Early Administration of Fluvastatin, but not at the Onset of Ischemia or Reperfusion, Attenuates Myocardial Ischemia-Reperfusion Injury Through the Nitric Oxide Pathway Rather Than Its Antioxidant Property

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Background Three-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) are known to attenuate myocardial ischemia-reperfusion (IR) injury. Fluvastatin (FV) has a potent free radical scavenging action, but it is unclear whether the timing of FV administration could affect its cardioprotective effect or if the antioxidant property of FV might attenuate IR injury.

Methods and Results IR was induced in rats by left coronary artery occlusion for 30 min followed by 24-h reperfusion. The rats were divided into 4 groups; oral FV group (10 mg/kg per day for 2 weeks before ischemia); iv, FV group (10 mg/kg) before ischemia; iv, FV group (10 mg/kg) before reperfusion; and control group. Oxidative stress was evaluated by myocardial 8-hydroxydeoxyguanosine (8-OHdG) content. The area at risk did not different among the 4 groups. Pretreatment with FV for 2 weeks significantly reduced the infarct size by 28% as compared with the control group, but FV administered just before ischemia or reperfusion did not. Myocardial 8-OHdG content was not affected by FV. The infarct-sparing effect of FV was completely abolished by N\textsuperscript{3}-nitro-L-arginine methyl ester or wortmannin.

Conclusions The present results indicate that pretreatment with FV, but not just before ischemia or reperfusion, attenuates IR injury primarily through the nitric oxide pathway, not through its antioxidant property. (Circ J 2006; 70: 1643–1649)

Key Words: Fluvastatin; Myocardial ischemia-reperfusion injury; Nitric oxide; Oxidative stress

Early reperfusion of an occluded coronary artery preserves myocardial viability and function by limiting the size of the myocardial infarct. However, despite early reperfusion, myocardial ischemia-reperfusion (IR) injuries, including no reflow, stunning and reperfusion arrhythmias, sometimes occur, thereby attenuating the cardioprotective effect of reperfusion therapy. Recent studies in experimental animals have demonstrated that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) attenuate IR injury independently of their lipid-lowering action. Statins have pleiotropic effects, including improvement of endothelial function by increased nitric oxide (NO) bioavailability and antioxidant and anti-inflammatory actions which may explain their attenuation of IR injury. The experimental result of cardioprotection by statins has therapeutic implication for patients with acute coronary syndrome who will be treated with reperfusion therapy; however, the time at which statin treatment was administered before IR varied from hours to days, and the results are conflicting. It is not clear whether acute administration of statins at the onset of ischemia or reperfusion will prevent or attenuate the IR injury.

Oxidative stress plays an important role in IR injury, and antioxidants such as superoxide dismutase and catalase could limit the infarct size in IR. Statins are known to decrease free radical generation in the vascular wall and myocardium which suggests that statins may protect the ischemic myocardium from IR injury via suppression of oxygen-derived free radicals produced upon reperfusion. Among the statins, fluvastatin (FV) has a potent free radical scavenging property derived from its chemical structure and the purpose of the present study was to elucidate the effects of acute administration of FV and the role of its antioxidant property on IR injury in rats.

Methods The experimental procedures followed the approved guidelines for animal experimentation at the University of Toyama.

Myocardial IR Male Wistar rats weighing 270–380 g (n=103) were intubated under ether anesthesia and ventilated using a rodent respirator. The heart was exposed by left thoracotomy and the left coronary artery was ligated 2–3 mm from its origin.
with a 5-0 suture. After 30 min, the ligature was removed and reperfusion was visually confirmed. The chest wall was closed and the rat was allowed to recover for 24 h. Sham rats were operated similarly but without IR.

Pretreatment
The rats undergoing ligation were randomized into 4 groups before IR according to the study protocol: (1) FV (10 mg/kg per day, Tanabe Seiyaku, Saitama, Japan) was administered orally by gavage for 2 weeks before ischemia (FVPO group), (2) intravenously (10 mg/kg) 5 min before ischemia (FVIV group), (3) intravenously 5 min before reperfusion (FVREP group), and (4) vehicle (0.1% carboxymethyl cellulose) was administered orally for 2 weeks (VEH group).

