Effect of fluvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, on nitric oxide-induced hydroxyl radical generation in the rat heart

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Abstract

We examined the effect of fluvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, on the production of hydroxyl radical ('OH) generation via nitric oxide synthase (NOS) activation by an in vivo microdialysis technique. The microdialysis probe was implanted in the left ventricular myocardium of anesthetized rats and tissue was perfused with Ringer’s solution through the microdialysis probe at a rate of 1 µl/min. Sodium salicylate in Ringer’s solution (0.5 nmol/µl/min) was infused directly through a microdialysis probe to detect the generation of 'OH. Induction of [K+]o (70 mM) or tyramine (1 mM), significantly increased the formation of 'OH trapped as 2,3-dihydroxybenzoic acid (DHBA). The application of N(G)-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, significantly decreased the KCl depolarization-induced 'OH formation, but the effect of tyramine significantly increased the level of 2,3-DHBA. When fluvastatin (100 µM), an inhibitor of low-density lipoprotein (LDL) oxidation, was administered to L-NAME-pretreated animals, both KCl and tyramine failed to increase the level of 2,3-DHBA formation. The effect of fluvastatin may be unrelated to K+ depolarization-induced 'OH generation, but the effect of tyramine significantly increased the level of 2,3-DHBA. When fluvastatin (100 µM), an inhibitor of low-density lipoprotein (LDL) oxidation, was administered to L-NAME-pretreated animals, both KCl and tyramine failed to increase the level of 2,3-DHBA formation. The effect of fluvastatin may be unrelated to K+ depolarization-induced 'OH generation. To examine the effect of fluvastatin on ischemic/reperfused rat myocardium, the heart was subjected to myocardial ischemia for 15 min by occlusion of the left anterior descending coronary artery (LAD). When the heart was reperfused, a marked elevation of the level of 2,3-DHBA was observed. However, in the presence of fluvastatin (100 µM), the elevation of 2,3-DHBA was not observed in ischemia/reperfused rat heart. Fluvastatin, orally at a dose of 3 mg/kg/day for 4 weeks, significantly blunted the rise of serum creatine phosphokinase and improved the electrocardiogram 2 h after coronary occlusion. These results suggest that fluvastatin is associated with a cardioprotective effect due to the suppression of noradrenaline-induced 'OH generation by inhibiting LDL oxidation in the heart. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Fluvastatin; 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor; Low-density lipoprotein; N(G)-Nitro-L-arginine methyl ester; Hydroxyl radical; Microdialysis

Abbreviations: LDL, low-density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; L-NAME, N(G)-nitro-L-arginine methyl ester; CPK, creatine phosphokinase; DHBA, dihydroxybenzoic acid; LAD, left anterior descending coronary artery; NOS, nitric oxide synthase; XO, xanthine oxidase

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1. Introduction

Oxidative modification of low-density lipoprotein (LDL) is a key event in early atherogenesis, which contributes to cholesterol accumulation in the arterial wall and the development of the atherosclerotic lesion [1,2]. Fluvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor [3,4], protects against the progression of atherosclerosis and it is known to be a potent inhibitor of LDL oxidation [5,6]. LDL is oxidized by transition metal ions, such as copper and iron [5,7]. Although free radical reactions are a part of normal metabolism, the overproduction of reactive oxygen species such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (•OH) may contribute to cellular injury [8]. Oxygen-derived free radicals have been implicated in the mediation of myocardial ischemia/reperfusion injury [9]. Although fluvastatin has been shown to have antioxidative properties [10-13], the effect of fluvastatin on nitric oxide (NO) is not well known. NO is a free radical that regulates a variety of biological functions and also has a role of in the pathogenesis of cellular injury [14-16]. Cytotoxic free radicals such as peroxinitrite (ONOO$^-$) and •OH may also be implicated in NO-mediated cell injury [17]. NO is synthesized from L-arginine by NO synthase (NOS) [18]. To confirm the cardioprotective effect of fluvastatin, we investigated the effect of N$^\text{G}$-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, on •OH generation by an in vivo microdialysis technique [19,20]. To achieve this goal, we measured •OH formation in in vivo hearts, with the use of a flexibly mounted microdialysis technique that we developed [19].

