Pravastatin Reduces Myocardial Infarct Size Via Increasing Protein Kinase C-Dependent Nitric Oxide, Decreasing Oxyradicals and Opening the Mitochondrial Adenosine Triphosphate-Sensitive Potassium Channels in Rabbits

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Background Statins reportedly protect against myocardial infarction, but the precise mechanism is unclear.

Methods and Results Rabbits underwent 30 min of coronary occlusion followed by 48 h of reperfusion. Pravastatin (1 or 5 mg/kg) or saline was intravenously administered 10 min before ischemia. Pravastatin (5 mg/kg) was also administered 10 min before reperfusion. N\textsuperscript{\textregistered}-nitro-L-arginine methylester (L-NAME, 10 mg/kg), chelerythrine (5 mg/kg) or 5-hydroxydecanoic acid sodium salt (5-HD, 5 mg/kg) was intravenously administered 10 min before pravastatin injection. The infarct size was determined. The myocardial interstitial levels of 2,5-dihydroxybenzoic acid (DHB) and nitrogen oxide (NOx), and the intensity of myocardial dihydroethidium staining were measured. Pre-ischemic treatment with pravastatin reduced the infarct size (34±5% and 24±4%, 1 and 5 mg/kg, respectively), but not pre-reperfusion treatment (42.1±3.7%), compared with the control (45±3%). This effect was blocked by L-NAME (42.6±4%), chelerythrine (50.9±3%) and 5-HD (52.7±2%). Pre-ischemic treatment with pravastatin increased myocardial NOx levels, and attenuated both the 2,5-DHBA level and the intensity of dihydroethidium staining during reperfusion. Chelerythrine abolished the increase in NOx levels by pravastatin.

Conclusion Pre-ischemic treatment with pravastatin reduces the myocardial infarct size via protein kinase C-dependent nitric oxide production, decreasing hydroxyl radicals and superoxide, and opening the mitochondrial adenosine triphosphate-sensitive potassium channels. (Circ J 2007; 71: 1622–1628)

Key Words: Infarct size; Mitochondrial KATP channel; Nitric oxide; Pravastatin; Protein kinase C

Treatmen t with 3-hydroxyl-methylglutaryl coenzyme A reductase inhibitors (statins) has been reported to decrease the incidence of myocardial infarction and death in patients with hypercholesterolemia. Statins have been reported to have pleiotropic effects on the cardiovascular system, beyond their ability to lower cholesterol. Indeed, one of these effects is to reduce the myocardial infarct size. It has been reported that oxygen-free radicals play an important role in ischemia and reperfusion injury and blockade of oxygen free radicals has been reported to reduce myocardial infarct size. Furthermore, protein kinase C (PKC), mitochondrial adenosine triphosphate-sensitive potassium (KATP) channels, nitric oxide (NO) and oxyradicals have been reported to be involved in the infarct size-reducing effect of ischemic preconditioning.

It is still unknown whether pravastatin reduces the infarct size by involving these factors. Therefore, in the present study, we examined whether pravastatin reduces the myocardial infarct size, and investigated the contribution of PKC, mitochondrial KATP channels, NO and oxyradicals to the effect of pravastatin, using a rabbit model of myocardial infarction.

Methods

In this study, all rabbits received care in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication 8523, revised 1985). The study protocol was approved by the Ethical Committee of Gifu University School of Medicine, Gifu, Japan.

Chemicals

Five-hydroxydecanoic acid sodium salt (5-HD) and N\textsuperscript{\textregistered}-nitro-L-arginine methylester (L-NAME) were purchased from Sigma Chemical Co (St Louis, MO, USA). Pravastatin was provided by Sankyo Co Ltd (Tokyo, Japan).

Surgical Preparation

Male Japanese White rabbits, each weighing 2.0–2.5 kg, were anesthetized with 30 mg/kg sodium pentobarbital and...
mechanically ventilated with room air. Surgical procedures were performed aseptically. A polyethylene catheter (0.9-mm lumen diameter) was inserted into the internal carotid artery and was advanced approximately 1 cm toward the heart for blood pressure monitoring with a fluid-filled pressure transducer connected to the end of the cannula. Drugs and saline were administered via an ear vein. After a left thoracotomy was performed in the third intercostal space, the heart was exposed and 4-0 silk thread snare was placed around the large arterial branch coursing down the middle of the anterolateral surface of the left ventricle. Coronary arterial occlusion and reperfusion were performed by pulling or releasing the snare.

