for prostate cancer [*6, 7*] are thought to be associated with decreased inflammation within prostate tumors attributable to the statin therapy [*8*]. Since elevated cholesterol has been linked with high-grade prostate cancer, prophylactic statin use may reduce the risk of high-grade prostate cancer due to their cholesterol lowering ability [*9*].

The common denominator for these destructive pathways is inflammation. Many studies have illustrated the anti-inflammatory properties of statins [*10–12*]. Inflammation is a cascade-like response to a stimulus that produces several byproducts including ROS and reactive nitrogen species (RNS) specifically superoxide, hydroxyl radical, and peroxynitrite [*13, 14*]. Among them, hydroxyl radicals ($^{\bullet}$OH) is one of the most reactive molecules in biological systems [*15, 16*]. However, it remains unknown whether statins are hydroxyl radical scavengers. Furthermore, their effects on oxidative-induced DNA damage are still lacking. In this study, electron paramagnetic resonance spectroscopy in combination with 5-(diethoxyphosphoryl)-5-methylpyrroline-*N*-oxide (DMPO)-spin-trapping technique was utilized to study hydroxyl radical-scavenging activities of pravastatin and fluvastatin (Fig. 1), two forms of statins being widely used to lower LDL cholesterol, in a hydrogen peroxide/Fe*(*II*)* system. We have demonstrated here for the first time that pravastatin and fluvastatin at physiologically relevant concentrations significantly decreased the formation of DMPO-hydroxyl adduct (DMPO-OOH) in hydrogen peroxide/Fe*(*II*)* system, indicating that both compounds could directly scavenge hydroxyl. Using φX-174 plasmid DNA as an in vitro system, we have further investigated the effects of these compounds on DNA oxidative damage. Pravastatin and fluvastatin at physiologically relevant concentrations had no direct protection against DNA oxidative damage.

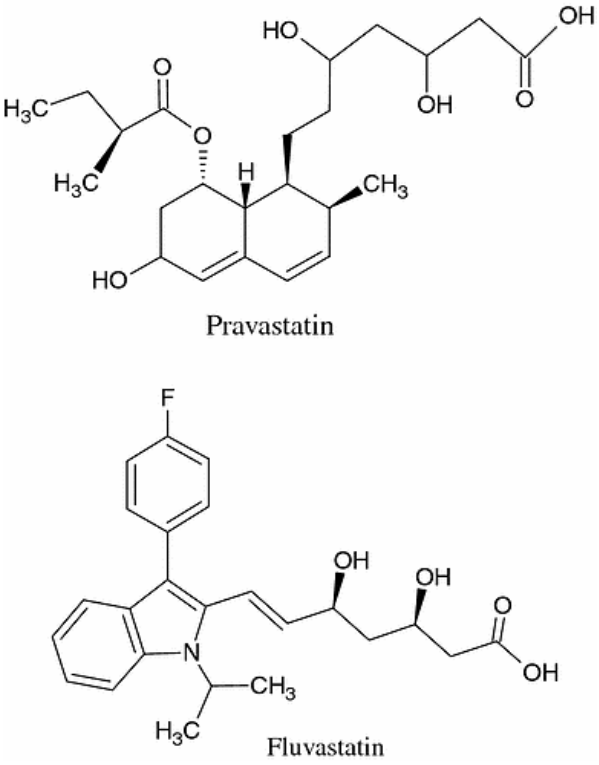

Fig. 1 Structures of pravastatin and fluvastatin

Materials and methods

Materials

φX-174 RF I plasmid DNA was from New England Bio-labs, Beverley, MA. Fluvastatin sodium ≥98% (HPLC), pravastatin sodium ≥98% (HPLC), hydrogen peroxide and other chemicals were purchased from Sigma (St. Louis MO).

