Effects of Hypercholesterolemia on Myocardial Ischemia-Reperfusion Injury in LDL Receptor–Deficient Mice

Wesley G. Girod, Steven P. Jones, Nola Sieber, Tak Yee Aw, David J. Lefer

Abstract—Hypercholesterolemia is a primary risk factor for atherosclerosis, coronary artery disease, and myocardial infarction. We subjected low density lipoprotein receptor–deficient (LDLr –/–) and control (wild-type) mice to 30 minutes of myocardial ischemia and 120 minutes of reperfusion. Myocardial infarction per area at risk (AAR) was noted under baseline conditions to be significantly \( (P < 0.05) \) smaller in the LDLr –/– mice compared with wild-type mice (24.7±3.2% and 38.8±4.3% of AAR, respectively). Subsequently, mice were fed a high-cholesterol diet (HCD) for 2 or 12 weeks, which resulted in significant increases in serum cholesterol levels in both LDLr –/– and wild-type groups. After 2 weeks of the HCD, the LDLr –/– mice demonstrated a significant elevation \( (P < 0.01) \) in myocardial necrosis per AAR (50.2±6.36% of AAR) compared with the normal-diet LDLr –/– group, whereas the short-term HCD-fed wild-type mice demonstrated no significant difference from baseline. In contrast, wild-type mice fed the HCD for 12 weeks revealed a significant \( (P < 0.05) \) decrease in necrosis per AAR, which was 22.5±3.2% of the AAR in comparison with that in the normal-diet wild-type mice (38.8±4.3% of AAR). LDLr –/– mice on the same long-term HCD showed a similar significantly \( (P < 0.05) \) decreased infarct size, which was 13.2±4.0% of the AAR. In additional experiments, we determined that myocardial tissue total glutathione (GSH) levels were reduced after 2 weeks of the HCD and were significantly increased after 12 weeks of the HCD in the LDLr –/– mouse heart. These data suggest that short-term cholesterol feeding renders the myocardium of LDLr –/– mice more susceptible to ischemia-reperfusion injury, whereas more long-term hypercholesterolemia confers cardioprotection in the LDLr –/– mouse heart. (Arterioscler Thromb Vasc Biol. 1999;19:2776-2781.)

Key Words: infarct ■ cholesterol ■ neutrophils ■ mutant mice

There is a large body of evidence indicating that the best-known risk factor for the development of coronary artery disease is elevated plasma lipid levels.1–3 Furthermore, a number of prospective studies have established that the risk of cardiac morbidity and mortality is directly related to the concentration of plasma cholesterol.2,3. Despite the development of a number of agents that effectively reduce serum cholesterol levels in patients, coronary atherosclerosis and subsequent myocardial infarction (MI) still represent a major health concern in our society. The majority of previous studies concerning the pathobiology of myocardial ischemia-reperfusion (MI-R) injury have been derived from animals that are otherwise normal. Although the data derived from these studies provide meaningful insights into the mechanisms of cellular injury after MI-R, the relevance of these findings to those human populations that are at greatest risk for developing myocardial reperfusion injury remains unclear.

Previous studies investigating the effects of MI-R in the setting of hypercholesterolemia have focused primarily on rabbit models of ischemia alone4,5 or on I-R.6–8 Osborne et al4 reported that creatine kinase release after 5 hours of regional ischemia was significantly greater in rabbits fed a high-cholesterol diet (HCD) for 10 to 12 weeks. Furthermore, a subsequent study5 of MI in hypercholesterolemic rabbits reported an increased severity of myocardial tissue injury that was significantly reversed by treatment with the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor lovastatin. More recently, hypercholesterolemic rabbits have been subjected to MI-R injury.6–8 Golino and colleagues6 demonstrated that MI size was dramatically increased in rabbits subjected to I-R after only 3 days of cholesterol feeding. It was also reported7 that platelet depletion markedly reduced MI size and the extent of no-reflow in rabbits fed a 2.0% cholesterol diet for 3 days. Enhanced MI-R injury in the setting of hypercholesterolemia was also demonstrated in isolated rabbit hearts after global I-R.8

