Hypercholesterolemia contributes to cardiac morbidity and mortality in man. Numerous therapeutic agents have been designed to decrease serum lipid levels and combat the subsequent deleterious effects of hypercholesterolemia. Although many drugs are successful in reducing serum cholesterol levels, none have been more effective and well tolerated than the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, commonly referred to as “statins.” Clinical trials have clearly demonstrated the safety and efficacy of statins in reducing the risk for coronary events in association with serum cholesterol reduction (1–3). However, more recent data have indicated that a portion of the cardiovascular benefits of statins may be unrelated to reduction of serum cholesterol.

In fact, a portion of the pleiotropic effects of statins may result from an increase bioavailability of nitric oxide (NO). Recent in vitro studies from Laufs et al. (4) have demonstrated that statins can upregulate endothelial NO synthase (eNOS) in cultured human endothelial cells. In addition, others have shown that statins enhance the activity of eNOS through protein kinase activation (5). The importance of endothelial-derived NO in cardiovascular homeostasis combined with the pervasive use of statins has driven numerous laboratories to investigate the possible effects of statins in experimental models.

Several reports have documented cytoprotective actions of statins in clinically relevant models of human disease. Endres et al. (6) recently described the neuroprotective effects of statins in a model of cerebral ischemia and reperfusion. In addition, others have demonstrated cardioprotective effects in normocholesterolemic (7), hypercholesterolemic (7), and diabetic (8) rodents subjected to myocardial ischemia–reperfusion injury. In addition, statins enhance angiogenesis via a protein kinase B/NO pathway in normocholesterolemic rabbits (5). All of these studies demonstrated significant cardiovascular benefits associated with statins in animal models of human disease in the absence of serum cholesterol reductions. Furthermore, these effects were apparently mediated by enhanced endothelium-derived NO.

Although we have previously demonstrated cardioprotective effects with other statins, it is unclear whether these effects would be shared with a novel, chemically distinct member of the statin family. Rosuvastatin is a synthetic statin that is currently in clinical trials for the treatment of hypercholesterolemia in man. Preliminary clinical evidence has indicated that rosuvastatin may be the most potent and efficacious statin to date (9). However, at present it is unknown whether this agent can upregulate endothelial cell eNOS function and enhance NO production. Alternatively, it is also possible that rosuvastatin can exert a greater degree of cardioprotection by greater eNOS stimulation and NO production compared to other statins. This issue is of vital...
importance given that all statins are not equipotent in terms of low density lipoprotein cholesterol reduction. Finally, questions still remain regarding the potential toxicity of synthetic statins. Consequently, the purpose of the present study was to investigate the effects of a new HMG-CoA reductase inhibitor, rosuvastatin, on endothelial NO production and in the setting of myocardial ischemia and reperfusion.

**MATERIALS AND METHODS**

**Mice.** Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine) were used as wild-type mice in the present study. Endothelial NO synthase-deficient mice (10) were obtained from Dr. Paul L. Huang of Harvard University. Briefly, the eNOS gene was replaced with a targeting vector with 5' and 3' flanking regions of homology, which replaced the HindIII to SalI fragment that contains exons encoding the nicotinamide-adenine dinucleotide phosphate ribose and adenosine binding sites (amino acids 1010–1144). These mice are completely deficient of eNOS protein. All animal experiments complied with the Guide for the Care and Use of Laboratory Animals and with state and federal regulations. One hundred fifty wild-type mice were randomized to each of the myocardial ischemia-reperfusion protocols. Mice received intraperitoneal injections of either saline vehicle (200 μl) or rosuvastatin (Crestor, Astra-Zeneca, London, UK). The time course study utilized a fixed dose of 0.5 mg/kg rosuvastatin and various durations of pretreatment. The dose-response group was performed using various doses of rosuvastatin given 18 h prior to myocardial ischemia and reperfusion. The vehicle group for the time-course study consisted of mice given the saline vehicle at random times prior to myocardial ischemia.