Protocol 1

Myocardial Infarct Size As shown in Fig 1, rabbits were randomly assigned to 10 groups: pravastatin-1 group (pravastatin 1mg/kg, n=10), pravastatin-5 group (pravastatin 5mg/kg, n=10), pravastatin-post group (pravastatin 5mg/kg, n=7), L-NAME + pravastatin group (pravastatin 5 mg/kg + L-NAME 10 mg/kg, n=9), 5-HD + pravastatin group (5-HD, a mitochondrial KATP channel blocker, 5 mg/kg + pravastatin 5 mg/kg, n=7), chelerythrine + pravastatin group (chelerythrine, a PKC inhibitor, 5 mg/kg + pravastatin 5 mg/kg, n=7), L-NAME group (L-NAME 10 mg/kg, n=7), 5-HD group (5-HD 5 mg/kg, n=7), chelerythrine group (chelerythrine 5 mg/kg, n=7) and the control group (n=10). In the control group, saline was administered intravenously 10 min before the 30 min of ischemia. The pravastatin groups were identical to the control group except that pravastatin was administered intravenously (1 mg/kg or 5 mg/kg) instead of saline. The pravastatin-post group was intravenously administered 5mg/kg of pravastatin 10 min before reperfusion. The 5-HD, L-NAME and chelerythrine groups were identical to the control group except that 5-HD, L-NAME or chelerythrine was injected intravenously 20 min before ischemia instead of saline. After the treatment, the coronary artery was occluded for 30 min and then reperfused. Hemodynamic parameters were recorded throughout the experiment until 20 min after reperfusion. Then the chest was closed and the rabbits were allowed to recover from anesthesia for 2 days’ survival.

Postmortem Study The rabbits were heparinized (100 U/kg) and killed with an intravenous overdose of pentobarbital (100–200 mg/kg) at the end of the study. The heart was excised and mounted on a Langendorff apparatus. The coronary branch was reoccluded and Evans Blue dye (0.2%, Wako Pure Chemical Industries, Ltd, Osaka, Japan) was injected from the aorta at 80 mmHg. After fixation in 10% phosphate-buffered formalin for 3 days, both the atrial and right ventricular free walls were removed. The left ventricle was cut into 7 transverse slices, each of which was weighed and photographed. The area at risk (AAR) (area without blue dye) was identified and traced from the enlarged projection (×10) of the photographic slide of each ventricular slice. The AAR and the infarct area were quantified by retracing these tracings on a digitizing tablet connected to a personal computer. The AAR and infarct area were calculated as a percentage of the total slice area and multiplied by the slice’s weight and then summed to obtain the total tissue weight of the AAR and infarct area. The infarct size was obtained as a percentage of the AAR as previously reported.

Protocol 2

Measurement of Myocardial Interstitial Nitrogen Oxide (NOx) Levels Thirty-two rabbits were assigned to the study investigating the effect of pravastatin on the level of myocardial interstitial NOx during ischemia and reperfusion. A microdialysis probe (PNF 1700; Asahi medical, Tokyo, Japan; 20 mm length, 0.31 mm OD, 0.2 mm ID; transverse type, 50,000 MW cut-off) for dialysate sampling was implanted in the risk region of the myocardium, which was served by the anterolateral coronary artery along the axis of the ventricular fibers and extended from the epicardial outer layer to the endocardial inner layer of the myo-
cardium. Probe placement was confirmed at autopsy. The microdialysis probe was perfused with Ringer’s solution at a rate of 10 μl/min. After a 60-min rest following the completion of instrumentation, the dialysate was sampled during 30-min pre-ischemia, during 30-min ischemia and during 30-min reperfusion with and without pravastatin (5 mg/kg, iv, at 10 min before ischemia, n=8, or at 10 min before reperfusion, n=8) or saline (control, n=8) or L-NAME (10 mg/kg, iv, at 10 min before reperfusion, n=8) or pravastatin (5 mg/kg, iv, at 20 min before ischemia) + pravastatin (5 mg/kg, iv, at 10 min before ischemia, n=8) being present. Dialysate samples were frozen at –83°C until further analysis. NOx levels were measured by Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical) as previously reported.13,14

