Hypercholesterolemia Enhances Oxidant Production in Mesenteric Venules Exposed to Ischemia/Reperfusion

Iwao Kurose, Robert E. Wolf, Matthew B. Grisham, D. Neil Granger

Abstract—It has been shown that hypercholesterolemia (HCh) exaggerates the microvascular dysfunction that is elicited by ischemia and reperfusion (I/R). The objective of this study was to determine whether oxidants contribute to the exaggerated inflammatory responses and enhanced albumin leakage observed in HCh rat mesenteric venules exposed to I/R (10 minutes of ischemia and 30 minutes of reperfusion). Intravital videomicroscopy was used to quantify the number of adherent and emigrated leukocytes, albumin extravasation, platelet-leukocyte aggregation in postcapillary venules, and the degranulation of adjacent mast cells. Oxidation of the fluorochrome dihydrorhodamine 123 (DHR) was used to monitor oxidant production by venular endothelium. I/R was shown to elicit an increased DHR oxidation in venules of both control and HCh rats, with the latter group exhibiting a significantly larger response. Treatment with either oxypurinol or superoxide dismutase largely prevented the leukocyte recruitment, platelet-leukocyte aggregation, mast cell degranulation, and enhanced DHR oxidation elicited by I/R in HCh rats. The enhanced albumin leakage was reduced by superoxide dismutase but not by oxypurinol. These results indicate that HCh amplifies the oxidant stress elicited by I/R and that interventions that blunt the oxidant stress effectively attenuate the leukocyte, platelet, and mast cell activation that result from I/R. (Arterioscler Thromb Vasc Biol. 1998;18:1583-1588.)

Key Words: vascular permeability ■ platelet aggregation ■ mast cell degranulation ■ leukocyte–endothelial cell adhesion

Hypercholesterolemia (HCh) has been implicated in the pathophysiology of coronary artery disease and other regional ischemic disorders (eg, stroke and mesenteric ischemia). Elevated plasma cholesterol concentrations appear to contribute to both the initiation of ischemia as well as the subsequent propagation of ischemic damage.1,2 Although the association between high plasma cholesterol levels and ischemic vascular disease is well documented, the mechanisms by which HCh enhances ischemic tissue injury remain poorly defined. However, the findings of several recent studies suggest that HCh renders the microvasculature more vulnerable to inflammatory responses3 elicited by cytokines,4 lipid mediators,4,5 or ischemia/reperfusion (I/R).6,7 It is now well recognized that reperfusion of ischemic tissues leads to an injury response that profoundly affects the venous segment of the microcirculation. Postcapillary venules typically respond to I/R with the recruitment a large number of rolling, firmly adherent, and emigrating leukocytes; the formation of platelet-leukocyte aggregates; and an increased extravasation of albumin. These events are generally accompanied by the degranulation of perivenular mast cells and the enhanced formation of reactive oxygen species, the latter of which has been implicated as a major initiating stimulus for the aforementioned inflammatory responses.6 We have recently shown that the cell-cell adhesion, mast cell degranulation, and albumin leakage elicited in postcapillary venules by I/R are all greatly exaggerated in hypercholesterolemic (HCh) animals.7 On the basis of these observations, we speculated that HCh may lead to an enhanced production of inflammatory mediators that results from a more profound oxidant stress in postcapillary venules after I/R.

The objectives of the current study were to (1) determine whether the oxidant stress elicited in postcapillary venules by I/R is altered by HCh and (2) define the contribution of xanthine oxidase (XO)–derived superoxide to the exaggerated leukocyte–endothelial cell adhesion, platelet-leukocyte aggregation, mast cell degranulation, and albumin extravasation observed in postcapillary venules of HCh rats exposed to I/R. These issues were addressed by applying intravital microscopic techniques to intact mesenteric venules of normal rats and rats rendered HCh by a 2-week dietary regimen. Oxidant production by postischemic venules was quantified from the oxidation of dihydrorhodamine 123 (DHR), an oxidant-sensitive fluorochrome previously applied to studies of the mesenteric microcirculation.10 Oxypurinol and superoxide dismutase (SOD) treatments were used to assess the contributions of XO and superoxide, respectively.