**Myocardial ischemia-reperfusion protocol.** The surgical protocol and infarct size determinations were performed similar to methods described previously (7,8,11–14). Briefly, the mice were orally intubated with polyethylene-90 (PE-90) tubing and connected to a rodent ventilator (model 683, Harvard Apparatus, Holliston, Massachusetts). Core body temperature was maintained at 37°C using a rectal thermometer and infrared heating lamp. The left anterior descending coronary artery (LAD) was visualized and ligated with 7-0 silk suture. Ischemia was confirmed by the appearance of hypokinesis and pallor distal to the occlusion. After 30 min of LAD occlusion, the ligature was removed and reperfusion was visually confirmed. The chest wall was closed and mice were allowed to recover in a temperature-controlled area.

The following day (at the end of 24 h of reperfusion), a tracheostomy was performed and the mouse was connected to the respirator. The right common carotid artery was cannulated for Evans blue infusion. The LAD was ligated and Evans blue (1.5 ml of 1.0% solution) was retrogradely infused into the carotid artery catheter to delineate the ischemic from the nonischemic zones. Ex vivo incubation in 2,3,5-triphenyltetrazolium chloride for 5 min at 37°C allowed differentiation between the viable and necrotic areas of the myocardium previously rendered ischemic. Each of the (five) 1-mm-thick slices was weighed, and the areas of infarction, risk, and left ventricle were assessed using computer-assisted planimetry (NIH Image 1.57).

**Measurement of vascular NO release.** Nitric oxide release from mouse aortic tissue was quantified using an isolated polarographic NO probe (Iso-NO Mark II, World Precision Instruments, Sarasota, Florida) as described previously (7,8,15). Briefly, mice were injected with either saline vehicle (250 μl, intraperitoneal) or rosuvastatin (0.5 mg/kg, intraperitoneal). After 18 h, mice were sacrificed and the aortae were carefully removed and placed in ice-cold Krebs-Henseleit solution. The aortae were cleaned of any adherent fat or connective tissue. The aortae were pinned endothelium-up in 24-well culture plates. Each aorta was submerged in 1 ml of Krebs-Henseleit solution and allowed to equilibrate at 37°C. A standard curve was performed and NO release from aortic tissue was assessed from three portions of each aorta. The solution was then removed from each well and replaced with fresh Krebs-Henseleit solution along with the NO synthase inhibitor, N-G-nitro-L-arginine methyl ester (l-NAME) (100 μM). After 30 min, NO release was measured from three portions of each aorta.

**Hemodynamic measurements.** Eighteen hours after injection of 0.5 mg/kg rosuvastatin, a separate group of wild-type mice were subjected to hemodynamic determination. Heart rate and mean arterial blood pressure were assessed via a polyethylene catheter inserted into the right common carotid artery. Care was taken to avoid damaging or excessively manipulating the vagus nerve along the carotid artery. The carotid catheter was connected to a World Precision Instruments blood pressure transducer and monitor. The monitor interfaced with a MacLab and personal computer from which the heart rate and mean arterial blood pressure were acquired. The data were taken from at least 50 cardiac cycles per mouse.

**Determination of eNOS messenger ribonucleic acid (mRNA) levels.** The reverse transcription–polymerase chain reaction (RT-PCR) was performed on whole hearts from wild-type mice receiving either 0.5 mg/kg rosuvastatin or vehicle 18 h prior to harvesting and rapidly frozen in
liquid nitrogen. Total ribonucleic acid was extracted from mouse heart using the acid guanidium-phenol-chloroform extraction method (16), and RT-PCR was performed as described previously (17).

**Statistical analyses.** All data were subjected to analysis of variance with the Scheffe post hoc test or Student unpaired t test where appropriate. All values are reported as mean ± SEM. Statistical significance was set at p < 0.05.

**RESULTS**

**Effect of rosuvastatin on serum cholesterol levels and hemodynamics.** To ascertain whether rosuvastatin would acutely alter serum cholesterol levels, we injected wild-type mice with either 5 mg/kg rosuvastatin or saline vehicle 18 h before harvesting blood samples. The mice given saline vehicle (n = 8) had serum cholesterol levels of 60.8 ± 2.5 mg/dl, and mice receiving rosuvastatin had serum cholesterol levels of 62.1 ± 3.0 mg/dl. Consequently, rosuvastatin did not alter serum cholesterol levels in mice. We also assessed peripheral hemodynamics in the mice given either rosuvastatin or vehicle (Table 1). Mean arterial blood pressure, heart rate, and rate-pressure product did not differ significantly between the vehicle and rosuvastatin-treated groups.

