Rosuvastatin reduces experimental left ventricular infarct size after ischemia-reperfusion injury but not total coronary occlusion

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The most compelling data with statins are those reported after ischemic injury with reperfusion, rather than with permanent coronary occlusion (9–11, 14, 30). A direct comparison of the effects of rosuvastatin between permanent coronary artery occlusion and transient ischemia followed by reperfusion has not been performed. Because of advances in early diagnosis and interventional techniques, a growing proportion of acute MI patients are treated with early reperfusion. We therefore performed a randomized and blinded evaluation of the effect of rosuvastatin on left ventricular (LV) infarct size and remodeling after permanent ischemia vs. transient, 60-min ischemia with reperfusion to establish the effect of rosuvastatin on infarct size in these two experimental model systems. We tested the hypothesis that rosuvastatin decreases infarct size in the reperfused myocardium in association with a reduction of neutrophil accumulation in the injured myocardium, an increase in NOS3 expression, and mobilization of stem cells.

MATERIALS AND METHODS

Randomization of mice, rosuvastatin treatment, and method of blinding. Animal studies were conducted in conformity with a protocol approved by the Harvard Standing Committee on Animals. Male C57BL/6 mice, 10–14 wk of age, were randomized to receive once-daily subcutaneous injections of 20 mg·kg−1·day−1 rosuvastatin dissolved in saline or saline alone. This dose was selected because it has previously been shown to reduce cerebral infarct size after ischemia-reperfusion (13). Dosing was begun 2 days before surgery and continued until the time of death. Rosuvastatin or saline was coded so that investigators performing all analyses were blinded, and coding was revealed on completion of sample processing and analyses.

Coronary artery ligation. Mice were anesthetized with pentobarbital sodium (90 mg/kg ip). A tracheotomy was performed via a midline cervical incision with a PE-90 endotracheal tube, and mechanical ventilation with room air was begun at 0.3–0.4 ml at 100 rpm (Harvard Apparatus). A thoracotomy was performed to expose the heart. For permanent coronary artery occlusion, the left anterior descending artery (LAD) was visualized and ligated with 7-0 silk suture with a slip knot to allow for reperfusion. The LV was reperfused after 60 min, and the ligature was kept in place for later determination of LV area at risk. Animals were placed on a heating pad for 3 h during recovery, and buprenorphine (0.03 mg im) was administered twice a day for pain relief until the time of death. A 13-MHz linear probe (Sequoia, Siemens, Mountain View, CA) was used for echocardiograms, which were performed 2 and 28 days after permanent coronary artery ligation. Mice were then euthanized, and the LV was harvested.

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weighed, and fixed in 4% formaldehyde. After paraffin fixation, consecutive slices were obtained, and MI size was measured (22). Tail cuff blood pressure was measured as described elsewhere (23).

**Determination of LV infarct size after ischemia-reperfusion.** Mice were anesthetized with intraperitoneal pentobarbital, and the heart was excised and mounted on an isolated heart perfusion apparatus. The isolated heart was perfused at 37°C and 60 mmHg retrograde through the aorta with Krebs buffer followed by 1% 2,3,5-triphenyltetrazolium chloride solution for 10 min to stain viable myocardium. The coronary artery was reoccluded with the suture that was left in place at the time of reperfusion. The heart was then perfused with filtered 10% phthalo cyanine blue in phosphate-buffered saline to define the LV area at risk. The LV was sliced in cross section into six sections and fixed in 4% buffered paraformaldehyde. Each section was weighed and photographed. Nonjeopardized (region outside the ligated area) LV tissue was identified by deep blue staining, ischemic but viable myocardium was identified by deep red staining, and necrotic LV tissue was identified by white coloration. A planimetry image analysis program developed in our laboratory was used by an investigator blinded to treatment group to quantify the three regions in each slice. The areas of the three regions from all slices were summed to calculate their respective volumes. Infarct size is expressed as percentage of LV volume at risk.