Electron paramagnetic resonance spin-trapping assay

Spin trap 5,5-dimethylpyrroline-*N*-oxide (DMPO)-spin was used to measure hydroxyl radicals generated by the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$) in the presence or absence of various concentrations of fluvastatin or pravastatin. EPR spectra were recorded at room temperature with a spectrometer (Bruker D-200 ER, IBM-Bruker), operating at X-band with a TM cavity and capillary cell, as described previously [*17*]. The EPR spectrometer settings were: modulation frequency, 100 kHz; X-band microwave frequency, 9.5 GHz; microwave power, 20 mW; modulation amplitude, 1.0 G (gauss); time constant, 160 s; scan time, 200 s; and receiver gain, 1×10^5. Reactants were mixed in test tubes to a final volume of 0.1 ml, and the reaction mixture was then transferred to a capillary tube for EPR spectral analysis at room temperature under conditions described above. Spectral simulations were performed on the EPR data by matching directly to the spectra as described previously [*18*].

Assay for oxidative DNA breaks

DNA damage was measured by the conversion of supercoiled φX-174 RF I double-stranded DNA to open circle and linear forms [*19*, *20*]. In summary, 0.2 μg DNA was incubated with H_2O_2 + Fe(II), SIN-1, fluvastatin or pravastatin in PBS at 37°C at a final volume of 24 μl for 30 min. Following incubation, loading dye was added and the samples were immediately loaded in a 1% agarose gel containing 40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA, and electrophoresed in a horizontal slab gel apparatus in Tris/acetate/EDTA gel buffer at 150 V for 60 min. After electrophoresis, the gels were stained with a 0.5 mg/ml solution of ethidium bromide for 30 min, followed by 30 min destaining in water. The gels were then photographed under ultraviolet illumination and quantified using the Alpha Innotech Imaging system (San Leandro, CA).

Statistical analysis

Statistical significance of the data was calculated by analysis of variance (ANOVA). Further statistical analyses for post hoc comparison were done by two-tailed *t* test. Standard deviation was used to illustrate deviation. All differences of $P \leq 0.05$ were considered significant.

Results and discussion

Hydroxyl-scavenging effects of pravastatin and fluvastatin

EPR spectroscopy in combination with the spin trap DMPO was employed to investigate the hydroxyl-scavenging ability of pravastatin and fluvastatin using the Fenton reaction, a typical system for hydroxyl generation. As shown in Fig. 2, a spectrum of 1:2:2:1 quartet with splittings at $a_N = a_H = 14.9$ G was observed. These splitting constants and the 1:2:2:1 quartet are indicative of the DMPO-OH adduct [*21*]. DMPO itself did not give rise to the formation of any detectable spin adducts (Fig. 2, line a), indicating the high purity of this spin trap used in this study. Reaction mixtures were then incubated with 1, 10, or 100 μM of pravastatin and fluvastatin to examine hydroxyl-scavenging ability of these compounds. As shown in Figs. 2a and 3a (lines c–e), the intensity of the signal increased, from top to bottom, with decreasing the concentrations of both compounds ranging from 100 to 1 μM indicating that the scavenging capacity of these compounds is concentration-dependent. The percent inhibition of EPR signal intensity by various concentrations of these compounds is presented in Figs. 2b and 3b. As shown in Fig. 2b, there were significant differences in EPR signal intensity in the presence of 1–100 μM pravastatin compared to solvent control (line b; vehicle control without pravastatin) ($P \leq 0.05$). Fluvastatin at 10–100 μM, while not 1 μM, significantly inhibited the intensity of the spectrum in a concentration-dependent manner indicating that higher concentrations of fluvastatin were necessary for the effective scavenging of hydroxyl radical (Fig. 3b) ($P \leq 0.05$).