**Rosuvastatin modulates eNOS mRNA.** The eNOS mRNA levels were assessed via RT-PCR on whole hearts from wild-type mice treated with either rosuvastatin (0.5 mg/kg) or vehicle 18 h before harvesting. Mice treated with rosuvastatin exhibited an approximately 50% increase in eNOS mRNA levels (Fig. 1).

**Induction of NO production by rosuvastatin.** Using a NO-specific electrode, we quantified the release of NO from the vascular endothelium of mice receiving either rosuvastatin (0.5 mg/kg) or saline vehicle (Fig. 2). Control vessels produced 24.1 ± 1.7 nM NO, whereas vessels from rosuvastatin-treated mice produced 38.8 ± 3.8 nM (p < 0.05 vs. control). Endothelial production of NO in both groups was virtually abolished by incubation with a high concentration of the NOS inhibitor L-NAME (5.6 ± 1.1 nM and 6.7 ± 1.5 nM, respectively). This finding also demonstrates that the NO being detected in our system is derived from NOS, further validating the system as a tool for authentic NO production.

**Temporal and dose-dependent effects of rosuvastatin on myocardial injury.** Using an 18-h pretreatment time point based on previous observations (7), mice were given various doses of rosuvastatin (0.1, 0.5, 1.0, 2.0, and 5.0 mg/kg). As shown in Figure 3A, all groups of mice exhibited similar areas-at-risk (AAR) for necrosis (NEC). Despite this similarity, attenuation of myocardial necrosis was observed with 0.5, 1.0, and 2.0 mg/kg but not 0.1 or 5.0 mg/kg of rosuvastatin (Fig. 3B). Survival was similar among all groups of mice (approximately 80%) except those given 5.0 mg/kg rosuvastatin (19%). The reason for this disparity was unclear, but it may be related to issues of toxicity in mice superimposed with the physiologic stress of a myocardial infarction (MI).

To establish a time course of possible cardioprotective effects by rosuvastatin, mice were treated with 0.5 mg/kg
Rosuvastatin immediately before initiating myocardial ischemia (0 h), 3 h before ischemia (3 h), or 6 h before ischemia (6 h). As shown in Figure 4A, all groups of mice had similar AAR for NEC. However, the data in Figure 4B demonstrate that rosuvastatin significantly attenuates myocardial injury in wild-type mice as soon as 6 h following injection. The cardioprotective effects are not evident if rosuvastatin is given at the time of ischemia (0 h) or 3 h prior to ischemia. The survival rates for these mice were not significantly different among the experimental groups (approximately 80%).

**Dependence of rosuvastatin on eNOS to attenuate myocardial injury.** Mice deficient in eNOS were given either saline or rosuvastatin (0.5 mg/kg) 18 h before onset of 30 min of myocardial ischemia followed by 24 h of reperfusion. As demonstrated in Figure 5, deficiency of eNOS abrogated the cardioprotective effects of rosuvastatin in mice. This finding indicates that the cardioprotective effects of rosuvastatin are dependent upon eNOS in this murine model of myocardial ischemia-reperfusion injury.