**Neutrophil and leukocyte immunohistochemistry.** Rosuvastatin- and saline-treated mice underwent ischemia-reperfusion surgery as described above and were killed 1 day [rosuvastatin (n = 8) and saline (n = 5)] and 2 days [rosuvastatin (n = 5) and saline (n = 8)] after reperfusion. Mice were anesthetized with intraperitoneal pentobarbital, and the heart was excised, rinsed in phosphate-buffered saline, fixed in buffered 4% paraformaldehyde, and sliced into six sections as described above. The fifth slice from the base of the LV was embedded in paraffin. Immunohistochemistry on 7-μm sections was performed with rat monoclonal anti-neutrophil MCA771G antibody (Serotec, Oxford, UK) and leukocyte common antigen (CD45, eBioscience, San Diego, CA) as primary antibodies and anti-rat IgG biotinylated monoclonal antibody as secondary antibody. Staining was generated with the Vectastain ABC Kit Peroxidase Rat IgG PK-4004 (Vector Laboratories). Positively stained cells from the infarcted myocardium were counted by an investigator blinded to treatment group.

**Peripheral blood flow cytometry.** At 24 h after reperfusion at the time of ex vivo heart perfusion, blood was collected from the inferior vena cava of rosuvastatin-treated (n = 5) and saline-treated (n = 5) mice. Whole blood samples were blocked with mouse CD16/32 for 10 min and stained for 30 min on ice with anti-mouse phycoerythrin (PE)-c-Kit (eBioscience), FITC-CD34 (eBiosciences), or FITC-Sca-1 as stem cell markers and PE- or FITC-conjugated IgG antibodies as isotype controls (eBiosciences). Red blood cells were lysed, and samples were washed twice in flow cytometry buffer. Gating on the PE or FITC channel was set at 1–3% on the isotype control samples, and the percentages of positive-staining cells were determined for each sample. Samples were run in duplicate.

**LV NOS3 mRNA and protein determination.** TriReagent (Sigma) was used to extract RNA from LV and lung tissue and Bio-Rad Protein Extraction Reagent (Bio-Rad) was used to extract protein from LV tissue of mice treated with rosuvastatin (n = 5) or saline (n = 3–5) for 5 days. NOS3 mRNA levels were determined by Northern blotting with a 32P-labeled cDNA fragment of mouse NOS3 (GenBank accession no. BC052636). Quantitative real-time PCR was used for quantitation of NOS3 RNA levels using CybrGreen (Qiagen) with the LightCycler system (Roche). Primers amplified a 176-nt product (nt 1157–1317) from mouse NOS3 (GenBank accession no. BC052636). Data were normalized to mouse tubulin mRNA levels. NOS3 protein levels were determined using Western analysis with anti-mouse NOS3 antibody (Lab Vision).

**Statistical analysis.** Values are means ± SE. Data were analyzed by Student’s t-test, except in Fig. 4B, where data were analyzed by analysis of variance.

**RESULTS**

**Rosuvastatin and myocardial remodeling after permanent coronary artery ligation.** MI size (Fig. 1A) was not different in rosuvastatin vs. saline groups after 28 days of permanent coronary artery ligation: 24 ± 3% vs. 23 ± 2% (P = not significant NS). Tail cuff blood pressure measurements showed no difference in mean arterial pressure between the saline and rosuvastatin groups: 96 ± 10 vs. 101 ± 12 mmHg (P = NS). LV internal diameters were similar in rosuvastatin-treated (n = 17) and saline-treated (n = 20) animals at end diastole 2 days after total coronary occlusion: 3.3 ± 0.2 and 3.5 ± 0.3 mm in untreated and treated groups, respectively. Thereafter, LV dilation occurred in both groups, as expected, after infarction, and there was no significant difference in ventricular dilation between the treated and untreated groups. LV internal diameter increased, as anticipated, at end diastole from day 2 to day 28 (9 ± 3% and 14 ± 3% in untreated and treated animals, respectively), but the difference in dilation between the untreated and treated groups was not significant. Fractional shortening was similar in treated and untreated animals 2 days after MI: 40 ± 7 and 45 ± 5% in untreated and treated groups, respectively (P = NS). At 28 days after coronary artery ligation, fractional shortening was similarly decreased in rosuvastatin- and saline-treated groups: 36 ± 12% and 33 ± 10% in saline- and rosuvastatin-treated groups, respectively (both P < 0.05 vs. baseline, P = NS between groups). These data suggest that rosuvastatin does not favorably alter myocardial remodeling or reduce infarct size after permanent coronary artery occlusion in this animal model.