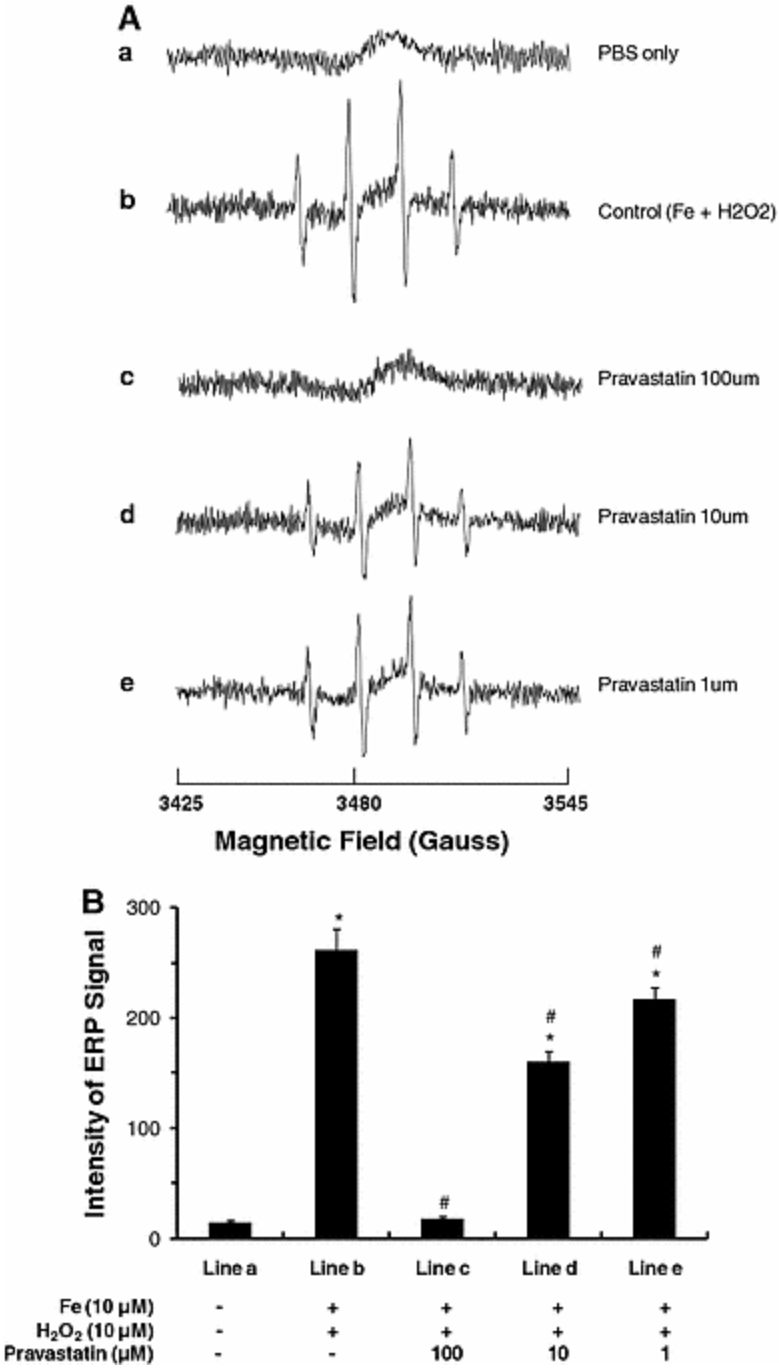

Fig. 2 Effect of pravastatin on hydroxyl radicals generated from the Fenton reaction. EPR spectra of DMPO-OH observed during the reaction of 200 μM ferrous sulfate and 200 μM H_2O_2 containing 100 μM DMPO, in the presence or absence of pravastatin. **a** *Line a* 100 μM DMPO alone without $FeSO_4$, H_2O_2, and test compounds; *line b* 200 μM $FeSO_4$ and 200 μM H_2O_2; *line c* 200 μM $FeSO_4$ and 200 μM H_2O_2, and 1 μM pravastatin; *line d* 200 μM $FeSO_4$ and 200 μM H_2O_2, and 10 μM pravastatin; *line e* 200 μM $FeSO_4$ and 200 μM H_2O_2, and 100 μM pravastatin. EPR measurement conditions were as described in "Materials and methods" section.

Data represent similar results from three independent experiments. **b** Signal intensity at 3480 G was expressed as mean ± SD from three separate experiments (*P < 0.05 vs. control)