Measurement of Hemodynamics and Myocardial Infarct Size
Hemodynamic study was performed 24 h after reperfusion. A 2Fr micromanometer-tipped catheter was inserted into the right carotid artery and advanced into the left ventricle (LV) to determine the pressure. With the rat anesthetized lightly with ether and breathing spontaneously, the heart was quickly excised and mounted on a Langendorff apparatus, and perfused with blue dye to stain perfused myocardium (ie, the area at risk would be unstained). The LV was sliced into 2-mm sections for incubation in triphenyl tetrazolium chloride for 10 min at 37°C to distinguish stained viable tissue from unstained infarcted area. Area at risk and infarct area were quantified using computer-assisted planimetry and the infarct size was determined by the following equation: infarct size (%) = (infarct area/area at risk) x 100.

Assessment of Myocardial Oxidative Damage
In a separate set of experiments, myocardial oxidative stress was assessed by 8-hydroxydeoxyguanosine (8-OHdG) content, a marker of oxidative DNA damage. After 30 min of reperfusion following 30 min coronary occlusion, the ischemic myocardium was dissected (approximately 300 mg), rapidly frozen in liquid N2, and stored at −80°C until later analyses. The frozen tissue was homogenized and the DNA was extracted by NaI method using a commercially available kit (DNA extractor WB kit, Wako, Osaka, Japan). After the DNA pellet was dissolved in distilled water, 50 μg of DNA was digested with nuclease P1 (Sigma-Aldrich, Tokyo, Japan) and alkaline phosphatase (Sigma-Aldrich), and then centrifuged at 14,000 g for 10 min through a 0.22-μm filter (Millipore, MA, USA) according to the manufacturer’s instructions. The 8-OHdG content in the extracted DNA solution was determined by enzyme-linked immunosorbent assay (ELISA) method (Highly Sensitive 8-OHdG ELISA kit, Japan Institute for the Control of Aging, Shizuoka, Japan). In the Langendorff-perfused hearts of rats, the amount of 8-OHdG is reported to increase significantly after 20 min of reperfusion following 30 min of ischemia. In our preliminary study, myocardial 8-OHdG content did not differ after 30 min or 2 h of reperfusion (data not shown). Accordingly, the myocardial 8-OHdG content was determined at 30 min of reperfusion in the present study.

Involvement of NO Pathway
To evaluate the contribution of the NO pathway to the cardioprotective effect of pretreatment with FV, Nω-nitro-L-arginine methyl ester (L-NAME, 15 mg/kg, Sigma-Aldrich) was administered intravenously 10 min before ischemia in the FVPO and VEH groups. Hemodynamic changes caused by L-NAME treatment were measured before ischemia and the area at risk and infarct area were determined after 24-h reperfusion in both groups. In addition, to clarify the influence of FV on endothelial NO synthase (eNOS) activation via phosphatidylinositide (PI) 3-kinase/Akt pathway, wortmannin (15 μg/kg), a specific inhibitor of PI3-kinase, was administered intravenously 15 min before ischemia in the FVPO and VEH groups.

Statistical Analysis
All data are expressed as mean ± SD. The differences between groups were tested with 1-way analysis of variance, followed by the Bonferroni test for multiple comparisons. A value of p<0.05 was considered statistically significant.
Results

Plasma Lipids and Hemodynamic Data (Table 1)
FV did not affect body weight, or the levels of TC and TG. In the VEH group, LV end-diastolic pressure was elevated and maximum values of the rate of change in LV pressure tended to decrease as compared with the sham rats. FV did not affect these indices significantly.

Area at Risk and Myocardial Infarct Size
Representative examples of the area at risk and infarct area of each group are shown in Fig 1. Pretreatment with FV (FVPO) significantly reduced infarct size by 28% as compared with VEH (46±11% vs 64±15%, p<0.05) despite a similar extent of area at risk (Fig 2). However, FV administered just before ischemia or reperfusion did not reduce the infarct size (FVIV, 66±8%; FVREP, 66±14%).