We also evaluated the effect of rosuvastatin on infarct size after permanent LAD occlusion in NOS3-deficient mice. MI size after permanent LAD occlusion tended to be greater in NOS3-deficient mice (n = 6) than in the wild-type saline-treated group: 33 ± 4% vs. 23 ± 2% (P = 0.08). However, infarct size in NOS3-deficient mice was not modified by treatment with rosuvastatin (34 ± 5%, n = 6, P = NS vs. NOS3-deficient saline-treated group). Thus rosuvastatin had no beneficial effect independent of NOS3 in the permanent occlusion model.

**Rosuvastatin and infarct size after ischemia and reperfusion.** We hypothesized that rosuvastatin may be preferentially protective after 60 min of ischemia and reperfusion compared with total occlusion given the participation of inflammation in ischemia-reperfusion. Therefore, we performed a blinded and randomized evaluation of rosuvastatin on infarct size. At 1 day after reperfusion, MI size was significantly reduced by 18% in mice treated with rosuvastatin compared with those treated with saline: 46 ± 4% vs. 56 ± 3% (P = 0.03; Fig. 1B). Area at risk was similar in saline-treated (n = 22) and rosuvastatin-treated (n = 18) mice (38 ± 3 and 43 ± 3, respectively, P = NS), indicating that placement of the ligature was similar between the groups (Fig. 1B). This demonstrates that rosuvastatin decreases the extent of myocardial necrosis when the myocardium is reperfused after coronary artery occlusion.

**Rosuvastatin and leukocyte infiltration after ischemia and reperfusion.** The beneficial effect of rosuvastatin, i.e., reduction of infarct size after ischemia-reperfusion, may be due to its...
regulation of early leukocyte and neutrophil infiltration to the injured myocardium (9). The number of neutrophils in the injured myocardium increased after ischemia-reperfusion but was similar between rosuvastatin- and saline-treated mice 1 day (465 ± 131 vs. 527 ± 136 neutrophils/section, P = NS) and 2 days (403 ± 71 vs. 438 ± 57 neutrophils/section, P = NS) after reperfusion (Fig. 2A). Similarly, the number of cells staining positive for leukocyte common antigen (CD45) in the injured myocardium increased after ischemia-reperfusion and was similar in rosuvastatin- and saline-treated mice 1 day (112 ± 30 vs. 99 ± 23 leukocytes/section, P = NS) and 2 days (306 ± 67 vs. 341 ± 34 leukocytes/section, P = NS) after reperfusion (Fig. 2B). These data suggest that the beneficial effect of rosuvastatin on infarct size after ischemia-reperfusion is not related to an inhibitory effect of rosuvastatin on leukocyte and neutrophil aggregation and adhesion in injured myocardium.