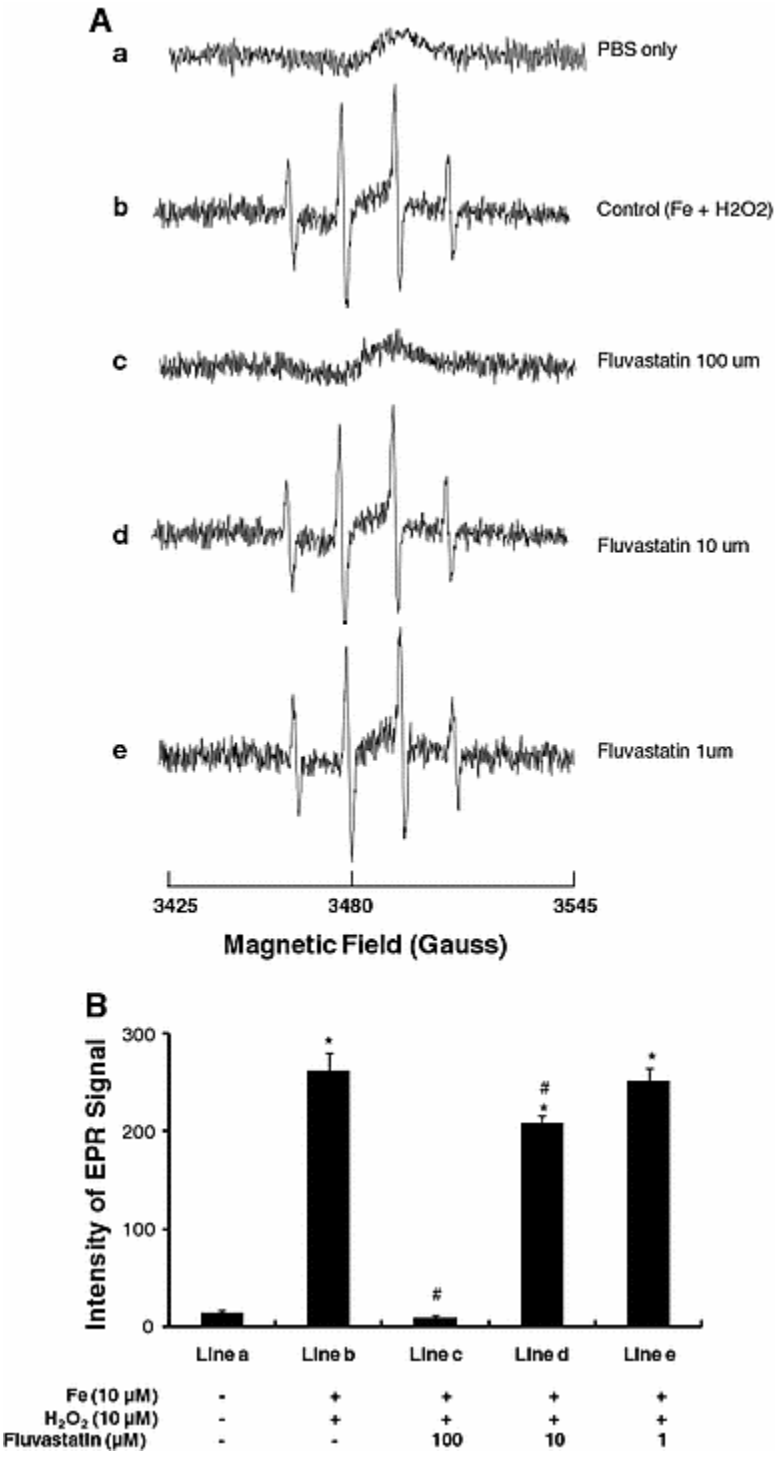

Fig. 3 Effect of fluvastatin on hydroxyl radicals generated from the Fenton reaction. EPR spectra of DMPO-OH observed during the reaction of 200 μM ferrous sulfate and 200 μM H_2O_2 containing 100 μM DMPO, in the presence or absence of fluvastatin. **a** *Line a* 100 μM

DMPO alone without $FeSO_4$, H_2O_2 and test compounds; *line b* 200 μM $FeSO_4$ and 200 μM H_2O_2; *line c* 200 μM $FeSO_4$ and 200 μM H_2O_2, and 1 μM fluvastatin; *line d* 200 μM $FeSO_4$ and 200 μM H_2O_2, and 10 μM fluvastatin; *line e* 200 μM $FeSO_4$ and 200 μM H_2O_2, and 100 μM fluvastatin. EPR measurement conditions were as described in "Materials and methods" section. Data represent similar results from three independent experiments. **b** Signal intensity at 3480 G was expressed as mean ± SD from three separate experiments (*$P < 0.05$ vs. control)

