Reduction of myocardial infarct size by fluvastatin

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Tiefenbacher, C. P., J. Kapitza, V. Dietz, C.-H. Lee, and F. Niroomand. Reduction of myocardial infarct size by fluvastatin. Am J Physiol Heart Circ Physiol 285: H59–H64, 2003. First published March 6, 2003; 10.1152/ajpheart.00782.2002.—Statins have a variety of cardioprotective properties following chronic treatment. In contrast, little is known about the acute effects. Reperfusion acutely injures the heart by activation of neutrophils as well as endothelial cells. Because statins are known to influence the processes pathogenetically involved, we hypothesized that acute application of statins attenuates the sequelae of cardiac reperfusion. In rats, myocardial infarction (MI) was induced by ligation of the left coronary artery followed by reperfusion. Myocardial blood flow (MBF) was determined by $H_2$ clearance and regional myocardial function (fractional thickening, FT) by pulsed Doppler. MI size was measured by triphenyltetrazolium chloride (TTC) staining, neutrophil extravasation by determination of myeloperoxidase (MPO) activity, and nitric oxide generation via measurement of cGMP. Treatment with fluvastatin, administered intravenously 20 min before the onset of ischemia, significantly attenuated the decline of FT and MBF at the end of the reperfusion period and significantly reduced MI size. Furthermore, fluvastatin induced a significant reduction of MPO activity and an increase of cGMP level compared with the control group. The effect of fluvastatin was completely abolished following pre-treatment of N$^\omega$-nitro-l-arginine methyl ester (l-NAME). These findings suggest that acute application of fluvastatin reduces MI size and attenuates reperfusion injury. We propose that the underlying mechanism is at least partially an inhibition of inflammation and endothelial dysfunction by preventing the activation and extravasation of neutrophils; toxic radicals; endothelial function; nitric oxide; neutrophils; toxic radicals

STATINS ARE LIPID-LOWERING SUBSTANCES that have been shown to reduce cardiovascular morbidity and mortality in a number of large clinical trials (23, 26, 27). Because cardioprotection by statins was also observed in patients with normal cholesterol levels, it was proposed that statins exert a broad spectrum of cholesterol-independent protective effects, including plaque stabilization; preservation of endothelial function; scavenging of free radicals; and antiproliferative, anti-inflammatory, and antiapoptotic effects (13–19, 21, 33). Many of these properties are mediated via the synthesis of nitric oxide (NO). A number of studies has shown an upregulation of NO synthase (NOS) expression as well as a stabilization of NO through scavenging of free toxic radicals (1, 8, 17, 31) during chronic treatment with statins. NO is an endogenous inhibitor of the expression of adhesion molecules that influence the binding and extravasation of activated neutrophils (19). Increased production of toxic radicals and increased adhesion and extravasation of neutrophils are two of the major pathomechanisms leading to the development of cardiac reperfusion injury (9, 20). So far, the beneficial effects of statins have been demonstrated in studies where treatment was performed over a period of at least hours to days. From the theoretical background, however, one could expect statins to have very acute effects on cardiovascular regulation. The aim of our study was, therefore, to assess in an in vivo model if acute application of a statin is beneficial regarding the sequelae of ischemia-reperfusion. We investigated the impact of intravenous application of fluvastatin immediately before the induction of cardiac ischemia on myocardial infarct size in an in vivo occlusion rat heart model.

MATERIALS AND METHODS

General preparation. Female Wistar-Furth rats of 215 ± 30 g body wt were anesthetized with intraperitoneal barbiturate (Inactin Byk-Gulden, 0.1 mg/100 g body wt ip) and artificially ventilated (small animal respirator, Harvard) with a volume of 2–3 ml at a rate of 60–75 breaths/min. Body temperature was maintained at 37°C by using a heated operating table. The carotid artery was cannulated for continuous measurement of mean arterial blood pressure with a Statham pressure transducer, and the jugular vein was cannulated to allow administration of fluid and drugs. The heart was exposed by removing an oblong portion of chest wall and sternum, and a tie was placed around the left coronary artery (LCA). The tie was then passed through a plastic cannula to allow ligation and reopening of the vessel.

The investigation conforms with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1985).