Myocardial 8-OHdG Content
Myocardial 8-OHdG content was significantly elevated in the reperfused myocardium as compared with the sham-operated rats. However, FV did not affect the myocardial 8-OHdG content after IR (Fig 3).

Influence of NO
L-NAME increased mean blood pressure (VEH, 97±10 to 151±17 mmHg, p<0.05; FVPO, 96±10 to 143±21 mmHg, p<0.05) and decreased heart rate (VEH, 383±41 to 332±
24 beats/min, p<0.05; FVPO, 377±45 to 334±41 beats/min, p<0.05). Changes in mean blood pressure and heart rate after L-NAME were not different between VEH and FVPO groups. L-NAME did not affect infarct size in the VEH group, despite changes in the mean blood pressure and heart rate, but completely abolished the infarct-sparing effect of FV in the FVPO group (Fig 4). Pretreatment with wortmannin also abolished the infarct-sparing effect of FV in the FVPO group (Fig 5).

**Discussion**

The major findings of the present study are as follows. First, pretreatment with FV reduced the myocardial infarct size after IR, independent of its lipid-lowering action. However, FV administered just before ischemia or reperfusion did not attenuate IR injury. Second, pretreatment with FV did not affect myocardial 8-OHdG content, which served as a marker of oxidative stress and was elevated in reperfused myocardium. Third, L-NAME and wortmannin completely abolished the infarct-sparing effect of FV.

These results indicate that early administration of FV, be-
fore ischemia, is required for attenuation of IR injury and the cardioprotective effect of FV is primarily mediated through increased NO bioavailability via the PI3-kinase/Akt pathway, not through its antioxidant property.

Antioxidant Property of FV

There were few in vivo studies that have determined quantitatively the antioxidant effect of statins in IR injury. Production of oxygen-derived free radicals is considered to be a determinant of IR injury and of these, hydroxyl radical (·OH) is highly reactive and plays a critical role in post-ischemic myocardial damage during reperfusion. The antioxidant action of statins is derived not only from the reduction of oxidized low-density lipoprotein, but from inhibition of NADPH oxidase, which generates superoxide anion (O₂⁻) in the vascular wall. FV has a potent antioxidant property as a free radical scavenger, because of its unique chemical structure but in the present study, however, FV did not reduce the myocardial content of 8-OhdG, which is produced by hydroxylation at the C-8 position of deoxyguanosine by ·OH and is currently used as a stable biomarker of oxidative DNA damage. Formation of 8-OHdG in the heart with IR progressively increases after reperfusion and is completely blocked by co-treatment with a free radical scavenger. A large amount of oxygen-derived free radicals are released from mitochondria, the vessel walls, and leukocytes during reperfusion and therefore the antioxidant action of FV might be insufficient for cardioprotection from IR injury. Hayasaki et al reported that a reduction in myocardial oxidative stress was associated with attenuation of IR injury in mice deficient in the CC chemokine receptor 2. The discrepancy between the study by Hayasaki et al and the present study regarding the antioxidative effect of FV might be elucidated. In the present study, the dose of FV was relatively high, but within the range commonly used in rat and mouse experiments. Suzumura et al reported that FV showed dose-dependent free radical scavenging action in vitro. To elucidate the antioxidative effect of FV we therefore used a high dose (10 mg/kg).