The potential vascular effects of statins have been documented in various animal models and clinical patients [*22–24*]. Statins were found to reduce generation of ROS by the inhibition of vascular NAD(P)H oxidase and myeloperoxidase and by the up-regulation of antioxidant enzymes such as catalase and paraoxonase [*25*]. Statins inhibit Rho-GTPase-dependent platelet–neutrophil interaction, which in part prevent neutrophil oxygen free radical release [*26*]. They also decrease respiratory burst activity of human neutrophils [*27*]. These previous studies indicate that the protective actions of statins could be due to their antioxidant activity, however, the exact mechanism(s) underlying their protective actions remains to be elucidated. We have demonstrated here for the first time that pravastatin and fluvastatin at physiologically relevant concentrations significantly decreased the formation of DMPO-hydroxyl adduct (DMPO-OH) in hydrogen peroxide/Fe(II) system, indicating that both compounds could directly scavenge hydroxyl radicals. Hydroxyl radicals play a pathogenetic role in a variety of diseases including inflammatory vascular disease and heart failure [*28–30*]. It is noted that the concentrations of pravastatin and fluvastatin used in this study could be physiologically relevant. For example, Siekmeier et al. [*31*] reported that the physiological concentration of fluvastatin in the cytoplasm could reach up to 5000 ng/ml (11.5 μM). Therefore, the potential capacities of statins in scavenging of hydroxyl radicals reported in this study could be important in ameliorating various diseases.

Effect of pravastatin and fluvastatin on hydroxyl radical-mediated DNA strand break

In φX-174 RF I plasmid DNA, induction of single-strand breaks to the supercoiled double-stranded DNA leads to formation of open circle DNA, while the formation of a linear form of DNA is indicative of double-strand breaks suggesting further damage to the open circle DNA [*32*]. The highly reactive hydroxyl radicals are known to damage a wide array of molecules in cells and critically participate in the induction of DNA damage. There is a pool of intracellular iron known as catalytic iron, which may contribute to hydroxyl generation through the Fenton reaction [*15*]. In this cortex, human studies have demonstrated a link between increased catalytic iron and many chronic diseases including cardiovascular diseases [*33–35*]. Thus, the Fenton reaction, involving Fe^{2+} and H_2O_2 for hydroxyl radical's generation was used to investigate whether pravastatin and fluvastatin can protect against DNA damage.

As shown in Fig. 4a, c (lane 2), incubation of DNA with H_2O_2 and Fe(II) for 30 min resulted in increased formation of open circle DNA, indicating that DNA breaks can be induced by the H_2O_2/Fe(II) system. Although pravastatin and fluvastatin at various concentrations exhibited

hydroxyl-scavenging activities, the addition of these compounds at the concentrations ranging from 1 to 100 μM to H_2O_2/Fe(II) has no significant inhibition of the conversion of supercoiled DNA to the open circle form (Fig. 4b, d). These results indicate that pravastatin and fluvastatin do not effectively protect against H_2O_2/Fe(II)-mediated DNA damage. The reason of this result is not clear because the mechanism(s) of DNA damage by Fenton reaction and action of these compounds remains unknown. One possible explanation is that both compounds could exhibited antioxidant activities by donating one of their own electrons to hydroxyl radicals and then become another radical that could further cause DNA damage.