Methods

Male Sprague-Dawley rats (110 to 135 g; Harlan Laboratories, Frederick, Md) were maintained on a purified laboratory diet (normal-diet rats) or an HCh diet (HCh rats) containing 1% cholesterol, 0.2% cholic acid, and 2.5% olive oil for 2 weeks,7 which

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resulted in cholesterol levels of 73.7±1.8 mg/dL or 343.7±21.8 mg/dL, respectively.

**Surgical Procedure**

The animals were initially anesthetized with pentobarbital (65 mg/kg body weight), and then a tracheotomy was performed to facilitate breathing during the experiment. The right carotid artery was cannulated and systemic arterial pressure was measured with a Statham P23A pressure transducer connected to the carotid artery cannula. Systemic blood pressure and heart rate were continuously recorded with a Grass physiological recorder (Grass Instruments). The left jugular vein and femoral artery were also cannulated for drug administration. All procedures involving the use of animals were approved by and in accordance with the guidelines of the Louisiana State University Medical Center Animal Care and Use Resources Committee.

**Intravitral Microscopy**

Rats were placed in a supine position on an adjustable acrylic microscope stage, and the mesentery was prepared for microscopic observation as described previously.3,10,11 In brief, the mesentery was draped over a nonfluorescent coverslip that allowed for observation of a 2-cm² segment of tissue. The exposed bowel wall was covered with Saran Wrap (Dow Chemical Co), and then the mesentery was superfused with bicarbonate-buffered saline (37°C, pH 7.4) that was bubbled with a mixture of 5% CO₂ and 95% N₂. An inverted microscope (TM-D2S, Diaphot, Nikon) with a 40× objective lens (Fluor, Nikon) was used to observe the mesenteric microcirculation. The mesentery was transilluminated with a 12-V, 100-W, DC-stabilized light source. A video camera (VK-C150, Hitachi) mounted on the microscope projected the image onto a color monitor (PVM-2030, Sony), and the images were recorded with a video cassette recorder (NV8950, Panasonic). A video time-date generator (WJ810, Panasonic) projected the time, date, and stopwatch function onto the monitor.

Single, unbranched venules with diameters ranging between 25 and 35 μm and lengths >150 μm were selected for study. Venular diameter (Dv) was measured either on- or off-line with a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, Tex). Red blood cell (RBC) centerline velocity (Vmean) was measured in venules by using an optical Doppler velocimeter (NV8950, Panasonic). A video time-date generator (WJ810, Panasonic) projected the time, date, and stopwatch function onto the monitor. The number of adherent leukocytes was determined off-line during playback of videotape images. A leukocyte was considered to be adherent to venular endothelium if it remained stationary for a period equal to or >30 seconds. Adherent cells were expressed as the number per 100-μm length of venule. The number of emigrated leukocytes was also determined off-line during playback of videotaped images. Any interstitial leukocytes present in the mesentery at the onset of the experiment were subtracted from the total number of leukocytes that accumulated during the course of the experiment. Leukocyte emigration was expressed as the number per field of view surrounding the venule. Platelet-leukocyte aggregates that were visible within postcapillary venules were quantified and expressed as a percentage of luminal area occupied by aggregates.1 This calculation was carried out by using each microvascular image stored in a computer-assisted digital imaging processor (NIH Image 1.35 installed in a Macintosh computer).

To quantify albumin leakage across mesenteric venules, 50 mg/kg IV of FITC-labeled bovine albumin (Sigma Chemical Co) was administered to the animals 30 minutes before each experiment.10,12 Fluorescence intensity (excitation wavelength, 420 to 490 nm; emission wavelength, 520 nm) was detected by using a silicon-intensified target camera (C-2400-08, Hamamatsu Photonics). The fluorescence intensity of FITC-albumin within 3 segments of the venule under study (I₀) and in 3 contiguous areas of perivenular interstitium (Iᵢ) were measured at various times after administration of FITC-albumin by using the computer-assisted digital imaging processor. An index of vascular albumin leakage was determined from the ratio of Iᵢ to I₀, at specific times during the reperfusion phase.

