Pravastatin Decreases Infarct Size Induced by Coronary Artery Ischemia/Reperfusion with Elevated eNOS Expression in Rats

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Summary
Our previous study showed that pravastatin prevents ischemia and reperfusion-induced lethal ventricular fibrillation in rats. This study explored whether pravastatin decreases myocardial infarct size and this effect is associated with endothelial nitric oxide synthase (eNOS) expression in myocardium. Rats were treated with ischemia (30 minutes) and reperfusion (60 minutes) after chronic oral administration of pravastatin, fluvastatin, or vehicle once daily for 22 days. Electrocardiograms and blood pressure were continuously recorded, myocardial infarct size was measured by TTC-staining, and eNOS expression was measured by western blot. The results showed that pravastatin and fluvastatin significantly reduced myocardial infarct size. No statistical differences were found in the areas at risk among all groups. However, a significant reduction in infarct size was observed in three pravastatin groups and one fluvastatin group compared to control. Both pravastatin and fluvastatin significantly increased eNOS protein expression in ischemic and non-ischemic tissues compared to control. Our results suggest that pravastatin decreases cardiovascular mortality beyond its cholesterol-lowering effect. Pravastatin is more potent than fluvastatin in reducing infarct size. These effects may be associated with elevation of eNOS expression.

Key words: Fluvastatin, Ventricular fibrillation

Many large clinical trials have shown that statins can reduce cardiovascular morbidity and mortality. This cardiovascular protection of statins can be easily associated with their cholesterol-lowering effects, because high cholesterol levels are actually related to cardiovascular risks including angina, myocardial infarction, and death. However, an increasing number of reports have revealed that statins have broad spectrum activity through which they either directly or indirectly exert their cholesterol-independent cardioprotective effects. These effects include preserving endothelial function, stabilizing arterial plaque, scavenging free radicals, and inhibiting proliferation, inflammation, and apoptosis. Furthermore, our previous studies have indicated that pravastatin effectively prevented coronary artery ischemia and reperfusion-induced ventricular fibrillation (VF) through suppressing neutrophils but without decreasing serum cholesterol levels in a rat model. These findings expanded the pleiotropic effects of statins on cardiovascular protection.

Cardiovascular diseases including acute myocardial infarction and severe arrhythmias are leading causes of death worldwide. In our previous studies, we found that hydrophilic pravastatin significantly inhibited lethal VF, but both hydrophilic pravastatin and lipophilic fluvastatin decreased myeloperoxidase (MPO) activity in ischemic myocardium. Therefore, this study was specifically designed to evaluate and compare the effects of hydrophilic pravastatin and lipophilic fluvastatin on infarct size induced by ischemia. We also investigated their effects on endothelial nitric oxygen synthase (eNOS) expression, which was shown to be associated with cardioprotective effects after myocardial ischemia.

Methods

Animal model of coronary artery ischemia/reperfusion injury: Sprague-Dawley rats (male, 370-440 g) were intraperitoneally anesthetized using pentobarbital sodium at a dose of 60 mg/kg, and artificial ventilation was established through endotracheal intubation. Systemic blood pressure and electrocardiogram (ECG) recordings were continuously monitored with a bioelectrical impedance device (MP150, Biopac Systems, Santa Barbara, CA). Normal arterial blood gas and pH were maintained through artificial ventilation of room air (tidal volume: 1.5 mL/100 g; 54 strokes/minute). A left thoracotomy was performed to open the chest, and the 4th and 5th ribs were sectioned at about 2 mm from the left margin of the sternum. The heart was gently exteriorized after the pericardium was

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carefully incised, and a 5/0 nylon suture was put under the left coronary artery. The heart was then placed back into the chest.

Two ends of the suture were pulled through a narrow plastic tube, and this tube was placed against the heart to induce myocardial ischemia and was maintained for 30 minutes by clamping the tube. The tube was removed to allow reperfusion of the heart, and related indexes were continuously recorded for 1 hour. Ischemia and reperfusion were confirmed according to a previous report.12

Animals were purchased from the Animal Laboratory for Research of Jilin University. The rats were kept in normal light-dark cycles, and were given food and water ad libitum. All experiments followed the Guideline for Animal Experiments at Beihua University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

**Definition of arrhythmias and ECG analysis:** Arrhythmias were defined according to the description of the Lambeth Conventions.15] Blood pressure tracings were used to determine ectopic activity, specifically polymorphic ventricular tachycardia (VT) and VF. VF was classified as lethal VF (terminal VF sustained ≥3 minutes) and non-lethal VF. VT time and incidence and lethal VF were recorded. Heart rate was recorded at three-time points: (1) just before chest opening, (2) just before occlusion, and (3) 30 minutes after occlusion. QT interval was only recorded just before the chest opening.