Rosuvastatin and stem cell mobilization. One of the potential pleiotropic effects of statins is the mobilization of progenitor cells from the bone marrow (3, 15, 27, 29). To test whether rosuvastatin can exert this effect under the experimental conditions in the present study, we analyzed peripheral blood from mice treated with rosuvastatin or saline and subjected to ischemia and reperfusion. We found no increase in rosuvastatin- vs. saline-treated mice in the percentage of circulating cells staining positive for the early stem and progenitor cell marker...
CD34 (3.7 ± 1.0% vs. 6.4 ± 2.0%, P = NS), the hematopoietic stem cell marker Sca-1 (4.0 ± 1.0% vs. 6.2 ± 1.2%, P = NS), and the stem cell factor receptor (CD117) c-Kit (5.1 ± 1.4% vs. 6.4 ± 1.4%, P = NS; Fig. 3). These data suggest that this dosing regimen of rosuvastatin does not result in the mobilization of endothelial progenitor cells.

Rosuvastatin and NOS3 protein and mRNA levels. Statins have been shown to improve endothelial function in hyperlipidemic patients and to upregulate the expression and activity of NOS3 (eNOS). To determine whether the beneficial effect of rosuvastatin on infarct size after myocardial ischemia and reperfusion is related to an increase in expression of NOS3, we examined gene expression and protein levels of NOS3 from LV and lung samples from rosuvastatin- and saline-treated mice. Levels of NOS3 protein were similar in LV tissue from rosuvastatin- and saline-treated mice (Fig. 4A, top), as were levels of LV NOS3 mRNA assessed by Northern blotting (Fig. 4A, bottom). Quantitative RT-PCR confirmed the Northern blotting results (Fig. 4B). These data suggest that the decrease in infarct size after ischemia and reperfusion in rosuvastatin-treated mice is not due to an increase in NOS3 mRNA and protein expression.

DISCUSSION

Progressive LV remodeling after MI leads to deterioration in contractile function associated with increased morbidity and mortality. Reperfusion strategies to restore blood flow limit ventricular remodeling after MI by salvaging jeopardized myocardium, which decreases the incidence of congestive heart failure and improves survival. In this study, we compared the effects of rosuvastatin on infarct size in the permanent occlusion and ischemia-reperfusion models of MI. An important finding of the present study is that rosuvastatin had no beneficial effect on infarct size in the permanent occlusion model but significantly decreased infarct size (necrotic area/area at risk) after 60 min of ischemia with reperfusion. The novel finding of this study is the beneficial effect of rosuvastatin in salvaging reperfused myocardium in the absence of an effect on neutrophil and leukocyte recruitment, a decrease in NOS3 expression, or stem cell mobilization, effects that have been reported in association with salvage after ischemia with statins.

Rosuvastatin is relatively hydrophilic; therefore, in contrast to lipophilic statins, it has reduced access to many cell types throughout the body by diffusion across cell membranes (18). Uptake of rosuvastatin is extremely high in hepatocytes, cells that express active transporters for anionic compounds such as...
This property, in association with the higher affinity of rosuvastatin for the active site of hydroxymethylglutaryl (HMG)-CoA reductase than of other statins (18), explains its selectivity for inhibition of HMG-CoA reductase in hepatocytes compared with fibroblasts (17). However, pleiotropic effects of rosuvastatin have clearly been documented. For example, Susic et al. (26) demonstrated that rosuvastatin reduced hypertension in genetically hypertensive rats independent of effects on cholesterol. Furthermore, rosuvastatin can correct nerve and vascular function in diabetic mice, also independent of effects on cholesterol (20). Intriguingly, in this study, cotreatment with mevalonate inhibited the beneficial effects of rosuvastatin, suggesting that, at least in this study, the benefit was independent of cholesterol lowering but via effects dependent on cholesterol biosynthesis pathway inhibition (20). This may explain why rosuvastatin has high selectivity for hepatocyte HMG-CoA reductase but still clearly has powerful pleiotropic effects relevant to cardioprotection (Fig. 5), such as the infarct size reduction seen in this study.