The oxidant-sensitive fluorescence probe DHR (Molecular Probes, Inc)13 was added to the mesenteric superfusate (10 μmol/L) in some experiments to monitor oxidative stress. The fluorochrome was visualized by using the same microscope and image analysis system described above. DHR fluorescence intensity was monitored in a region of mesentery that was equivalent to twice the area of the venule under observation. An image processor was used to monitor fluorescence intensity just before (baseline value, Iₜ=0) and after exposure of the mesentery to I/R (Iₜ=x). The ratio of Iₜ=x to Iₜ=0 was used as an index of oxidant stress in mesenteric tissue. DHR is oxidized to the fluorescent rhodamine 123 (RH 123) by intracellular and extracellular reactive oxygen species, such as H₂O₂, superoxide anion, and hydroxyl radicals (DHR is more sensitive to H₂O₂ and secondary H₂O₂-dependent reaction products than to O₂⁻). Because RH 123 binds to the mitochondrial inner membrane, any RH 123 formed by the oxidation of DHR is stable within endothelial cells and other cell types.9 RH 123 is known to be a mitochondrial membrane potential–sensitive fluorescence probe11 that responds to mitochondrial ATP production. Therefore, in some experiments, RH 123 (Sigma) rather than DHR was superfused onto the mesenteric preparation (10 μmol/L) as a control group.

**Experimental Protocols**

After all parameters measured on-line were in a steady state, images from the mesenteric preparation were recorded on videotape for 10 minutes. Immediately thereafter, the superior mesenteric artery was ligated with a snare created from polyethylene tubing. On the basis of findings from a previous study,1 an ischemic period of 10 minutes (or 0 minutes for sham experiments) was used to elicit the acute inflammatory responses. Reperfusion was induced by gently removing the arterial ligature. All measured variables were recorded at 10 minutes for 30 minutes after the onset of reperfusion. In some experiments, SOD was injected intravascularly (local intra-arterial) to yield a final concentration of 15 000 U/kg (1 mg = 3000 U), and the same protocol was used.14 When SOD was administered, both renal pedicles were ligated to prevent excretion of the protein by the kidneys. In another series of experiments, oxypurinol (an XO inhibitor, 50 mg per rat) was administered by gavage at 12-hour intervals beginning 2 days before the experiment.14 The microvascular and inflammatory responses to these treatment regimens were compared between rats placed on normal and HCh diets by using the measurements obtained 30 minutes after reperfusion.

**Statistics**

The data were analyzed by standard statistical analysis, ie, 1-way ANOVA and Fisher’s (post hoc) test. All values are reported as mean±SE, from 6 to 9 rats, and statistical significance was set at P<0.05.

**Results**

The Table summarizes the differences in basal and postischemic (30 minutes) values for RBC velocity and wall shear rate in mesenteric venules of sham-operated rats placed on either a normal or an HCh diet. The basal values for these hemodynamic parameters did not differ between groups, and in both instances, 10 minutes of ischemia followed by 30 minutes of reperfusion resulted in lower values for venular RBC velocity and wall shear rate that did not differ between groups. Treatment with either SOD or oxypurinol effectively restored wall shear rate and RBC velocity to basal values after I/R in rats on the HCh diet. Except for wall shear rate after SOD treatment, the hemodynamic parameters were not sig-
significantly altered by the drug treatments in rats placed on a normal diet.

The changes in leukocyte–endothelial cell adhesion (adherence and emigration), platelet-leukocyte aggregation (luminal area occupied by platelets), and albumin leakage elicited in mesenteric venules of normal and HCh rats after I/R were similar to those reported in a recent study from our laboratory.7 The results indicate that HCh results in an exacerbation of the number of adherent leukocytes, platelet-leukocyte aggregates, and degranulated mast cells, whereas similar responses are noted for leukocyte emigration and albumin leakage.