**Protocol 3**

*Measurement of Myocardial Interstitial Hydroxyl Radicals* Twenty-four rabbits were assigned to the study investigating the effect of pravastatin on the level of myocardial interstitial hydroxyl radicals during ischemia and reperfusion. A microdialysis probe was implanted in the risk region of the myocardium as in Protocol 2. The microdialysis probe was perfused with 1 mmol/L salicylic acid dissolved in Ringer’s solution at a rate of 10 μl/min. After a 60-min rest following the completion of instrumentation, the dialysate was sampled during 30-min pre-ischemia, during 30-min ischemia and during 30-min reperfusion at an interval of 10 min in the presence of saline (n=8) or pravastatin (5 mg/kg, iv, at 10 min before ischemia, n=8, or at 10 min before reperfusion, n=8). Dialysate samples were frozen at –83°C until further analysis. The measurement of hydroxyl radicals is based on the reaction between salicylic acid and hydroxyl radical; 1 mmol/L salicylic acid can trap approximately 10% of the theoretically generated hydroxyl radical15 producing 2,3-dihydroxybenzoic acid (DHBA), 2,5-DHBA and catechol as the derivatives in proportions of 49, 40 and 11%, respectively.15 In the present study, we used 2,5-DHBA as an indicator of hydroxyl radical production as previously reported.13,14

The column used in the present study was an MCM C18 column (6×250; 5-120A; MC Medical Inc, Tokyo, Japan). The 2,5-DHBA was measured using high-performance liquid chromatography coupled with electrochemical detection, as described previously.13,14

**Protocol 4**

*Detection of Superoxide Anion Production in the Heart* Ten rabbits were assigned to the study investigating the effect of pravastatin on the levels of superoxide in the myocardium during reperfusion. Rabbits were subjected to 30-min ischemia and 10-min reperfusion with saline (n=5) or pravastatin (5 mg/kg, iv, at 10 min before ischemia, n=5 or at 10 min before reperfusion, n=5) being present 10 min before ischemia, after which the heart was immediately removed and sectioned into 7 transverse slices parallel to the atrioventricular ring. Next, the tissues were embedded in OCT compound (Miles Scientific) and snap-frozen in liquid nitrogen. The OCT compound samples were sectioned at 6-mm thickness with a cryostat for detection of the superoxide levels in situ by dihydroethydium because it reacts with superoxide anions to form ethidium bromide, which in turn intercalates with DNA to provide nuclear fluorescence as a marker of superoxide anion generation.16 Dihydroethidium (2 mmol/L) was applied to each tissue slice, which in turn intercalates with DNA to provide nuclear fluorescence as a marker of superoxide anion generation. The tissue slices were observed under a fluorescence microscope with a 490-nm long-pass filter.

**Statistical Analysis**

All values are presented as mean±SE. Risk and infarct size were compared among the groups by 1-way analysis of variance (ANOVA) combined with Bonferroni’s post hoc test for multiple comparisons. The difference in hemodynamics, 2,5-DHBA levels and NOx levels over the time course between the control and the drug-treated groups was assessed by 2-way repeated measures ANOVA. Differences with p<0.05 were considered statistically significant.
Pravastatin Reduces MI Size

Fig 2. (a) Area at risk (AAR) as a percentage of left ventricle (LV). There was no significant difference among the groups. (b) Infarct size as a percentage of AAR. Pravastatin reduced the infarct size in a dose-dependent manner. The infarct size-reducing effect of pravastatin was blocked by pretreatment with Nω-nitro-L-arginine methylester (L-NAME), chelerythrine or 5-hydroxydecanoic acid sodium salt (5-HD). L-NAME, chelerythrine or 5-HD alone did not modify the infarct size as compared with the control group. *p<0.05 vs control. IA, infarct area.

Fig 3. Changes in myocardial interstitial nitrogen oxide (NOx) levels. *p<0.05 vs control.
Results

Hemodynamic Parameters

There was no significant difference in mean blood pressure or heart rate among the groups (Table 1).