2. Materials and methods

2.1. Animal preparation

Adult male Wistar rats weighing 300–400 g were kept in an environmentally controlled room (20–23°C, 50–60% humidity, illuminated from 7.00 to 19.00 h) and fed with food and water ad libitum. The rats were anesthetized with chloral hydrate (400 mg/kg, i.p.; Sigma, St. Louis, MO, USA) and the level of anesthesia was maintained by intraperitoneal injection of chloral hydrate (20 mg/kg). After intubation, the rat was mechanically ventilated with room air supplemented with oxygen. The chest was opened at the left fifth intercostal space, and the pericardium was removed to expose the left ventricle. The heart rate, arterial blood pressure, and electrocardiogram (ECG) were monitored and recorded continuously. At the end of the experiments the rats were killed by an overdose of anesthetic. All procedures in dealing with the experimental animals met the guideline principles stipulated by the Physiological Society of Japan and the Animal Ethics Committee of the Oita Medical University.

2.2. Microdialysis technique

Details of the flexibly mounted microdialysis technique and its application to measure biological substances in the interstitial space have been described previously [19]. We created a suitable microdialysis probe. The tubes of the dialysis probe (approx. 15 cm long) were supported loosely at the midpoint on a rotatable stainless steel wire, so that their movement was totally synchronized with a rapid up-and-down movement of the tip caused by the heart beats. The probe was implanted from the epicardial surface into the left ventricular myocardium to a depth of 3 mm and perfused through an inlet tube. The synchronized movement of the tip of the microdialysis probe with the beating ventricle minimized the tissue injury that would otherwise be caused by friction between the probe and the muscle tissue. The tip of a microdialysis probe (3 mm length and 220 μm o.d. with the distal end closed) was made of dialysis membrane (cellulose hollow fiber of 10 μm thick with 50000 molecular weight cutoff). Two fine silica tubes (150 μm o.d.) were inserted from the open end into the tip of the microdialysis tube consisting of a cylinder-shaped dialysis membrane which served as the inlet for the perfusate and the outlet for the dialysate, respectively. The inlet tube was connected to a microinjection pump (Carnegie Medicine, CMA/100 Stockholm, Sweden), and the outlet tube was led to a high performance liquid chromatography (HPLC) pump.
2.3. Experimental protocol

To find out the most appropriate perfusion rate, we bathed the microdialysis probe in vitro in a 37°C Ringer’s solution that contained 1.0 µM noradrenaline and the samples were collected at different perfusion speeds (0.5–5 µl/min). Based on the data collected from such experiments, we chose the most pertinent perfusion rate of 1.0 µl/min in the present measurements of noradrenaline and the relative recovery of noradrenaline under this perfusion rate was estimated to be 17.0 ± 0.7%. Oxygen free radicals are very reactive, and the non-enzymatic 'OH adduct of salicylate, 2,3-dihydroxybenzoic acid (DHBA), provides an assay of 'OH formation [20,21]. The 'OH captured as the hydroxylated derivative of salicylic acid was measured by HPLC with an electrochemical (EC) procedure. For trapping 'OH radicals [20,21] in the myocardium, sodium salicylate in Ringer’s solution (0.5 nmol/µl/min) was perfused by a microinjection pump and the basal level of 2,3-DHBA during a definite period of time was determined. Fluvastatin, KCl or tyramine was dissolved in Ringer’s solution for perfusion through a microdialysis probe into the myocardium. After a 30 min washout with Ringer’s solution, fluvastatin was introduced through the probe. Under a constant supply of fluvastatin, KCl or tyramine was perfused. Samples (1.0 µl/min) were collected into small collecting tubes containing 0.1 N HClO4 and assayed immediately for 2,3-DHBA by HPLC-EC. In the case of the L-NAME-treated rats, L-NAME (5 mg/kg) was injected intravenously into the rats before the experiments. In order to ascertain the protective effect of fluvastatin on myocardial infarction and reperfusion damage, the serum creatine phosphokinase (CPK) assay was measured. We divided the animals into two groups of five rats each: a non-treatment group (control group) and a fluvastatin-pretreated group. In the fluvastatin-pretreated group, fluvastatin was orally administered once a day in a dose of 3 mg/kg for 4 weeks. Blood was collected from catheterization of the carotid artery analyzed for CPK using Iatrontec CK rate (A) (Iatron Laboratories).