Determination of myocardial blood flow. Myocardial blood flow (MBF) was measured by the hydrogen clearance technique using platinum electrodes inserted into the myocardial tissue for measuring tissue H$_2$ pressure (P$_{H2}$). A discussion of the basic theory of determining tissue blood flow by inert gas clearance and demonstration of the applicability of H$_2$ as an indicator has been given earlier (2). In brief, the target tissue is saturated with hydrogen at a constant P$_{H2}$ by adding a constant fraction of H$_2$ (30%) in inspired air. Thereafter, H$_2$ concentration was monitored by the indicator technique using platinum electrodes inserted into the myocardial tissue for measuring tissue H$_2$ pressure (P$_{H2}$). The hydrogen produced in the tissue is removed by the gas-exchange system, and the H$_2$ concentration in the arterial and venous blood is measured. The hydrogen clearance rate is calculated from the difference in H$_2$ concentration between the arterial and venous blood and the rate of blood flow in the myocardial tissue. The myocardial blood flow is calculated from the hydrogen clearance rate and the hydrogen flow rate in the myocardial tissue.

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is removed from the breathing air and the tissue H2 is washed out by H2-free blood draining the tissue yielding an exponential concentration decline. The tissue blood flow (in ml/min g−1) is derived from the half-time (t1/2) of Pt2 decay as tissue blood flow = In2/t1/2. The exponential fit of the measured Pt2 curve for determination of flow was done with a computer-aided device. The H2 curves deviated slightly from a monoeponential shape only at the beginning (100–80%) and at the end (5–0%). Therefore, for evaluation of MBF, only the midportion of the curves (80–100%) has been used. The logarithmic regression coefficient of all curves was >0.98. The electrodes used in this study were constructed from 50-μm epoxide-insulated platinum wires with a 300- to 500-μm noninsulated Pt2-sensing tip. The electrodes were inserted into the myocardium for ~2 mm. Two electrodes were positioned in the ischemic area determined visually after a short ligation of the LCA before insertion of the electrodes and one in a nonischemic control area. The electrodes were polarized against an intrarectally positioned silver-silver chloride reference electrode by −300 mV.

The H2-clearance method has been validated in isolated perfused rat hearts (Langendorff model): at different perfusion rates, reliable values for myocardial blood flow could be obtained. In the in situ perfused rat heart, measurements of myocardial blood flow could be reliably reproduced with 5–10% changes.

**Determination of regional left ventricular function.** Regional left ventricular function was assessed by determination of myocardial systolic thickening fraction by use of the pulsed Doppler technique with a single epicardial transducer (Crystal Biotech) as described previously (11). The Doppler crystal (probe size validated for rats) was placed on the ischemic area of the left ventricular wall and sutured to the epicardium with a fine stitch (Prolene, 7.0). Myocardial wall thickening was evaluated by integrating the velocity of myocardial layers passing through a sample volume at a selected depth, which was determined by characteristic acoustic signals and by characteristic echo signals on an oscilloscope. Myocardial thickening fraction was then estimated by dividing systolic excursion by sample volume depth. Measurements could be reliably reproduced with 5–10% changes of myocardial thickening fraction.

**Determination of myeloperoxidase activity.** Tissue-associated myeloperoxidase (MPO) activity was determined by the method of Suzuki as modified by Sircjš et al. (29). At the end of the experiments, tissue obtained from the ischemic area of the left ventricle and from a control area of the right ventricle was homogenized manually in 1.1 ml of 0.02 M potassium phosphate buffer (PBS, pH 7.4) with the help of a glass homogenizer. One milliliter of the suspension was then centrifuged at 12,000 g for 5 min at 5°C to pellet the insoluble cellular debris. The pellet was then rehomogenized in an equivalent volume of 0.05 M PBS (pH 6) containing 0.05% hexadecyltrimethylammonium bromide (HTAB) and 0.01 M EDTA. MPO activity was assessed by measuring H2O2-dependent oxidant of 3,3',5,5'-tetramethylbenzidine (TMB). Extinction was measured in a spectrophotometer (model 24, Beckmann) at 655 nm. Horseradish peroxidase (Boehringer Mannheim; Mannheim, Germany) was used as a standard to allow the measured MPO activity to be related to the known activity of the standard.