Infarct Size

The mean percentages of the AAR (percent of left ventricle) were 28.5±2.7, 27.0±2.2, 28.2±2.6, 29.8±2.6, 31.2±2.5, 29.0±2.4, 29.4±2.6, 30.2±2.5, 31.0±2.4 and 26.8±2.3% in the control group, pravastatin-1 group, pravastatin-5 group, L-NAME + pravastatin group, 5-HD + pravastatin group, chelerythrine + pravastatin group, L-NAME group, 5-HD group, chelerythrine group and pravastatin-post group, respectively (Fig 2a). No significant difference was seen among these groups. As shown in Fig 2b, the infarct size as a percentage of the AAR was significantly reduced in the pravastatin-1 group (34.0±5.0%, n=10) and the pravastatin-5 group (24.3±4.0%, n=10) compared with the saline control group (45.0±3.0%, n=10). However, the pravastatin-post group did not show a reduction in the infarct size (36.0±3.8%, n=7). Pretreatment with L-NAME (n=9), chelerythrine (n=7) and 5-HD (n=7) completely abolished the infarct size-reducing effect of pravastatin-5 (42.6±4%, 50.9±3%, 52.7±2%, respectively). L-NAME, chelerythrine or 5-HD alone did not affect the infarct size (40.6±3.0%, n=7; 50.6±3.0%, n=7; 45.2±3.0%, n=7; respectively).

Effect on Myocardial Interstitial NOx Levels

As shown in Fig 3, myocardial interstitial levels of NOx, an indicator of NO, were significantly increased during 30 min of ischemia and 30 min of reperfusion in the pravastatin group as compared with the control group. This increase in the NOx levels was completely abolished by pretreatment with chelerythrine, a selective PKC inhibitor. However, pravastatin administered at 10 min before reperfusion did not decrease the myocardial NOx levels during 30 min of ischemia and 30 min of reperfusion.

Effect on Myocardial Interstitial 2,5-DHBA Levels

As shown in Fig 4, myocardial interstitial levels of 2,5-DHBA, an indicator of hydroxyl radicals, were significantly increased at 30 min of ischemia and 10 min after reperfusion compared with the pre-ischemic period. Pretreatment with pravastatin significantly attenuated the increase in the myocardial interstitial 2,5-DHBA levels during ischemia and reperfusion. However, pravastatin treatment at 10 min before reperfusion did not affect the increase in the myocardial interstitial levels of 2,5-DHBA during ischemia and reperfusion.

Effect on Superoxide Levels in the Myocardium

As shown in Fig 5, the intensity of dihydroethidium staining in the AAR of the myocardium, an indicator of superoxide, was significantly attenuated in the pravastatin group, but not in the pravastatin-post group compared with the control group.

Discussion

Our findings demonstrate that pre-ischemic treatment with pravastatin (1) reduced the infarct size, which was abolished by a selective PKC inhibitor, chelerythrine, and a NOS inhibitor, L-NAME and a mitochondrial KATP channel blocker, 5-HD, (2) increased the levels of myocardial interstitial NOx during reperfusion, and (3) inhibited the increase in myocardial interstitial levels of 2,5-DHBA, an indicator of hydroxyl radicals and decreased the intensity of dihydroethidium, an indicator of superoxide during reperfusion.
Pravastatin Reduces MI Size

In the present study, pre-ischemic, but not pre-reperfusion, treatment with pravastatin reduced the infarct size. Pravastatin did not affect the hemodynamic parameters, such as mean blood pressure and heart rate, suggesting that the infarct size-reducing effect of pravastatin was not caused by a decrease in oxygen consumption.

The infarct size-reducing effect of pravastatin was abolished by pretreatment with a selective PKC inhibitor, chelerythrine, a NOS inhibitor, L-NAME, and a mitochondrial KATP channel blocker, 5-HD, suggesting that the infarct size-reducing effect of pravastatin was caused by activation of PKC, the production of NO and the opening of mitochondrial KATP channel.