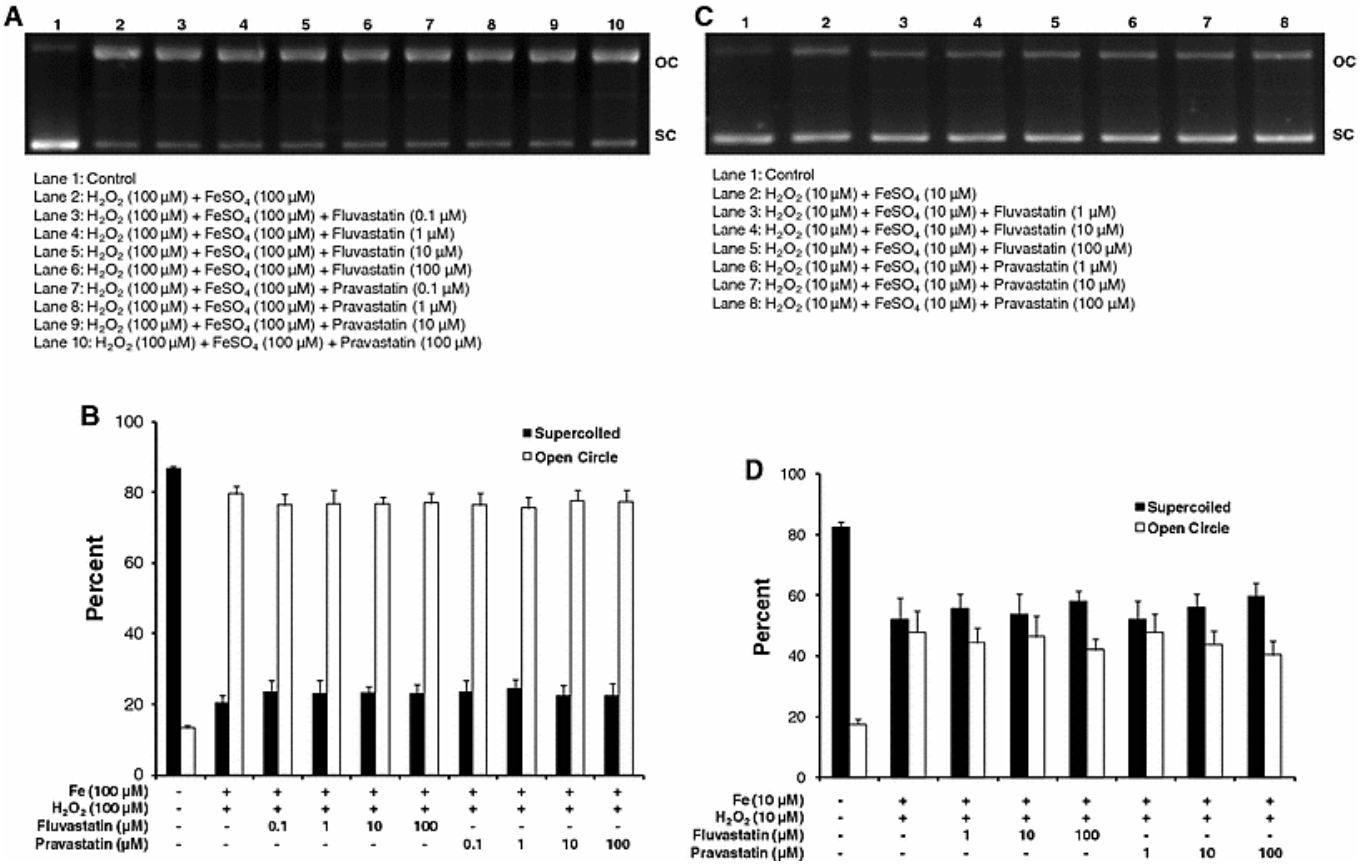

Fig. 4 Effects of pravastatin and fluvastatin on H_2O_2/Fe(II)-mediated DNA strand breaks. The φX-174 plasmid DNA was incubated with 100 μM H_2O_2 + 100 μM $FeSO_4$ (**a**, **b**) or 10 μM H_2O_2 + 10 μM $FeSO_4$ (**c**, **d**) in the presence or absence of the indicated concentrations of pravastatin or fluvastatin in PBS at 37°C for 30 min. (**a**, **c**) A representative agarose gel picture, demonstrating effects of increasing concentrations of pravastatin and fluvastatin on DNA damage; (**b**, **d**) quantitative analysis of DNA damage demonstrated in **a** and **c**, respectively. The DNA strand breaks were determined as described in the "Materials and methods" section. The control was φX-174 plasmid DNA incubated in PBS in the absence of any added agents. *OC* open-circular DNA conformation; *SC* supercoiled DNA conformation. Data represent mean ± SD of three independent experiments

Effect of pravastatin and fluvastatin on SIN-1-*mediated DNA strand break*

Peroxynitrite is a potent oxidant capable of causing DNA damage. Statins are reported to show anti-atherosclerotic effects mediated by peroxynitrite [*36*]. At a physiological pH, SIN-1 is known to undergo auto-oxidation to produce equal molar nitric oxide and superoxide, leading to the formation of peroxynitrite [*37*,*38*]. This system is often used in the examination of peroxynitrite on biological systems [*20*, *37*]. Thus, SIN-1 was used to further investigate whether pravastatin and fluvastatin can protect DNA damage caused by peroxynitrite. As shown in Fig. 5, a significant formation of open-circular form of DNA was observed following incubation of the plasmid DNA with 250 μM SIN-1 for 30 min (Fig. 5). However, the addition of pravastatin and fluvastatin at the indicated concentrations do not effectively protect against SIN-1-mediated DNA damage suggesting that those compounds do not directly scavenge peroxynitrite generated from SIN-1 autooxidation or inhibit the auto-oxidation of SIN-1 to produce peroxynitrite.