**Determination of myocardial infarct size and area at risk.** Myocardial infarct size was determined by TTC staining. At the end of the myocardial infarction (MI) protocol, the LCA was briefly reocluded and Evans blue dye solution (3 ml, 2%) was injected into the left ventricle to allow perfused (stained blue) and nonperfused (unstained) areas of the heart to be distinguished. Thereafter, the animals were euthanized, the hearts were excised, and the left ventricle was divided into six slices perpendicular to the apex–base axis. To distinguish between the area at risk and infarcted myocardium, slices were incubated in triphenyltetrazolium chloride (TTC, 0.5 mg/ml for 20 min at 37°C). In the presence of intact dehydrogenase enzyme systems, TTC forms red-colored precipitates while areas of necrosis lack dehydrogenase activity and therefore do not stain. Tissue slices were photographed, and the area at risk, infarcted, and noninfarcted area were then determined using a computer-based system (Adobe photoshop and NIH Image). Area at risk and infarcted area were expressed as a percentage of the left ventricle.

**Determination of cGMP.** Systemic cGMP levels were determined from rat plasma by ELISA (Amersham).

**Experimental protocol.** After the preparation and a stabilization period of 30 min, four control measurements of MBF and FT were obtained before any intervention. The mean value of these four baseline measurements was taken as 100% in all groups. All subsequent measurements were related to this baseline value. Mean arterial blood pressure (MAP) and heart rate (HR) were continuously determined before interventions. After determination of baseline values, a bolus of fluvastatin (2 mg/kg) was applied followed by a continuous intravenous infusion of 1 mg·kg−1·h−1 in the intervention group or saline (0.9%, 2 ml/h) in the control group, respectively. Administration of fluvastatin was started 20 min before ischemia and continued throughout the experiment. Additional four control measurements of MBF and FT were taken to rule out possible effects of fluvastatin on baseline values. The dose of fluvastatin used in this study is equivalent to that used in similar investigations (12, 17). One group of animals was additionally pretreated with L-NAME (0.5 mg/kg), an inhibitor of NO synthesis. The provided dose of L-NAME has been suggested by others as being effective in inhibiting NO synthesis (25). In our experiments, this dose did not influence the sequelae of ischemia but significantly reduced the plasma cGMP level. Higher doses of L-NAME induced a significant increase in systemic blood pressure (SBP).

After control measurements, the LCA was occluded for 50 min and reperfused for 60 min. MBF and FT were measured during ischemia and at 1, 10, 20, 30, and 60 min of reperfusion. At the end, hearts were infused with Evans blue dye to mark the area at risk, tissue samples were taken for MPO determination, and hearts were fixed in glutaraldehyde before TTC staining. For determination of cGMP, blood samples were taken at the beginning and at the end of the experiment.

**Statistical analysis.** The data are expressed as means ± SE. To test for significance between means, as appropriate, the data were analyzed by Student’s t-test for paired data or the (repeated) analysis of variance followed by the Bonferroni t-test. For all tests, a probability value of P < 0.05 was considered indicative of a statistically significant difference.

**RESULTS**

In all groups, HR remained fairly constant throughout the experiments. Baseline MAP was similar in all groups and at the end of the experimental protocol, there was an average reduction by 15% to 95 mmHg (not significant). There was no significant change in HR, MAP, MBF, and FT due to intravenous infusion of fluvastatin or L-NAME (Table 1).
Systolic thickening fraction. Ligature of the LCA caused a dramatic decrease of fractional thickening (FT) from initially 20.7 ± 1.6% to 7.3 ± 1% during ischemia. Reopening of the vessel induced visible tissue reperfusion, but there was no significant increase of FT, and after 60 min of reperfusion, FT was more or less unchanged (8.3 ± 1.3%; Fig. 1). In animals that were treated with fluvastatin shortly before the onset of ischemia, there was also a significant reduction of FT from initially 20.9 ± 0.9% to 8.7 ± 1.3% during ischemia, and this is not different from the control group. Interestingly, however, there was gradual recovery of FT during reperfusion and at the end of the reperfusion period, the reduction of FT was significantly lower in the fluvastatin-treated group compared with the control group (15.8 ± 1.2%; P = 0.002). Additional pretreatment with 1-l-NAME completely abolished the effect of fluvastatin (7.5 ± 0.4% at the end of reperfusion; P = 0.003) (Fig. 1).