In recent years, a number of mutant mice have been developed in which genes that regulate lipoprotein metabolism and circulating cholesterol levels have been manipu-
lated. The LDL receptor knockout (LDLr−/−) mouse is one of the earliest gene-targeted strains developed for atherosclerosis research. The LDLr−/− mouse closely resembles the condition of familial hypercholesterolemia in humans and has been widely utilized for experimental studies of hypercholesterolemia and how this genetic alteration leads to atherosclerosis.9–11 The LDLr−/− mouse develops profound hypercholesterolemia and arterial lesions when placed on a high-fat diet.9,10 Thus, the LDLr−/− mouse appears to be an ideal animal model for the investigation of the effects of hypercholesterolemia on MI. In the present study, we investigated the effects of acute and prolonged hypercholesterolemia on MI-R injury in both wild-type and LDLr−/− mice.

Methods

Transgenic Mice

This study focused on the LDL receptor–deficient (ie, LDLr−/−) mouse as an animal model of hypercholesterolemia. Male LDLr−/− mice, as previously described,9 and male wild-type (C57BL/6) mice (age and weight matched) were purchased from Jackson Laboratories (Bar Harbor, Me). All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services publication No. (NIH) 86-23, revised 1985. Animal Resources Program, DRR/NIH, Bethesda, Md), approved by the Council of the American Physiology Society, and with federal and state regulations. All experimental procedures were approved by the Louisiana State University Medical Center Animal Care and Use Committee.

LDLr−/− (n=9) and wild-type (n=10) mice were maintained on standard laboratory rodent chow No. 8640 (normal diet; ND) purchased from Harlan Teklad. A second group of LDLr−/− (n=6) and wild-type (n=6) mice were placed on an HCD (Teklad 90221, Harlan Teklad) containing 1.25% cholesterol and 15.8% fat for 2 weeks, and a third group of LDLr−/− (n=6) and wild type (n=6) mice were fed the same HCD but for a longer time (12 weeks).

Surgical Procedures

Animals were initially anesthetized with sodium pentobarbital (100 mg/kg IP) before any surgical procedure. Anesthesia was maintained via supplemental doses of sodium pentobarbital (30 mg/kg IP) as needed. Mice were secured to the operating table by taping the extremities. A 4-0 silk ligature was placed behind the upper incisors and tied tautly to extend the neck. A midline incision was made from the xiphoid process to the submentum. The salivary glands were separated from the midline to allow access to the trachea. A tracheotomy was then performed and a section of polyethylene-90 tubing was inserted into the animal’s trachea and connected via a loose junction to a Harvard respirator (model 683 rodent respirator, Harvard Apparatus). The respirator’s tidal volume was set at 1.4 mL/min and the rate at 120 strokes/min, and it was supplemented with 100% O2. The right carotid artery was then cannulated with polyethylene-10 tubing to monitor mean arterial pressure. The arterial cannula was connected to a blood pressure transducer and a BP-1 (World Precision Instruments) blood pressure monitor.

After an equilibration period of 10 minutes, a thoracotomy was performed. With the use of electrocautery (model 100, Geiger Instrument Co, Inc), an incision was made to the left of the sternum. The pericardial sac was then removed. Ligation of the left anterior descending (LAD) coronary artery was performed using a 7-0 silk suture attached to a BV-1 needle (Ethicon, Inc). A small piece of polyethylene tubing was used to secure the ligature without damaging the artery. The chest wall was approximated and covered with Parafilm wax paper to prevent desiccation.