Neutrophil and leukocyte recruitment after ischemia and reperfusion may be reduced with statin treatment (24). Enhanced neutrophil and leukocyte recruitment has been attributed to decreased NO release from activated endothelial cells. This endothelial dysfunction with decreased NO release results from the formation of isoprenoid intermediates from substrate-activated HMG-CoA reductase activity. These isoprenoid intermediates act as posttranslational modifiers of proteins, including Rho, to allow membrane translocation and activation.
...of Rho kinase, which decreases eNOS expression and activity (24, 31). This mechanism (decreased NO and eNOS due to activation of Rho kinase by isoprenoid intermediates) is unlikely to be operative in the ischemia-reperfusion model used in the present study, inasmuch as cholesterol levels were normal in these mice. In agreement with this finding, we observed increased neutrophil and leukocyte infiltration into the myocardium after ischemia-reperfusion injury, but it was not affected by treatment with rosuvastatin. Thus mechanisms other than that described above account for neutrophil and leukocyte recruitment in this model.

Although the degree of neutrophil accumulation in the ischemic-reperfused myocardium was not altered by rosuvastatin treatment, rosuvastatin may have altered neutrophil function, for example, through an attenuation of neutrophil generation of reactive oxygen species (ROS) (4). Statins decrease NADPH oxidase-related ROS generation through inhibition of prenylation and translocation of cytosolic rac1 to the membrane subunits of NADPH oxidase. Lipophilic and hydrophilic statins have this effect in the heart (16). Thus, because ROS generation during ischemia-reperfusion contributes to cell death, the beneficial effect of rosuvastatin on myocyte death and infarct size may have been through a decrease in neutrophil and/or myocyte ROS generation early after activation following ischemia-reperfusion (2). Results from animal studies have shown that neutrophil depletion has beneficial effects in preventing ischemia-reperfusion injury. However, neutrophil depletion in humans has not been very successful (21; for review see Ref. 28), probably because neutrophils also have beneficial functions in promoting healing and scar formation, and the balance between beneficial and detrimental effects may differ in animal models and humans. Statins may favorably alter neutrophil function to allow their beneficial effects to dominate in the setting of ischemia and reperfusion.

Statins can induce the differentiation of endothelial progenitor cells from peripheral blood mononuclear cells cultured under endothelial cell growth conditions through a mechanism involving phosphatidylinositol 3-kinase/Akt signaling similar to vascular endothelial growth factor (VEGF) (3, 15). Atorvastatin has been shown to increase the expression of VEGF receptor 2 (KDR) on peripheral blood CD34 cells without increasing the total number of CD34+ cells, demonstrating the potential for atorvastatin to induce the differentiation of peripheral blood progenitor cells toward an endothelial cell phenotype (27). The number of bone marrow-derived cells that also stained for endothelial markers was increased by simvastatin, which was associated with accelerated reendothelialization and significant reductions in neointimal thickening in balloon-injured arterial segments in rats (29). Cultured splenocytes and bone marrow cells harvested from mice fed simvastatin for 3 wk showed an increase in the number of cells staining positive for 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate-labeled acetylated LDL/lectin compared with saline-treated mice (29). In the present study, we did not observe an increase in the number of peripheral blood cells staining positive for CD34, c-Kit, and Sca-1 in response to rosuvastatin with the dosing regimen used. However, we cannot rule out the possibility that expansion in culture under endothelial cell growth conditions is required to observe this effect with rosuvastatin. Furthermore, because studies have shown in humans and mice that longer dosing times with statins are required to achieve bone marrow mobilization of cells that differentiate into the endothelial cell phenotype, this effect is unlikely to account for the reduction in infarct size by rosuvastatin in the present study. Recent studies have shown that peripheral blood cells expressing the monocyte marker CD14 (CD14+ cells that are usually CD34−) may function as pluripotent progenitor cells able to differentiate into endothelial cells when cultured with VEGF, as well as into macrophages, epithelial cells, neuronal cells, and liver cells when cultured with appropriate growth factors (7, 8, 32). The differentiation of CD14+ cells into specific lineage cells is accompanied by a decrease in CD14 expression and an increase in lineage markers. Simvastatin treatment in patients with hypercholesterolemia was associated with a decrease in CD14 expression in peripheral blood (25). It is attractive to consider the possibility that statins can induce this special subset of CD14+ peripheral blood cells to differentiate into endothelial progenitors as a mechanism contributing to improved endothelial function with statins. Peripheral blood CD14 expression was not measured in the present study, and a possible beneficial effect of statins on this phenomenon needs to be resolved in future studies.