2.4. Preparation of ischemic rats

After microdialysis probe implantation in the ischemic zone, the left anterior descending coronary artery branch (LAD) was clamped by a thread through a tube surrounding the coronary artery. The heart was subjected to regional ischemia for 15 min by the occlusion of LAD followed by reperfusion for 60 min.

2.5. Analytical procedures

The dialysate samples were immediately injected for analysis into an HPLC-EC system equipped with a glassy carbon working electrode (Eicom, Kyoto, Japan) and an analytic reverse-phase column on an Eicompak MA-5ODS column (5 µm 4.6×150 mm; Eicom). The working electrode was set at a detector potential of 0.75 V. Each liter of mobile phase contained 1.5 g 1-heptanesulfonic acid sodium salt (Sigma), 0.1 g Na2EDTA, 3 ml triethylamine (Wako Pure Chemical Industries, Japan) and 125 ml acetonitrile (Wako) dissolved in H2O. The pH of the solution was adjusted to 2.8 with 3 ml phosphoric acid (Wako).

![Fluvastatin (L-NAME) vs KCl](image-url)  
Fig. 1. Effect of fluvastatin on KCl-induced 'OH formation after treatment with L-NAME. Animals treated with KCl (hatched bar; 70 mM) after the application of L-NAME (50 mg/kg i.v.) plus fluvastatin (solid bar; 100 µM) (closed circles) were compared with animals treated with KCl only (open circles). Differences between the time courses of 2,3-DHBA were studied with the Mann-Whitney U-test. Values are means ±S.E.M. for six animals; *P < 0.05 versus pre-KCl value.
2.6. Materials

Fluvastatin sodium (XU-62-320) was donated by the Discovery Research Laboratory, Tanabe Seiyaku (Saitama, Japan). Tyramine-HCl and sodium salicylate and its metabolites were purchased from Sigma. These drugs were dissolved in Ringer’s solution containing 147 mM NaCl, 2.3 mM CaCl₂ and 4 mM KCl, pH 7.4. L-NAME was purchased from Dojindo Laboratories (Kumamoto, Japan).

2.7. Statistical analysis

All values are presented as means ± S.E.M. Differences between the time courses of the levels of 2,3-DHBA were studied by means of the Mann-Whitney U-test. The significance of difference was determined using ANOVA with Fisher’s post hoc test. A P value of less than 0.05 was regarded as statistically significant.

3. Results

3.1. Effect of fluvastatin on high KCl-induced *OH formation after L-NAME treatment

The *OH captured as the hydroxylated derivative of salicylic acid was measured by the HPLC-EC procedure. The basal level of 2,3-DHBA in the heart dialysate from control animals following infusion of sodium salicylate (0.5 mmol/µl/min) was 0.033 ± 0.007 µM. Time-dependent changes in the formation of 2,3-DHBA were monitored in the dialysate from rat heart. Fluvastatin alone did not affect the level of 2,3-DHBA in the absence of sodium salicylate (data not shown). The effects of fluvastatin, an inhibitor of LDL oxidation, on extracellular potassium ion concentration, [K⁺]o (70 mM)-induced *OH formation were examined. Equivalent increases in the osmotic concentration of the Ringer’s solution by adding sucrose (150 mM) did not affect the level of 2,3-DHBA (data not shown). The introduction of high KCl (70 mM) was begun. KCl significantly increased *OH formation trapped as 2,3-DHBA at 15–30 min after the beginning of KCl application (n = 6, P < 0.05). However, no increase in 2,3-DHBA level in the absence of KCl was observed. After removal of KCl from the perfusate, the level of 2,3-DHBA significantly decreased. In the presence of fluvastatin (100 µM), when corresponding experiments were performed on animals pretreated with L-NAME (5 mg/kg i.v.), KCl (70 mM) failed to increase the level of 2,3-DHBA at 180 min application of fluvastatin (Fig. 1). However, in the absence of fluvastatin, L-NAME inhibited [K⁺]o-induced 2,3-DHBA formation and the results are summarized in Fig. 3A.