MI-R Protocols

The protocol for the MI-R experiments is depicted in Figure 1. One group each of wild-type mice on the ND (n=10) and of LDLr−/− mice also on the ND (n=9) was subjected to 30 minutes of LAD coronary artery occlusion and 120 minutes of reperfusion. In subsequent studies, a second group of wild-type (n=6) and LDLr−/− (n=6) mice was subjected to 2 weeks of the HCD before undergoing 30 minutes of LAD occlusion and 120 minutes of reperfusion. A third group of wild-type (n=6) and LDLr−/− (n=6) mice was subjected to 12 weeks of the HCD before being placed through the same MI-R model as the previous groups.

Determination of Area at Risk and Infarct Size

At the conclusion of the 2-hour period of reperfusion, the LAD was religated with 7-0 silk sutures. Evans blue dye (1.3 mL of a 1.0% solution, Sigma Chemical Co) was retrogradely injected into the carotid artery catheter to delineate the in vivo area at risk (AAR). The heart was excised and fixed in a 1.5% solution of agarose gel (Seaplaque, FMC BioProducts). After the gel had solidified, the heart was sectioned perpendicular to the long axis in 1-mm slices by using a McIlwain tissue chopper (Brinkman Instruments Inc). The 1-mm slices were placed in individual wells of a 6-well cell-culture plate (Cell Wells, Corning Glass Works) with the basal side exposed. Each slice was then counterstained with 3.0 mL of 1.0% 2,3,5-triphenyltetrazolium chloride (Sigma) solution for 5 minutes at 37°C. The right ventricle was excised and each slice was weighed and visualized under an Olympus SZ4045 (Olympus America Inc) dissecting microscope equipped with a Sony CCD Iris color video camera (Sony Electronics Inc). The left ventricular area, AAR, and area of infarction for each slice were then determined by computer planimetry using National Institutes of Health Image (version 1.57) software. The size of the MI was determined by the following previously described equation: weight of infarction=(A×WT1)+(A×WT2)+(A×WT3)+(A×WT4)+(A×WT5), where A is the percent of infarcted area as determined by planimetry from 5 sections numbered 1 to 5, and WTi is the weight of the corresponding numbered section.

Hematology of Peripheral Blood

Total white blood cell, neutrophil, and platelet counts were performed by LabCorp, Inc (BioVet Division). Whole-blood samples from LDLr−/− ND (n=6), 2-week HCD (n=6), and 12-week HCD (n=7) and from wild-type ND (n=12), 2-week HCD (n=7), and 12-week HCD (n=5) mice were obtained from the carotid artery and collected into pediatric (≤1 mL) lavender-topped, evacuated, EDTA-containing tubes (Microtainers, Becton Dickinson and Co).

Cholesterol Measurement

Blood was obtained from wild-type mice on the ND (n=12), the 2-week HCD (n=7), and the 12-week HCD (n=5) as well as from LDLr−/− mice fed an ND (n=6), a 2-week HCD (n=6), and a 12-week HCD (n=7) through a polyethylene-10 tubing cannula placed in the right common carotid artery. Once collected, blood was centrifuged in an Eppendorf microtube at 14 000 rpm for 10 minutes to isolate the plasma. Total, LDL, and HDL cholesterol fractions were assayed using enzymatic determination kits from Sigma Diag-
TABLE 1. Serum Lipid Levels for Wild-Type and LDLr –/– Mice

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cholesterol, mg/dL</th>
<th>Triglycerides, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>77±3</td>
<td>23±9</td>
</tr>
<tr>
<td>2-wk HCD</td>
<td>144±9*</td>
<td>25±17</td>
</tr>
<tr>
<td>12-wk HCD</td>
<td>295±18*</td>
<td>284±105†</td>
</tr>
<tr>
<td>LDLr –/–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>358±13</td>
<td>495±52</td>
</tr>
<tr>
<td>2-wk HCD</td>
<td>2479±213‡</td>
<td>5397±784‡</td>
</tr>
<tr>
<td>12-wk HCD</td>
<td>3180±70‡</td>
<td>4964±735‡</td>
</tr>
</tbody>
</table>

Mice were maintained under ND, 2 weeks of an HCD, or 12 weeks of an HCD conditions. 