**DISCUSSION**

A number of recently developed therapeutic agents target the rate-limiting enzyme, HMG-CoA reductase, of endogenous cholesterol synthesis. These agents, commonly referred to as statins, significantly reduce serum cholesterol and triglyceride levels in man. Consequently, individuals taking statins have a decreased risk for coronary events. Many investigators and clinicians have assumed the cardiovascular benefits resulted solely from serum cholesterol reduction. More recent clinical and experimental observations indicate that statin treatment may impart cardiovascular effects that do not directly result from serum cholesterol reduction. In the present study, we provide compelling experimental mechanistic evidence to support these clinical
reduction, we presently demonstrate that rosuvastatin in-
family, rosuvastatin. In the absence of serum cholesterol
into a pleiotropic effect of a newer member of the statin
ation could be useful therapeutic agents.
emia, agents that protect or promote vascular NO produc-
eNOS in protecting the myocardium subsequent to isch-
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tion in animal models (25). Furthermore, complete
absence of eNOS exacerbates myocardial ischemia—
reperfusion injury in mice (33). Considering the vital role of
eNOS in protecting the myocardium subsequent to isch-
emia, agents that protect or promote vascular NO produc-
tion could be useful therapeutic agents.
Accordingly, the present study provides valuable insight
into a pleiotropic effect of a newer member of the statin
family, rosuvastatin. In the absence of serum cholesterol
reduction, we presently demonstrate that rosuvastatin in-
creases eNOS mRNA levels in the heart. In association with
enhanced mRNA levels, rosuvastatin significantly augments
the bioavailability of endothelial-derived NO. Although the
enhanced NO production was demonstrated using intact
vascular endothelium, the cell type(s) responsible for the
increase in myocardial eNOS mRNA levels is unknown.
Rosuvastatin may act upon endothelial cells, cardiac myo-
cytes, and/or interstitial fibroblasts. Our use of whole heart
homogenates precludes the determination of the cell type
involved. However, possible future investigations could
identify the source(s). Nevertheless, this enhancement of
NO production attenuates myocardial cell injury following
myocardial ischemia—reperfusion injury. Previous studies
have shown that other statins (simvastatin and pravastatin)
similarly enhance the production of NO from the vascular
endothelium and attenuate myocardial injury following
ischemia and reperfusion in normocholesterolemic (7), hy-
percholesterolemic (7), and diabetic (8) mice. However, it is
unclear whether these endothelial and cardioprotective ef-
fects are shared by other statins (e.g., atorvastatin, ceriva-
statin). This is especially important considering the highly
varied chemical structures of the many members of the
statin family.
Mechanisms of eNOS induction. Numerous studies have
addressed the possible mechanism(s) of enhanced eNOS
activity with statins. Two of the more common explana-
tions of this mechanism involve prolongation of eNOS mRNA
half-life and activation of the protein kinase Akt. One study
demonstrated that simvastatin can increase the half-life of
eNOS mRNA in endothelial cell cultures (4). We presently
report similar findings in blood vessels from mice treated
with rosuvastatin. This increase in eNOS mRNA can result
in elevated eNOS protein and ultimately increased NO
production, as demonstrated in the present study.
The second theory of the mechanism of enhanced NO
production by statins concerns activation of the protein
kinase Akt (5). Subsequent to Akt activation, eNOS be-
comes phosphorylated and thereby increases production of
NO. Unlike the effects on eNOS mRNA levels, which may
take hours, Akt activation can occur within minutes and
lead to a virtually immediate increase in eNOS activity. In
the present study, we do not demonstrate effects consistent
with this (early Akt) mechanism. However, our data do not
exclude the possibility of Akt activation in mouse hearts
following acute rosuvastatin administration. It is conceiv-
able that Akt does activate eNOS in our model. However,
activated eNOS protein may be irreversibly damaged upon
ischemia and reperfusion of the myocardium, thereby ob-
scuring the possible cardioprotective effects dependent upon
Akt activation. We cannot presently make any conclusions
concerning the role of Akt activation in the present model
of murine ischemia—reperfusion injury. Ultimately, it is
possible that both mechanisms work synergistically to po-
tentiate the cardioprotective actions of statins during isch-
emia and reperfusion. Nevertheless, further studies are
warranted to elucidate these possibilities.

Figure 5. The role of endothelial nitric oxide synthase (eNOS) in the
attenuation of myocardial ischemia—reperfusion injury following injection
with rosuvastatin. Mice deficient in eNOS were administered either saline
vehicle (n = 5) or 0.5 mg/kg rosuvastatin (n = 5) 18 h before being
subjected to myocardial ischemia and reperfusion. Rosuvastatin failed to
significantly (p = NS) alter the extent of myocardial injury in eNOS-
deficient mice. AAR = areas-at-risk; LV = left ventricle; NEC = necrosis.

findings using a new synthetic and highly potent HMG-
CoA reductase inhibitor in a murine model of ischemia—
reperfusion injury. This study may be especially timely
considering the fact that rosuvastatin is currently being used
in clinical trials.