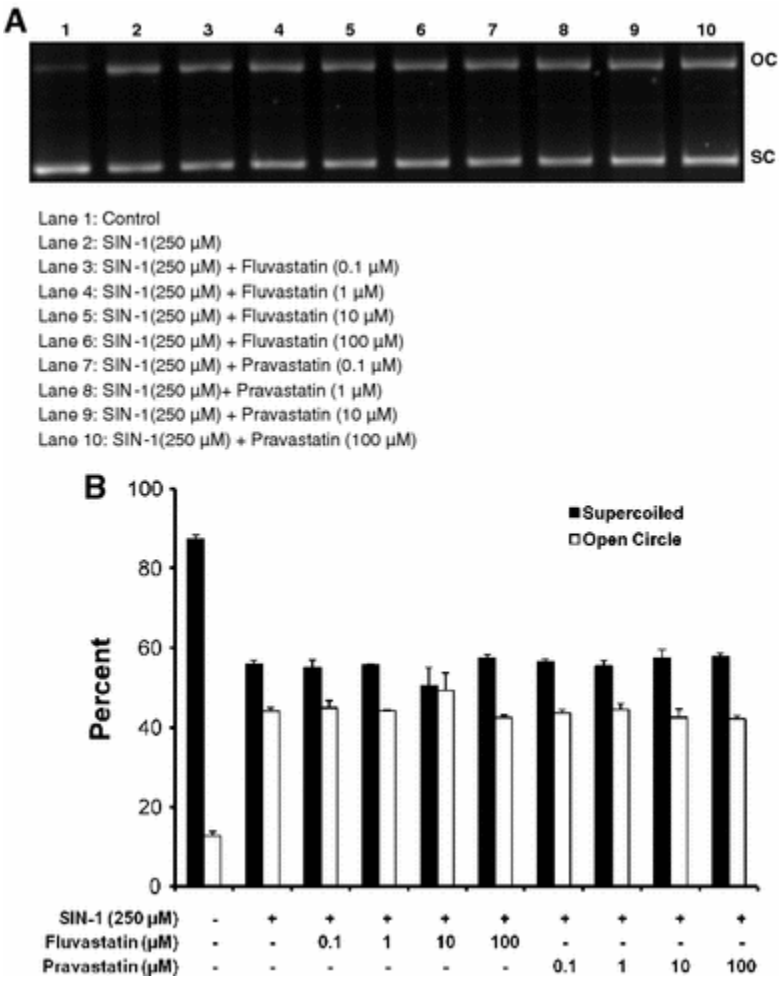

Fig. 5 Effects of fluvastatin or pravastatin on SIN-1 mediated DNA strand breaks. The φX-174 RF I plasmid DNA was incubated with 250 μM SIN-1 in the presence or absence of the indicated concentration of fluvastatin or pravastatin in PBS at 37°C for 30 min. The DNA strand breaks

were determined as described in “Materials and methods” section. **a** A representative agarose gel picture, demonstrating effects of increasing concentrations of pravastatin and fluvastatin on DNA damage; **b** quantitative analysis of DNA damage demonstrated in **a**. *OC* open-circular DNA conformation; *SC* supercoiled DNA conformation. Data represent mean ± SD of three independent experiments

In summary, this study demonstrates for the first time that pravastatin and fluvastatin at physiologically relevant concentrations exhibited hydroxyl radicals scavenging activities using a sensitive and specific technique of EPR spin-trapping method. Substantial evidence suggests that hydroxyl radicals are involved in the vascular pathology associated with atherosclerosis. Thus, the potential capacities of statins in scavenging of hydroxyl radicals activity reported in this study could be important in ameliorating various diseases. Further studies are essential to elucidate the exact mechanism of their protective actions observed in human clinical trials and animal models.

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