*P<0.05 vs wild-type ND. 
†P<0.01 vs wild-type ND. 
‡P<0.01 vs LDLr –/– ND.

Neutrophil Accumulation

Midventricular tissue slices (1 mm in thickness) were prepared from all of the hearts subjected to the MI-R protocol after the completion of all experimental procedures. The tissue sections were immediately fixed and stored in a 10% neutral buffered formalin solution (Sigma Diagnostics). The tissue slices were paraffin embedded, cut into 10-μm sections, and placed on slides. The tissue specimens were then stained with Gill’s No. 3 hematoxylin and eosin. The slides were then viewed microscopically, and the number of neutrophils (polymorphonuclear cells) per high-power field was determined. For each of the hearts examined, the number of neutrophils was counted in 6 fields of 3 separate tissue sections.

Myocardial Tissue Glutathione Measurement

In additional experiments, wild-type (C57BL/6) and LDLr –/– mice were fed an ND (n=5 each for wild type and n=6 for LDLr –/–) or the HCD for 2 weeks (n=9 for wild type and n=6 for LDLr –/– mice) or 12 weeks (n=8 for wild type and n=7 for LDLr –/– mice). Mice were anesthetized with sodium pentobarbital (100 mg/kg IP), and the hearts were excised and rapidly snap-frozen in liquid nitrogen. The heart tissue was derivatized with iodoacetic acid and Sanger’s reagent, and total glutathione (GSH) levels were quantified by high-performance liquid chromatography as described previously. 13

Statistical Analyses

The infarct size, AAR, left ventricle size, leukocyte counts, cholesterol levels, and hemodynamic data were analyzed with an ANOVA coupled with post hoc analysis with Scheffe’s test for significance. All statistics were calculated with StatView 4.5 (Abacus Concepts). All values are reported as mean±SEM. Statistical significance was set at P<0.05.

Results

Plasma Lipid Levels

Plasma total cholesterol and triglyceride levels for wild-type and LDLr –/– mice are presented in Table 1.

Circulating Blood Cells

Data for circulating white blood cells and neutrophils are presented in Table 2. High-cholesterol feeding for 12 weeks’ duration resulted in a significant (P<0.05) increase in the number of circulating white blood cells, neutrophils, and platelets in wild-type mice compared with baseline conditions. Similarly, hypercholesterolemia significantly (P<0.05) increased the number of white blood cells and neutrophils at 12 weeks after initiation of the HCD when compared with baseline. In contrast, circulating platelet levels were significantly (P<0.05) reduced in the LDLr –/– mice after 12 weeks of high-cholesterol feeding.

Hemodynamic Data

Mean arterial blood pressure and heart rate were recorded throughout the infarct size determination protocol, and the rate-pressure product was calculated. Summary hemodynamic data for wild-type mice are presented in Table 3, and LDLr –/– hemodynamic data are also presented in Table 3. There were no significant differences in heart rate, blood pressure, or rate-pressure product at any time during the experimental protocol in wild-type mice. In LDLr –/– mice, heart rate was significantly (P<0.05) increased in the 2-week HCD group at baseline compared with the ND group. Heart rate was also significantly higher at 120 minutes of reperfusion in the ND and the 2-week HCD groups compared with the 12-week HCD mice. In addition, the rate-pressure product was significantly less in the 12-week HCD group compared with the ND group at 30 minutes of ischemia.