One of the most characterized effects of statins independent of cholesterol lowering is the ability to increase the expression and activity of eNOS (NOS3). Absence of NOS3 in mice subjected to permanent coronary artery ligation is associated with increased mortality, greater myocardial remodeling, decreased fractional shortening, and impaired diastolic function compared with wild-type mice, effects that are not prevented...
by normalization of blood pressure in NOS3-deficient mice (23). In the ischemia-reperfusion model, NOS3 deficiency was associated with greater infarct size and more neutrophil infiltration in the postischemic myocardium (10). These effects were attributed to decreased bioavailability of NO in NOS3-deficient mice, resulting in an increased expression of endothelial adhesion molecules and a greater inflammatory response leading to necrosis. Statins inhibit leukocyte adhesion and promote eNOS production by inhibiting Rho kinase geranylation and, possibly, by directly activating eNOS expression and activity through activation of Akt (24). In contrast to several reports describing an increase in eNOS mRNA expression and activity with statin treatment in the ischemia-reperfusion model in association with a reduction in infarct size, we found no increase in eNOS mRNA and protein, although infarct size was significantly decreased in mice treated with rosuvastatin. Although we cannot explain this discrepancy, it is possible that local increases in NO production in the endothelium due to statin treatment that are not detected in whole tissue extracts contributed to the reduction in infarct size after ischemia-reperfusion in our model. In support of this, infarct size in NOS3-deficient mice in our study tended to be greater than in wild-type mice, in agreement with a published report (10).

It is well accepted that, with regard to damage after ischemia and reperfusion, timing is critical. The earlier the myocardium is reperfused after ischemia, the greater the myocardial salvage; conversely, the longer the period of ischemia, the less the myocardial salvage. In general, longer periods of ischemia lead to greater necrosis, rather than apoptosis, a form of cell death that is amenable to therapeutic intervention and prevention. An increase in neutrophil accumulation after 30 min vs. 20 min was observed after the same duration of reperfusion by Jones et al. (10). Apoptosis after ischemia-reperfusion measured by in vivo annexin staining in mouse hearts was also increased after 30 min vs. 15 min of ischemia (5). This same study also showed that apoptosis during reperfusion increases to an extent dependent on the time of ischemia. In the present study involving ischemia and reperfusion, we used 60 min of ischemia followed by 24 h of reperfusion. This duration of ischemia was within the critical time window for myocardial salvage by rosuvastatin, whereas no beneficial effect of rosuvastatin was observed in the group subjected to permanent ligation. Additional studies are required to determine the duration of time of both ischemia and reperfusion for which rosuvastatin has a beneficial effect on infarct size as well as on the additional end points that were measured in the present study.

In summary, rosuvastatin had no beneficial effect on infarct size after permanent coronary artery ligation. In contrast, rosuvastatin treatment resulted in a significant decrease in infarct size after 60 min of ischemia-reperfusion. The decrease in infarct size was observed in the absence of an effect of rosuvastatin on neutrophil leukocyte infiltration, progenitor cell mobilization, and eNOS mRNA and protein expression. Despite its limited bioavailability, rosuvastatin treatment of reperfused myocardium after ischemic injury decreased myocardial cell death. The protective effect of rosuvastatin may have been on apoptotic cell death, because the predominant form of cell death early after ischemia-reperfusion injury is apoptotic, and this form of cell death is amenable to reduction.

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**REFERENCES**