NO and MI. Ischemia and subsequent reperfusion of the
myocardium induces numerous deleterious events that ulti-
mately contribute to cellular injury, ventricular dysfunction,
and possibly death (18,19). Of these injurious events,
depressed endothelial NO production may be a critical
component of vascular and myocardial injury. Nitric oxide
is a crucial mediator of cardiovascular homeostasis; it does so
by maintaining normal blood vessel tone (10,20), attenuat-
ing leukocyte-endothelial cell interactions (21,22), and de-
creasing platelet activity (20,23). Certain pathologic events
such as myocardial ischemia have been shown to signi-
ficantly depressed NO production of endothelium-derived
NO (24). In addition, it appears that depressed NO
production is a pivotal step in the mechanism of the develop-
ment of cardiovascular disease in the setting of
hypercholesterolemia and type II diabetes mellitus. Supple-
mentation with authentic NO or NO-donating agents
following myocardial ischemia—reperfusion injury signi-
ficantly attenuated cardiac injury and/or contractile dysfunc-
tion in animal models (25–32). Furthermore, complete
absence of eNOS exacerbates myocardial ischemia—
reperfusion injury in mice (33). Considering the vital role of
eNOS in protecting the myocardium subsequent to isch-
emia, agents that protect or promote vascular NO produc-
tion could be useful therapeutic agents.

Accordingly, the present study provides valuable insight
into a pleiotropic effect of a newer member of the statin
family, rosuvastatin. In the absence of serum cholesterol
reduction, we presently demonstrate that rosuvastatin in-
Potential factors contributing to cardioprotection. The cardioprotective effects of rosuvastatin observed in the present study could be explained by alterations in serum cholesterol levels. However, we presently exclude this possibility by demonstrating that acute administration of rosuvastatin did not alter serum cholesterol levels. This is not surprising given extensive clinical experience with statins that indicates cholesterol reduction is not an immediate event. Mice also have constitutively low serum cholesterol levels and are generally unresponsive to statin therapy. Furthermore, these findings are consistent with two previous reports that did not demonstrate an acute reduction of serum cholesterol levels in mice receiving statins (7,8).

One of the most salient findings of the present study is the ability of rosuvastatin to induce the formation of NO from the vasculature and attenuate postischemic myocardial injury. We found that the beneficial effects of rosuvastatin administration were time- and dose-dependent in the present murine model. Furthermore, our studies in the eNOS-deficient mice clearly demonstrate that the protective effects of rosuvastatin are dependent upon eNOS. Although the ultimate defensive mechanism of the enhanced NO presently described is unknown, many groups have reported a number of mechanisms by which NO attenuates postischemic myocardial injury in a number of animal models. One of the primary mechanisms involves anti-inflammatory effects of NO in the postischemic myocardium. Given the powerful vasorelaxing properties of NO (20), one may also argue that the cardioprotective effects presently reported result from decreased afterload via lowered blood pressure. Although this is an important issue, the level of increased NO production we presently report did not apparently alter the hemodynamic status of the mice. However, it is possible that a transient vasorelaxation occurred but was offset by reflex sympathetic activity. Furthermore, the outside possibility remains that rosuvastatin favorably affected the hemodynamics only during ischemia, thereby decreasing the ultimate injury to the myocardium. In addition, NO can also scavenge oxidants or affect myocardial metabolism. However, we can neither support nor discount any of the possible protective mechanisms of NO in the present study. It is hoped this important issue can be addressed in future studies.

Summary and conclusions. The present data reveal that a novel HMG-CoA reductase inhibitor, rosuvastatin, enhances NO production and attenuates myocardial ischemia–reperfusion injury in an eNOS-dependent manner. These beneficial effects were observed within 6 h of a single, clinically relevant dose of rosuvastatin and in the absence of serum cholesterol reduction. Our findings provide important evidence regarding direct vascular effects of this novel statin in the setting of acute MI. This study also emphasizes the vital role of endothelial-derived NO in the cardiovascular system and develops a foundation from which future studies may decipher the intracellular mechanisms of the ever-expanding role of statins in the pathophysiology of ischemic heart disease.

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