Myocardial Neutrophil Accumulation

Neutrophil infiltration into the I-R myocardium was determined in wild-type (Figure 2A) and LDLr –/– (Figure 2B) animals. Neutrophil accumulation induced by MI-R was unchanged in wild-type mice after 2 weeks of high-cholesterol feeding and was significantly (P<0.05) reduced after 12 weeks of an HCD. After 2 weeks of an HCD, the extent of neutrophil infiltration into the I-R myocardium was significantly (P<0.01) increased in LDLr –/– mice. In contrast, postischemic neutrophil infiltration into the ischemic zone returned to levels significantly (P<0.05) lower than baseline in the LDLr –/– animals after 12 weeks of high-cholesterol feeding.
In wild-type animals (Figure 4A), we observed a significant ($P<0.05$) reduction in total GSH levels 2 weeks after initiation of an HCD in the LDLr $^{-/-}$ mouse heart. Continued HCD feeding of LDLr $^{-/-}$ mice for 12 weeks attenuated the infarct size to 13.2$\pm$4.0% of the AAR ($P<0.05$ versus ND LDLr $^{-/-}$).

Myocardial AAR and Infarct Size

Figure 3A represents AAR data for the left ventricle and infarct size in wild-type mice fed an ND, a 2-week HCD, and a 12-week HCD. We observed no significant differences in the size of the myocar-dial AAR placed at risk by coronary artery occlusion. There was no significant difference in infarct size between ND wild-type hearts (38.8$\pm$4.3%) and 2-week-HCD wild-type hearts (42.6$\pm$3.9%). However, 12-week-HCD wild-type hearts (22.5$\pm$3.2%) presented significantly ($P<0.05$) smaller areas of necrosis per AAR compared with ND wild-type hearts (38.8$\pm$4.3%).

AAR and infarct data for LDLr $^{-/-}$ mice are presented in Figure 3B. Although all 3 groups of LDLr $^{-/-}$ hearts experienced similarly sized areas of ischemia (AAR per left ventricle), 2 weeks of high-cholesterol feeding resulted in significantly ($P<0.01$) larger infarcts (50.2$\pm$5.4% of the AAR) compared with the ND LDLr $^{-/-}$ hearts (24.7$\pm$3.2% of the AAR). Continued HCD feeding of LDLr $^{-/-}$ mice for 12 weeks attenuated the infarct size to 13.2$\pm$4.0% of the AAR ($P<0.05$ versus ND LDLr $^{-/-}$).

Myocardial Tissue GSH Levels

In additional studies, myocardial GSH levels were measured as an index of the oxidant status of the hearts. These data are depicted in Figure 4. In wild-type animals (Figure 4A), subject to either 2 or 12 weeks of an HCD, we did not observe any significant changes in GSH levels. In contrast, we observed a significant ($P<0.05$) reduction in total GSH levels 2 weeks after initiation of an HCD in the LDLr $^{-/-}$ hearts (Figure 4B). Furthermore, with prolonged hypercholesterolemia (to 12 weeks in duration), we observed a significant ($P<0.05$) increase in total GSH levels in the LDLr $^{-/-}$ mouse heart.

**Discussion**

Elevated serum cholesterol has long been associated with an increased risk for coronary artery disease and the develop-
In the present study, we examined the extent of myocardial tissue injury in hypercholesterolemic murine hearts subjected to acute MI-R. We investigated MI-R injury in both wild-type and LDLr –/– mice fed either an ND, 2 weeks of an HCD, or 12 weeks of an HCD. Myocardial total GSH levels remained unchanged in wild-type animals after either 2 or 12 weeks of high-cholesterol feeding. In the LDLr –/– animals, total GSH levels were significantly reduced at 2 weeks of the HCD and were significantly elevated at 12 weeks of the HCD.

Figure 4. Myocardial tissue GSH levels (nmol/g tissue) in wild-type (A) and LDLr –/– (B) mice fed either an ND, 2 weeks of an HCD, or 12 weeks of an HCD. Myocardial total GSH levels were measured in normocholesterolemic and hypercholesterolemic animals. These data may offer support to the notion that species differences between the mouse and rabbit might help to reconcile some of the differences that were noted.