Earlier studies reported that statins had cardioprotective effects on the myocardial ischemia–reperfusion injury by increasing the release of NO. Those results were supported by the finding that the infarct size-reducing effect of statins was not observed after pretreatment with L-NAME or in the eNOS knockout mouse. NO has been reported to increase coronary flow, regulate the endothelial adhesiveness for monocyte, inhibit platelet aggregation, and deactivate neutrophils and attenuate the activation of sympathetic nerve activity. These effects of NO might be beneficial against myocardial ischemia–reperfusion injury. As a matter of fact, we previously demonstrated that S-nitroso-N-acetylcysteamine (SNAP), a NO donor, significantly reduced the myocardial infarct size in a rabbit model with 30 min of ischemia and 48 h of reperfusion. In the present study, pravastatin actually increased myocardial interstitial NOx levels during reperfusion as compared with the control group, suggesting that pravastatin increases cardiac NO levels during reperfusion. Interestingly, NO is reported to mediate an early and late phase ischemic preconditioning effect in rabbits, which suggests that NO is a common mediator of pravastatin and ischemic preconditioning in reducing the infarct size. Although pravastatin increased the myocardial interstitial NOx levels during reperfusion, this increase was attenuated by pretreatment with chelerythrine, a PKC inhibitor, which suggests that NO production by pravastatin is caused by activation of PKC upstream. Therefore, it is likely that NO production by pravastatin is PKC-dependent. On the other hand, there are reports that NO has a cardiodepressive action after ischemia–reperfusion. However, whether NO acts as a cardiodepressant agent or as a cardioprotective agent depends on the level of oxidative stress in the myocardium. Because NO has an extremely high affinity for superoxide radicals, it rapidly loses its biological activity through reaction with them. If the increased production of NO is in balance with a moderate increase in oxygen radicals, then NO will exert beneficial effects. However, if oxyradicals are produced in excess of NO, as in prolonged ischemic injury, then deleterious effects will be induced. Therefore, the balance between NO and free radicals is crucial in modulating the outcome of an ischemic insult. In the present study, pravastatin attenuated the production of hydroxyl radicals and produced NO and so the balance between NO and free radicals may have been appropriate for creating a beneficial effect on the heart.

In the present study, the infarct size-reducing effect of pravastatin was also blocked by a mitochondrial KATP channel blocker, 5-HD, or a selective PKC inhibitor, chelerythrine, suggesting that the infarct size-reducing effect of pravastatin was caused by the opening of the mitochondrial KATP channel or by activation of PKC, both of which are considered to be mediators of the early ischemic preconditioning effect on infarct size. Therefore, it is reasonable to consider that pravastatin activated PKC and then opened the mitochondrial KATP channel, because PKC activation is reported to open the mitochondrial KATP channel.

Production of oxygen-derived free radicals, such as superoxide and hydroxyl radicals, is regarded as an important factor in reperfusion injury. In the present study, we investigated whether pravastatin reduces the myocardial interstitial levels of 2,5-DHBA, an indicator of hydroxyl radicals, as well as the intensity of dihydroethidium staining, an indicator of superoxide, in the AAR in the myocardium. We found that pre-ischemic, but not pre-reperfusion, treatment with pravastatin attenuated both the 2,5-DHBA levels and the intensity of dihydroethidium staining in the myocardium during reperfusion. This suggests that one of the mechanisms of pravastatin for reducing the myocardial infarct size is attenuation of superoxide and hydroxyl radicals during reperfusion. Indeed, we have previously reported that SNAP, a NO donor, attenuated the increase in myocardial interstitial levels of 2,5-DHBA during reperfusion and reduced the infarct size in a rabbit model of myocardial infarction. This is consistent with the concept that pravastatin attenuates hydroxyl radicals via enhanced production of NO and thus reduces the infarct size. NO has been reported to have an extremely high affinity for superoxide radicals, reacting with them and scavenging those produced by reperfusion. Indeed, in the present study, pre-ischemic, but not pre-reperfusion, treatment with pravastatin increased the myocardial levels of NOx, an indicator of NO, and decreased the intensity of dihydroethidium staining of the myocardium, suggesting that pravastatin enhanced the production of NO and decreased the production of superoxide. This effect of pravastatin may also have reduced the myocardial infarct size.

In conclusion, pre-ischemic treatment with pravastatin reduces the myocardial infarct size through the activation of PKC, opening the mitochondrial KATP channels, increasing the production of NO and reducing the levels of hydroxyl radicals and superoxide in the myocardial interstitium. The development of infarction may be inhibited, even when acute myocardial infarction occurs, in patients under treatment with pravastatin.

References