Myocardial blood flow. MBF, initially 3.6 ± 0.4 ml·g⁻¹·min⁻¹, was reduced to 0.7 ± 0.2 ml·g⁻¹·min⁻¹ during ischemia indicating effective coronary occlusion. After the ligature was reopened, there was only a slight increase of MBF and after 60 min of reperfusion, MBF was 1.5 ± 0.1 ml·g⁻¹·min⁻¹, which is a reduction to 40% of baseline (Fig. 2). In fluvastatin-treated animals, MBF was initially not different from the control group (3.5 ± 0.2 ml·g⁻¹·min⁻¹), and the reduction of flow during ischemia was also not different from the untreated group (1 ± 0.2 ml·g⁻¹·min⁻¹). In contrast, during reperfusion, there was a gradual increase of flow in hearts from fluvastatin-treated animals, and at the end of the reperfusion episode, MBF was 2.1 ± 0.3 ml·g⁻¹·min⁻¹, which is significantly more than in the control group (P = 0.04). l-NAME pretreatment prevented the protective effect of fluvastatin (end of reperfusion: 1.3 ± 0.2 ml·g⁻¹·min⁻¹; P = 0.03) (Fig. 2).

Infarct size. In controls, mean area at risk of the left ventricle was 40.6 ± 7%, and the infarcted area was 28.2 ± 5.1%. Thus the infarcted area was 69.5 ± 5.1% of the area at risk. (Fig. 3). In fluvastatin-treated animals, the area at risk of the left ventricle was similar to the control group (41.4 ± 6.6%), but the infarcted area was only 22.8 ± 4.6%. This represents an infarcted area of 53.5 ± 4.7% from the area at risk, which is significantly less compared with the control group (P = 0.02). Again, the fluvastatin effect was completely prevented by additional application of l-NAME (infarcted area: 29.1 ± 4.1% infarcted area from area at risk: 72.4 ± 8.6%; P < 0.01) (Fig. 3).

MPO activity. In all groups, MPO activity in tissue from the ischemic area was significantly increased.

![Fig. 1](image1.png)  
**Fig. 1.** Regional myocardial function in control (n = 8), fluvastatin (fluv)-treated (n = 10), and fluvastatin + N⁶-nitro-l-arginine methyl ester (l-NAME)-treated (n = 6) animals. Data are presented as means ± SE. *P < 0.05, fluvastatin vs. control. †P < 0.05, fluvastatin vs. fluvastatin + l-NAME.

![Fig. 2](image2.png)  
**Fig. 2.** Myocardial blood flow (MBF) in control (n = 8), fluvastatin-treated (n = 10), and fluvastatin + l-NAME-treated (n = 6) animals. Data are presented as means ± SE. *P < 0.05, fluvastatin vs. control. †P < 0.05, fluvastatin vs. fluvastatin + l-NAME.
compared with nonischemic tissue from the right ventricle indicating extravasation of neutrophils into the ischemic tissue. However, in fluvastatin-treated animals, MPO activity in tissue from the ischemic area was significantly lower compared with the control group and the fluvastatin + L-NAME-treated group (fluvastatin: 0.23 ± 0.06 U/g vs. control: 0.47 ± 0.2 U/g and fluvastatin + L-NAME: 0.48 ± 0.08 U/g; P < 0.01; Fig. 4).

**cGMP level.** At baseline, the level of cGMP in peripheral blood was not different between groups. At the end of the reperfusion episode, cGMP was decreased in the control and the fluvastatin + L-NAME-treated group. In contrast, there was a significant increase of cGMP in fluvastatin-treated animals (P < 0.01) (Fig. 5).

**DISCUSSION**

**Summary.** Intravenous application of fluvastatin started 20 min before the onset of ischemia reduced infarct size accompanied by an improvement of regional myocardial perfusion and regional myocardial function in the present study. In addition, fluvastatin treatment led to a significant decrease of MPO activity in the ischemic tissue and a rise in systemic cGMP level, indicating reduced extravasation of activated neutrophils and increased availability of NO. Furthermore, after pretreatment with L-NAME, an inhibitor of NOS, the fluvastatin effect was completely abolished. Thus the results of this study indicate that acute application of fluvastatin ameliorates reperfusion injury in the heart via increasing NO activity.

**Physiological and pathophysiological implications.** Reperfusion injury is mediated via increased extravasation of activated neutrophils into the myocardial tissue, leading to the release of different cytokines and chemically active compounds finally inducing tissue inflammation (9, 20). The data of the present study clearly show that statins prevent the extravasation of neutrophils into the myocardial tissue during reperfusion. This is supported by data from other investigations. Statin therapy for 18 h increased the vascular release of NO and decreased endothelial expression of P-selectin on endothelial cells and CD18 on leukocytes in rats. The result was a decreased adherence of activated neutrophils to endothelial cells, thus improving the hemodynamic outcome following ischemia-reperfusion (18). Similar beneficial effects were found following cerebral ischemia-reperfusion (7). Furthermore, statins have been shown to directly reduce CD 11b expression on monocytes and to suppress monocyte-endothelial adhesion in patients independent of the cholesterol-lowering effect (5, 33).