**Exclusion criteria:** Exclusion criteria in this study included: arrhythmias occurring before coronary artery occlusion, atrioventricular block during the first 5 minutes of ischemia, and mean arterial blood pressure < 60 mmHg before ligation. A total of 100 rats were used in this study. Of these, four were excluded due to low blood pressure before occlusion, five for consecutive VT, and four for VF before occlusion. No statistical difference was found in the distribution of these nine animals excluded for VT or VF among groups.

**Determination of myocardial infarct size and risk area:** The left coronary artery was re-occluded at the end of the experiment. A volume of 0.5 mL of 3% methylene blue was injected into the left ventricle from the aorta to differentiate the ischemic (unstained) and non-ischemic (stained blue) areas. The entire ventricular area was then sectioned into four 2-3-mm thick slices from the apex to the base and incubated in 0.1% 2, 3, 5-triphenyltetrazolium chloride (TTC) at 37°C for 20 minutes.16 TTC staining was used to differentiate necrotic and non-necrotic tissue. In the presence of an intact dehydrogenase enzyme system, TTC forms red-colored precipitates, whereas necrotic tissue lacking dehydrogenase activity remains unstained (shown as Figure 2). Ischemia and infarct areas were determined by computerized planimetry. Three percentages of cardiac tissue slices were measured including area ratios of ischemia/left ventricle, infarct/ischemia, and infarct/left ventricle.

**Treatment protocols:** Pravastatin (0.02, 0.2, and 2 mg/kg), fluvastatin (2 and 4 mg/kg) or vehicle (distilled water) were orally administered daily for 22 consecutive days. These doses were chosen according to our previous study on coronary artery ischemia/reperfusion using the same rat model.13 The rats were intraperitoneally anesthetized with pentobarbital sodium 2 hours after the last administration, and subjected to 30-minute ischemia followed by 60-minute reperfusion. ECG and blood pressure were recorded during the 30-minute ischemia period. Arrhythmias were analyzed using ECG. The hearts were harvested to determine the eNOS expression immediately after reperfusion.

**Measurement of eNOS expression:** Four rats were used in each group. Ischemic (stained blue) and non-ischemic tissues (stained red) were taken from each heart and used to assay eNOS expression. Proteins were extracted according to a previous report.16 Proteins (50 μg) were separated by SDS-PAGE (10%, BIO-RAD, Hercules, CA) and electroblotted onto a nitrocellulose membrane. eNOS expression was detected by a monoclonal antibody (Santa Cruz Biotech, Inc. Santa Cruz, CA). β-actin was used as an internal control. Protein bands were visualized with the chemiluminescence detecting system and quantified with Integrated Optical Intensity.

**Drugs:** Pentobarbital sodium was purchased from Beijing Chemical Reagent Company (Beijing, China). Pravastatin and fluvastatin in powder form were provided by Sanyko Company (Tokyo, Japan) and dissolved in distilled water before administration.

**Statistical analysis:** Data are expressed as means ± standard error of the means. Treatment effects on hemodynamic indexes and QT intervals were examined by Student’s t-test. Fisher’s exact probability test was used to compare differences in incidence of arrhythmias among groups. A P value < 0.05 was considered statistically significant.

**Results**

**Effects of pravastatin and fluvastatin on incidence of total VF and lethal VF:** Eighty-seven rats were used in this set, of which 19 were included in the control group; 14, 14, and 12 were in the 0.02, 0.2, and 2 mg/kg pravastatin groups, respectively; and 14 and 14 were in the 2.0 and 4.0 mg/kg fluvastatin groups, respectively. VF occurred in some rats during the 30-minute ischemia. All rats with VF suffered VT, although not all VT observed in this study triggered VF. The incidence of total VF (both non-lethal and lethal) was 84% and lethal VF was 58% in controls during the ischemic period. The incidences of total VF ([68%, 57%, and 67%]) and lethal VF ([36%, 21%, and 17%]) were decreased after treatment with pravastatin ([0.02, 0.2, and 2 mg/kg]). The incidences of lethal VF were significantly decreased after treatment with pravastatin (0.2 and 2 mg/kg) compared to controls (both [P < 0.05]). However, no statistical differences were found in the incidences of total VF ([93% and 100%]) and lethal VF ([43% and 36%]) after treatment with 2.0 and 4.0 mg/kg fluvastatin compared to controls (both [P > 0.05]; Figure 1).