An additional finding of interest in the present study is the response of both wild-type and LDLr –/– mice to MI-R at 2 weeks of an HCD and a significant reduction in myocardial necrosis after 12 weeks. This result is somewhat unexpected, in light of the fact that serum cholesterol levels are markedly elevated in LDLr –/– mice compared with wild-type mice when both animals are fed an ND.

An additional finding of interest in the present study is the response of both wild-type and LDLr –/– mice to MI-R at 2 weeks of an HCD and a significant reduction in myocardial necrosis after 12 weeks. This result is in sharp contrast to the majority of previous studies regarding hypercholesterolemia.14–18 Previous reports have uniformly indicated that hypercholesterolemia results in larger infarct development in rabbit hearts exposed to ischemia5,7 or ischemia in combination with reperfusion.6–8 These results are somewhat contradictory to our observations, in that we observed significantly less reperfusion injury in the LDLr –/– mice compared with wild-type mice under baseline conditions. In addition, our studies of LDLr –/– mice indicate that more prolonged exposure to hypercholesterolemia actually protects the heart from reperfusion injury. It is conceivable that species differences between the mouse and rabbit might help to reconcile some of the differences that were observed.

More specifically, we observed that hypercholesterolemia in LDLr –/– mice induces a biphasic response to MI-R injury, in which the extent of infarction is elevated at 2 weeks and then significantly reduced at 12 weeks after initiation of the high-fat diet. This response is unique and does not parallel what has been reported previously in other studies. This may be due in part to the extremely high levels (ie, >2000 mg/dL) of cholesterol that are obtained in these mice after induction of a high-fat diet. In contrast, in previous rabbit studies, circulating cholesterol levels in hypercholesterolemic animals were increased to ~300 mg/dL, which is significantly lower than the cholesterol levels attained in the present study. Exposure to extremely high levels of circulating lipids may render the heart more tolerant to ischemia and reperfusion after more prolonged periods of time.

Additional experiments were performed in which the levels of myocardial tissue GSH were measured in normocholesterolemic and hypercholesterolemic animals. These data may...
provide insight into the oxidative stress of the myocardium in response to the various diet regimens. Our data indicate that exposure to acute hypercholesterolemia (ie, 2 weeks) significantly reduces total GSH levels in the LDLr –/– mice. This suggests that the defensive antioxidant enzyme levels of the heart have been reduced and that the cardiac myocytes may be more susceptible to myocardial reperfusion injury, which we observed as an increase in infarct size. In sharp contrast, more prolonged hypercholesterolemia (ie, 12 weeks) resulted in a significant increase in myocardial GSH levels in the LDLr –/– mouse, and this may be partially responsible for the reduction in myocardial infarct size that was observed. Thus, it may be possible that whereas acute hypercholesterolemia results in enhanced injury in the setting of MI-R, prolonged exposure to high circulating levels of cholesterol may serve to “precondition” the murine myocardium and reduce tissue injury resulting from subsequent I-R. Clearly, additional studies are required to fully elucidate the mechanisms responsible for this bimodal response in the LDLr –/– mouse. One might also speculate that understanding how hypercholesterolemia can actually protect the murine myocardium may lead to the development of novel therapeutic strategies for the treatment of MI in humans.

Acknowledgments

We gratefully acknowledge the technical support of Micah B. Strange. We thank DeRoyal Surgical (Powell, TN) and Ethicon Surgical (Somerville, NJ) for the generous donation of surgical supplies. This research was supported by National Institutes of Health, Lung, and Blood Institute Grant (T.A.) and DK43785 (T.A.). This research was also supported by National Institutes of Surgical (Somerville, NJ) for the generous donation of surgical aorta.

References

Effects of Hypercholesterolemia on Myocardial Ischemia-Reperfusion Injury in LDL Receptor–Deficient Mice
Wesley G. Giord, Steven P. Jones, Nola Sieber, Tak Yee Aw and David J. Lefer

doi: 10.1161/01.ATV.19.11.2776

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/19/11/2776

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/