Because a basic mechanism for the development of reperfusion injury is a reduced availability of NO that induces the expression and cellular presentation of adhesion molecules and causes endothelial dysfunction, we propose that the beneficial effect of statin therapy in this setting is the stabilization of NO. There are a number of investigations showing that statins acutely and chronically improve the availability of NO via several mechanisms. Statins increase the release of NO (18), induce expression of NOS (16), inhibit the hypoxia-mediated inhibition of NOS activity (17), and prevent the scavenging of NO by reducing the generation of toxic radicals (31). NO inhibits endothelial-
leukocyte interaction by preventing the upregulation and the release of several adhesion molecules such as P-selectin, VCAM-1, and ICAM-1 (3). Thus an acutely increased availability of NO would lead to reduced cellular presentation of adhesion molecules and finally lead to reduced neutrophil adhesion and extravasation, thereby reducing reperfusion injury and preserving myocardial tissue.

Another beneficial effect of statins mediated via an increased availability of NO is the acute improvement of the vasodilatory potential of resistance vessels leading to an increase of tissue perfusion. In our study, this is reflected by the finding that myocardial blood flow was statistically better in statin-treated animals during reperfusion compared with the control group. This hypothesis is further supported by findings from our laboratory that fluvastatin pretreatment for 15 min improves the response to endothelium-dependent vasodilators in coronary arterioles from patients with atherosclerosis and that this effect can be prevented by pretreatment with 1-NAME, an inhibitor of NO synthesis (31).

Both the improvement of vasodilatory capacity of resistance vessels and the inhibition of neutrophil extravasation would explain the findings of our study that statins acutely improve cardiac performance following ischemia-reperfusion.

The exciting news of the present investigation is the acuteness of the statin effect. To date, in most clinical studies claiming a “rapid” statin effect, treatment has been performed for 4–6 wk (6, 10, 12, 24). In the Myocardial Ischaemia Reduction with Aggressive Cholesterol Lowering study (28), patients with unstable angina or non-Q-wave acute myocardial infarction were randomly assigned to oral treatment with atorvastatin or placebo between 24 and 96 h after hospital admission. There was a significant reduction of recurrent ischemic events in the first 16 wk after hospital admission in the statin-treated group. At present, however, there are no clinical studies providing acute intravenous statin therapy in the setting of an acute myocardial infarction. In in vivo animal experiments, cardioprotective statin effects have been demonstrated after treatment for 7–10 days (16), and, in most cell culture experiments, statins were given for at least 1 wk (30). However, there are some experiments in cell cultures showing an influence on leukocyte migration following treatment for 30 min (14). Furthermore, in a study by Jones et al. (13), pretreatment with simvastatin attenuated infarct size as well as myocardial dysfunction after 30 min of ischemia and 24 h as well as 6 mo of reperfusion in mice. In contrast to our findings, in this study an effect of statin treatment on infarct size could be found only when treatment was performed for at least 3 h, however, not 1 h before the onset of ischemia. There might be several reasons to explain the discrepancy to our findings, namely, differences in dosage (1 vs. 2 mg/kg), the application form (bolus vs. bolus + continuous intravenous infusion), and the provided statin metabolite (simvastatin vs. fluvastatin), as well as species differences (mouse vs. rat). In addition, we investigated only female animals that might have different mechanisms regarding myocardial protection due to the hormonal status. Similar to our findings, Di Napoli et al. (4) demonstrated a reduction of reperfusion injury by simvastatin given 20 min before ischemia. In contrast to our study, the improvement of myocardial function and vascular hyperpermeability was achieved by reducing inducible NOS expression and, subsequently, NO generation. However, our experiments were performed in an in vivo rat model in the presence of circulating blood and blood cells. Because it is known that reperfusion injury is mainly mediated via activated neutrophils and platelets, our approach might be more physiological. Still, the difference in mechanistic findings independent from diverging study characteristics as mentioned above needs further evaluation.

Our study is the first to show in an in vivo animal model that statins acutely improve cardiac injury. From our findings, statins are not only a class of substances to be used for chronic treatment but might also be valuable in the setting of acute disease management such as in myocardial ischemia. Thus there is need for clinical studies to investigate if acute application of statins will improve the outcome of patients.

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REFERENCES