**Effects of pravastatin and fluvastatin on ischemia and infarct size induced by ischemia/reperfusion:** Ischemia and infarct areas were measured in 38 hearts, and 6 hearts were used for control group. The experiment included 5,
Effects of pravastatin and fluvastatin on incidence of total VF and lethal VF during ischemia. Pravastatin dose-dependently decreased the incidence of total VF and lethal VF. The incidence of lethal VF was significantly decreased after treatment with 0.2 and 2 mg/kg pravastatin compared to controls (both \( P < 0.05 \)). No statistical differences were found between total VF and lethal VF incidence after treatment with 2.0 and 4.0 mg/kg fluvastatin compared to controls (both \( P > 0.05 \)).

![Figure 1](image)

Discussion

In our two previous studies, we compared the effects of hydrophilic pravastatin with lipophilic fluvastatin on ischemia/reperfusion-induced lethal VF and MPO activity in ischemic myocardium of rats. It is very interesting that even though both pravastatin and fluvastatin decreased MPO activity, only pravastatin prevented ischemia/reperfusion-induced lethal VF. In this study, we used the same rat model of ischemia/reperfusion as our last experiment, but examined different parameters, including infarct size and eNOS expression. The present results show that both statins can decrease infarct size and increase eNOS expression. Similar to our previous results, only pravastatin demonstrated an anti-arrhythmic effect. This difference can be explained by our previous report. In brief, the favorable effects of lipophilic fluvastatin on
ischemic heart disease may be attenuated by its unfavorable effects on myocardial energy production and contractile function. Pravastatin has the advantage of being hydrophilic, which hinders its ability to enter myocardial cells.

Clinically, ischemic heart disease is one of the main causes of mortality, and ischemia/reperfusion is the primary cause of arrhythmias and infarction. A few types of arrhythmias including VT, ventricular tachyarrhythmias, and VF were reported as the primary causes of sudden cardiac death, which accounted for 50% of cardiovascular disease related mortality.\textsuperscript{9,20,21} Infarction can result in gradual deterioration in left ventricular systolic function and eventually lead to clinically overt heart failure and even death.\textsuperscript{22} In our previous two studies, we compared the effects of pravastatin and fluvastatin on arrhythmias induced by ischemia/reperfusion.\textsuperscript{12,13} Therefore, in this study, we focused on the effects of these two statins on other severe consequence of ischemia/reperfusion, such as infarction.

The results of the present study demonstrated that both statins reduced infarct size but had different effects on incidence of lethal VF.

Ischemia and reperfusion in our rat model can be divided into three stages. In the first ‘mild ischemic stage’ (first 4 minutes after occlusion), the myocardium is only slightly injured and a normal physiological property is maintained. Almost no arrhythmias are observed during this stage. In the second ‘moderate or vulnerable stage’ or ‘arrhythmia triggering window’ (4–8 minutes after occlusion), the myocardium is severely injured and arrhythmias are easily triggered, especially VT and lethal VF. In the third ‘incapable stage’ (>8 minutes after occlusion), the myocardium is necrotic and few arrhythmias are observed. In fact, even in the third stage, there may be some regions along the ischemia and non-ischemia borders that can still be classified as first or the second stages. Therefore, reperfusion after a long period of ischemia can result in ischemia/reperfusion injuries, which mainly include ar-
Table. Effects of Pravastatin and Fluvastatin on Heart Rate, Blood Pressure and QT Interval in Anesthetized Rats

| Groups            | n  | Heart rate (beats/minute) | Mean blood pressure (mmHg) | QT interval (ms) |
|-------------------|----|--------------------------|-----------------------------|-----------------|
|                   |    | Just before thoracotomy  | Just before occlusion       | Just before thoracotomy |
| Control (0.02 mg/kg) | 14 | 429 ± 10                 | 399 ± 15                    | 126 ± 4         |
| Pravastatin (0.2 mg/kg) | 14 | 419 ± 10                 | 385 ± 15                    | 123 ± 4         |
| Pravastatin (2 mg/kg)   | 12 | 411 ± 12                 | 360 ± 10                    | 116 ± 6         |
| Fluvastatin (2 mg/kg)  | 14 | 391 ± 11                 | 410 ± 15                    | 111 ± 3         |
| Fluvastatin (4 mg/kg)  | 14 | 404 ± 9                  | 379 ± 14                    | 115 ± 4         |

Values are mean ± SEM. *P < 0.05 compared with just before occlusion. **P < 0.01 compared with just before occlusion.

Figure 3. Effects of pravastatin and fluvastatin on eNOS protein expression. eNOS expression was normalized to β-actin expression. Control for ischemic tissues was arbitrarily taken as 1 unit and other values are expressed as relative units to control. In ischemic tissue, pravastatin (0.2 and 2.0 mg/kg) and fluvastatin (4.0 mg/kg) significantly increased eNOS expression compared to the control (all P < 0.01). In non-ischemic tissue, eNOS expression was significantly increased in the pravastatin and fluvastatin groups (P < 0.05 or P < 0.01). n = 4 rats per each group.