The introduction of tyramine (1 mM) significantly increased the level of 2,3-DHBA at 15–30 min after the beginning of tyramine application (n = 6, *P < 0.05) (Fig. 2). After removal of tyramine from the perfusate, the level of 2,3-DHBA significantly decreased. In the presence of fluvastatin (100 µM), when corresponding experiments were performed on animals pretreated with L-NAME (5 mg/kg i.v.), the same results were obtained. However, tyramine (1 mM) failed to increase the level of 2,3-DHBA formation at 180 min after application of fluvastatin and the results are summarized in Fig. 3B.
3.2. Effect of fluvastatin on •OH formation in ischemia/reperfusion

The presence of •OH was confirmed in ischemia/reperfused rat heart. The heart was subjected to regional ischemia for 15 min (210 min after probe implantation) by LAD occlusion followed by reperfusion for 60 min. Sodium salicylate (0.5 nmol/μl/min) was infused for 90 min to trap •OH, which was formed by ischemia/reperfusion of myocardium. After the dialysate probe was implanted in the left ventricular myocardium, the level of 2,3-DHBA remained unchanged until reperfusion. When the heart was reperfused, a marked elevation of the level of 2,3-DHBA was observed in the heart dialysate. This elevation was not observed outside the ischemic area. However, in the presence of fluvastatin (100 μM), no elevation of 2,3-DHBA in ischemia/reperfusion was observed at 180 min after application of fluvastatin (Fig. 4). When corresponding experiments were performed with rats orally treated with fluvastatin (at a dose of 3 mg/kg/day for 4 weeks), the same results were obtained (data not shown). To confirm myocardial ischemia and reperfusion...
fusion damage, the changes in serum CPK after reperfusion were examined. Oral fluvastatin blunted the rise in CPK (228 ± 32 versus 142 ± 19 U/ml, n = 5, P < 0.05).

4. Discussion

The present study indicated that fluvastatin, a HMG-CoA reductase inhibitor, is associated with the cardioprotective effect by suppressing noradrenaline-induced ·OH generation in the heart, with the use of a flexibly mounted microdialysis technique [19]. With this technique it is feasible to make stable and long-term measurements of ·OH. The control of 2,3-DHBA formation in the heart dialysate was about 10% higher than in the in vitro perfusion reagent background (data not shown). The drugs were administered through the microdialysis probe. Accordingly, the concentration profile of the administered compounds in the surrounding interstitial space is unknown; in general, the extracellular concentration of a compound given through the probe would never reach the concentration present in the dialysis probe [22]. This is an unavoidable limitation of the microdialysis technique that should be kept in mind when interpreting the experimental data.

Although the beneficial effect of fluvastatin was antioxidative properties [11,12], the exact mechanism is not clearly demonstrated until now. In the present study, to test a possible link between LDL oxidation and ·OH production, an HMG-CoA inhibitor (fluvasstatin) was used. Antioxidant substances have been shown to attenuate myocardial dysfunction [23–25]. Oxidative modification of LDL is thought to contribute to the production of oxygen-derived free radicals [26]. It is known that reactive oxygen species such as ·OH may be related to the oxidation of LDL [27]. Therefore, we examined whether inhibition of LDL oxidation can reduce ·OH generation. High [K+]o (70 mM) significantly increased the level of 2,3-DHBA (Fig. 1). To determine whether L-NAME has a radical scavenging or antioxidant effect, we examined the effect of L-NAME on K+ depolarization-induced ·OH generation. The application of L-NAME (5 mg/kg i.v.) significantly decreased the level of 2,3-DHBA by the action of high [K+]o. Therefore, it is possible that [K+]o-induced depolarization evokes ·OH generation via NOS activation. However, when corresponding experiments were performed on animals pretreated with fluvastatin (100 µM), the same results were obtained (Figs. 1 and 3A). Moreover, we confirmed that fluvastatin reduced ·OH formation by the action of K+ depolarization (data not shown). These results indicate that LDL oxidation may not be mediated with NOS activation. Although L-NAME did not affect the tyramine-induced ·OH formation, the effect of L-NAME on [K+]o-induced ·OH formation was abolished (Fig. 3A,B). The precise mechanism of depolarization-induced ·OH generation is obscure. It is known that NOS inhibition may inhibit depolarization-induced NOS activation by reducing Ca2+ influx through the blockade of Na+ and Ca2+ channels [28]. The reactive oxygen species causes excessive Na+ entry through the fast Na+ channel, leading to intracellular Ca2+ overload through the Na+-Ca2+ exchange system [29]. Intracellular Ca2+ overload is then considered to lead to cell death under physiological conditions such as ischemia/reperfusion injury [30,31]. It is known that O2− and NO rapidly react to form the stable peroxinitrite (ONOO−) and then its decomposition generates ·OH [17,32]. However, this idea is under discussion [33].