Improvement of NO Bioavailability by Statins

Earlier studies reported that statins had cardioprotective effects on IR injury because of increased release of NO via eNOS bioavailability. Those results were supported by data that the infarct-sparing effect of statins was not observed in eNOS-deficient mice or with pretreatment with L-NAME, a finding consistent with the present result. The mechanisms by which statins increase eNOS activity are related to their ability to stabilize eNOS mRNA and/or to activate the PI3-kinase/Akt pathway. Jones et al reported that simvastatin did not attenuate the infarct size in the mouse heart when given less than 3 h before myocardial ischemia which is consistent with the present result that administration of FV just before ischemia or reperfusion did not reduce the infarct size. By contrast, the concentration of Akt phosphorylation by statins peaked at approximately 1 h and declined by 3 h after exposure in a cell-culture system. The bolus administration of FV in the present study might be insufficient for maintaining eNOS activation via the PI3-kinase/Akt pathway during reperfusion. Sanada et al also reported that statins had an acute cardioprotective effect via activation of PI3-kinase/Akt in a dog model of IR. They suggested that there was an optimal dose for each statin for the infarct-sparing effect. Therefore, the intravenous injection of FV in the present study may not have been the optimal dose as compared with its oral administration for 14 days.

Mechanism of Cardioprotection by Statins

There are several possible mechanisms for the cardioprotective effect of FV in the present study. First, enhancement of the NO level by statins potentiates a vasodilatory effect of resistant vessels, leading to preservation of tissue perfusion after IR. Second, statins inhibit leukocyte–endothelial cell interactions and reduce the leukocyte extravasation which suppresses the release of proinflammatory cytokines and chemically active compounds that induce tissue inflammation and injury. Third, the cardioprotective effect of statins may be mediated through an alteration of autonomic nerve function. Chronic treatment with simvastatin reduced sympathetic overactivity and plasma norepinephrine levels in animals with chronic heart failure. The suppression of sympathetic activity during IR by statins may protect the ischemic myocardium from norepinephrine-induced myocyte injury.

Effect of FV on Plasma Cholesterol Level in Normocholesterolemic Rats

Pretreatment with FV did not reduce the level of plasma cholesterol in our study using normocholesterolemic rats and it is well known that plasma lipids do not change in normocholesterolemic rats treated with statins. Although statins effectively inhibit HMG-CoA reductase in the rat, the plasma cholesterol level does not change, or even increases, with statin therapy because hepatic expression of the enzymes involved in its biosynthesis is markedly up-regulated.

Clinical Implications

Some clinical reports have revealed the cardioprotective effect of statins in IR injury. Patients on statins prior to the onset of acute myocardial infarction had smaller myocardial infarcts. Wong et al showed that statin use within 2 weeks of the onset of acute myocardial infarction improved myocardial perfusion after thrombolysis. However, it has not been elucidated whether acute statin treatment given at the onset of acute myocardial infarction might also reduce the myocardial damage. Our results did not show a cardioprotective effect of FV when administered at the onset of myocardial ischemia or reperfusion; however, our study suggests that pretreatment with statins would possibly reduce the extent of myocardial injury during a coronary event.

Study Limitations

First, we did not determine the dose–response effect of FV for protection against IR injury. Recent studies show that higher doses of statins do not reduce the infarct size and suggest that there could be optimal doses for each statin to elicit the acute cardioprotective effect in IR injury. Second, we did not elucidate the time period required for statin treatment before IR to produce the cardioprotective effect. The optimal timing of statin administration in IR...
injury needs to be studied in further investigations. Third, hemodynamic alterations by L-NAME might have affected infarct size. However, there was no significant difference in infarct size between the VEH and VEH+L-NAME groups (Fig 4). An earlier study reported that 15 mg/kg of L-NAME did not cause a significant change in infarct size as compared with control animals. Therefore, the hemodynamic changes after L-NAME treatment might not have influenced infarct size in our study. Finally, we did not determine myocardial eNOS protein or plasma NO levels after treatment with FV. However, the present results using L-NAME and wortmannin strongly suggest an infarct-sparing effect of FV through the NO-dependent pathway.

Conclusions

Pretreatment with FV before ischemia, but not at the onset of ischemia or reperfusion, limited myocardial necrosis in IR injury. This cardioprotective effect is primarily mediated through a NO-dependent action, not through an antioxidant action. Earlier administration of statins before the onset of ischemia to augment NO production might be required for the cardioprotective effect to occur.

References