Nitric oxide synthase has three forms: eNOS, neuronal nitric oxide synthase (nNOS), and the inducible nitric oxide synthase (iNOS). Of these, eNOS mainly produces vascular nitric oxide (NO), which plays multiple roles in regulating the vasculature. These effects include suppressed vascular smooth muscle cell proliferation and decreased platelet aggregation and adhesion. NO also modulates vasodilation in response to tachycardia and exercise and flow-induced dilatation of large human arteries. Therefore, decreased plasma eNOS levels are an important indicator of endothelial dysfunction. Promoting eNOS activation and NO production, can improve myocardial ischemia after infarction.

The effect of NO on ischemia/reperfusion damage or
myocardial dysfunction is not always beneficial. Werrick showed that NO donors can prevent reperfusion injury.40 NOS inhibition and gene silencing of iNOS and eNOS aggravated reperfusion injury, and NO regulated intracellular calcium signaling and protein kinase C expression, as well as antagonized neutrophil-related injuries.30-34 However, NO is also an origin of peroxynitrite, which exerts cytotoxic effects in the ischemic myocardium. Moreover, there is a dose-dependent effect of NO in relation to ischemia/reperfusion in the myocardium. A picomolar range increase in NO produced by elevated eNOS activity can protect and/or restore coronary endothelial function. However, a nanomolar range increase in NO caused by elevated iNOS activity during reperfusion aggravates lipid peroxidation and cell injury.32-34

Statins are beneficial for restoring coronary endothelial function impaired by hypercholesterolemia.31 In addition, statins reduce plasma level of low density lipoprotein and alleviate atherosclerosis.36 Endothelium-dependent vasodilation was restored in hypercholesterolemia patients who received statin treatments.8,35,36 However, we previously reported that pravastatin and fluvastatin did not change normal baseline plasma cholesterol levels.13 The present study showed that pravastatin and fluvastatin significantly increased eNOS protein levels in rats. One study demonstrated that pravastatin administration for three days reduced infarct size following ischemia and reperfusion in both normo- and hyper-cholesterolaemic rabbits, and also enhanced eNOS expression. In contrast, lipophilic simvastatin reduced infarct size and activated eNOS only in normocholesterolaemic animals.39

Regarding the effect of statins on myocardial infarction, one earlier study showed that acute application of fluvastatin (2 mg/kg) reduced myocardial infarct size, attenuated reperfusion injury, and also reduced MPO activity.40 Another report showed that acute and chronic administration of atorvastatin also significantly reduced infarct size and contractile dysfunction.40 However, Kocsis, et al. showed that lovastatin failed to reduce infarct size in rat hearts.41 Several studies revealed the relationship between the reduction of infarct size and eNOS expression, neutrophils, and cholesterol levels. In particular, one study showed that atorvastatin limited infarct size in wild-type mice but not in eNOS and iNOS knockout mice, suggesting that eNOS and iNOS are involved in the protective effects of atorvastatin.42 Similarly, a previous study demonstrated that acute administration of atorvastatin significantly reduced myocardial ischemia/reperfusion injury in an eNOS-dependent manner. In wild-type mice, atorvastatin significantly reduced myocardial infarct size and this beneficial effect completely disappeared in eNOS knockout mice.43 Another recent report indicated that ischemia-reperfusion injury-induced myocardial infarction could not be decreased by aliskiren in eNOS knockout mice.44 In contrast, pretreatment with the NOS inhibitor, L-NAME, did not reduce the infarct size induced by ischemia-reperfusion injury.45 These studies indicate that eNOS activation mediates protection against ischemia-reperfusion injury. Two other studies indicated that statins significantly reduced infarct size and contractile dysfunction in isolated ischemic rat hearts, suggesting that reduced infarct size is not mediated by infiltrating neutrophils. Acute and chronic statins have been shown to exert protective effects even when cholesterol levels are normal,12,13,14,41,42 indicating that statins can have effects that are cholesterol independent.

In conclusion, this study showed that pravastatin, but not fluvastatin, demonstrated anti-arrhythmic effects. However, both pravastatin and fluvastatin decreased infarct size and increased eNOS expression, which are likely the underlying protective mechanisms of these statins. The results of our experiments may not exactly predict clinical outcomes, but we speculate that statins exert their cardiovascular protection through their pleiotropic effects. However, the reduction in infarct size is independent of cholesterol reduction, neutrophil suppression, and hemodynamic changes, and may be dependent on up-regulation of eNOS protein levels. Both inhibition of arrhythmias and reduction in infarct size observed in our studies are undoubtedly beneficial to cardiovascular protection.

Disclosures

Conflicts of Interest: The authors declare that they have no conflict of interest.

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