The interaction between depolarization and oxygen-free radicals in myocardium is not clear. However, the release of noradrenaline was induced by nerve depolarization [34]. Noradrenaline may also have a deleterious effect on the myocardium by serving as a source of free radicals [35]. It is known that noradrenaline is released from cardiac sympathetic nerve terminals [36,37]. Therefore, when tyramine (1 mM), a catecholamine releaser [38], was infused through a microdialysis probe, a marked elevation in the level of 2,3-DHBA was observed. When tyramine was administered to animals pretreated with L-NAME (5 mg/kg i.v.), L-NAME did not affect the tyramine-induced ·OH formation (Fig. 3B). However, in the presence of fluvastatin (100 M), tyramine failed to increase 2,3-DHBA formation. These results indicated that LDL oxidation may be related to noradrenaline-induced ·OH generation, but LDL oxidation may be unrelated to ·OH generation via NOS activation.

The therapeutic effect of fluvastatin is controversial, mainly due to a potent inhibitor of LDL oxida-
tion with which to test this hypothesis experimentally. Several experimental studies have shown that oxygen radicals contribute to myocardial damage induced by ischemia/reperfusion [39,40]. It is well known that ischemia induces depolarization [41,42]. Therefore, we confirmed the effect of fluvastatin on ischemia/reperfused rat heart. Fluvastatin (100 μM) attenuated \( \cdot OH \) generation induced by ischemia/reperfusion (Fig. 4). When rats were orally treated with fluvastatin (at a dose of 3 mg/kg/day for 4 weeks), the same results were obtained (data not shown). Based on the present studies, it is possible that LDL oxidation mediates ischemia/reperfusion-induced \( \cdot OH \) generation via depolarization in ventricular muscle. According to the reaction pathway in Fig. 5, \( \cdot OH \) may be formed in vivo during enzyme oxidation. The enzyme xanthine oxidase (XO) catalyzes the conversion of hypoxanthine to xanthine and simultaneously generates \( O_2^- \) [43]. XO resulting from xanthine dehydrogenase during ischemia [8] is thought to be a potential source of \( O_2^- \) in rat myocardium. It has an extremely short half-life [20] and rapidly undergoes dismutation yielding \( H_2O_2 \). Furthermore, it undergoes a Fenton-type reaction in the presence of iron and yields highly cytotoxic \( \cdot OH \) [44,45]. Radicals can cause tissue injury. Myocardial rat heart [46,47] injury was determined by measuring the level of CPK from the ventricular, a technique reported previously to be correlated with histologic determination of infarct size rat hearts. The protective effect of fluvastatin on myocardial ischemia and reperfusion damage was examined in rat hearts. In the group pretreated with fluvastatin (orally at a dose of 3 mg/kg/day for 4 weeks), serum CPK was significantly lower than that in the control group \( (n=5, P<0.05) \). It is possible that inhibition of LDL oxidation may reduce \( \cdot OH \) generation and hence ameliorate myocardial injury. The ECG manifestations of ischemia after ligation in fluvastatin appeared to improve. The abnormal Q waves of the fluvastatin-pretreated group were significantly less than in the control group \( (P<0.05, n=5) \) (data not shown). Although the precise mechanism of \( \cdot OH \) generation in the ischemic heart is obscure, we previously found a concomitant increase of noradrenaline and \( \cdot OH \) generation on myocardial injury [19]. Further investigation is necessary to confirm the relationship between free radical generation and LDL oxidation. This finding shows that LDL oxidation may be relevant to ischemia/reperfusion-induced \( \cdot OH \) generation. These results suggest that inhibition of LDL oxidation is associated with a cardioprotective effect due to the suppression of noradrenaline-induced \( \cdot OH \) generation.

The results of the present study may be useful in elucidating the actual mechanism of free radical formation in heart disorders. These experiments in cardiac microdialysis have versatile applications and of-
fer new possibilities for the in vivo study of cardiac physiology. In the future, cardiac microdialysis heart perfusion experiments using the hydroxylation of salicylate to detect "OH generation may be useful in answering some of the fundamental questions concerning the relevance of oxidant damage in the pathogenesis of heart disorders, such as infarction.

References