Figure 1 summarizes the time course of changes in DHR oxidation in mesenteric venules of rats on a normal or HCh diet after I/R. In both groups of animals, DHR oxidation was unaffected by ischemia alone. However, a progressive increase in oxidant production was noted after reperfusion in both groups of animals, such that a 6.3±1.4-fold increase was detected at 30 minutes after reperfusion in rats placed on a normal diet and a 12.1±3.6-fold increase in rats on an HCh diet. Nonischemic rat mesentery superfused for 50 minutes with DHR exhibited an ∼50% increase in spontaneous DHR oxidation. These findings indicate that HCh leads to a more intense I/R-induced oxidant stress in rat mesenteric venules.

Figure 2 summarizes the effects of treatment with either SOD or oxypurinol on I/R-induced recruitment of adherent and emigrated leukocytes in postcapillary venules of rats placed on a normal or HCh diet. The upper panel illustrates that HCh rats exhibited a more profound recruitment of adherent leukocytes than did normal rats and that treatment with either SOD or oxypurinol effectively blocked the I/R-induced leukocyte adhesion in both normal and HCh animals. These findings indicate that this exacerbated component of the inflammatory response in HCh rats involves an exaggeration of the same mechanisms that induce I/R-induced leukocyte adhesion in normal rats. The lower panel of Figure 2 illustrates that although the number of emigrated leukocytes observed after I/R did not differ between normal and HCh rats, both SOD and oxypurinol were effective in preventing the emigration process in both groups.

Figure 3 demonstrates that I/R elicited more profound increases in platelet-leukocyte aggregation and degranulated mast cells in HCh rats than in their normocholesterolemic counterparts. The data also show that the exaggerated platelet-leukocyte aggregation and mast cell degranulation observed in HCh rats are significantly blunted after treatment with either SOD or oxypurinol.

Figure 4 summarizes the changes in venular albumin leakage and DHR oxidation induced by I/R in normal and HCh rats. As shown previously,7 10 minutes of ischemia...
followed by 30 minutes of reperfusion results in a comparable albumin leakage response in both normal and HCh rats. We noted, however, that although SOD was equally effective in attenuating the leakage response in both groups of animals, oxypurinol exerted a beneficial effect in the normocholesterolemic rats only. DHR oxidation, a measure of oxidant stress in mesenteric venules, was more profoundly affected by I/R in HCh rats. Both SOD and oxypurinol were effective in blunting the oxidant stress elicited by I/R in normal as well as HCh rats.

Discussion
The results of the current study support evidence already in the literature that indicates that HCh results in an exaggerated inflammatory response to various stimuli. For example, the HCh associated with LDL receptor knockout mice appears to exacerbate the leukocyte–endothelial cell adhesion elicited in postcapillary venules by lipid mediators (platelet-activating factor or leukotriene B4) or tumor necrosis factor, when compared with the responses detected in wild-type mice. Furthermore, we have previously shown, using the same experimental model as in the current study, that I/R elicits a more profound inflammatory response, characterized by increased leukocyte adherence and emigration and platelet-leukocyte aggregation, in HCh rats than in their normocholesterolemic counterparts. The results of the current study extend our previous experience with this model of HCh by providing novel information with important implications concerning the potential role of oxidants in mediating the exaggerated inflammatory responses to I/R.

One of the major objectives of this study was to determine whether HCh animals exhibit an oxidant stress in postcapillary venules exposed to I/R. This objective appeared to have merit based on 2 lines of evidence already in the literature: (1) enhanced oxygen-radical production has been implicated as an initiator of the leukocyte recruitment in postcapillary venules of normocholesterolemic animals and (2) an enhanced superoxide production has been demonstrated in arterial vessels derived from HCh animals. Because mechanical denudation of endothelial cells from these vessels resulted in a reduction of superoxide production, it was proposed that endothelial cells are the major source of reactive oxygen metabolites in these vessels. A dependence of the enhanced superoxide production on the elevated serum cholesterol level is supported by the observation that dietary correction of the HCh in rabbits restores superoxide production to normal levels in the isolated arterial vessels. The results of the current study provide evidence for an enhanced oxidant production by the venous segment of the microcirculation in HCh animals. Although the previous studies on arterial vessels indicate that there is an elevated production of superoxide by endothelial cells under basal conditions, our findings suggest that the exaggerated oxidant stress of HCh that can be detected in postcapillary venules is manifested after I/R, but not under basal conditions.

The ability of SOD to attenuate the increased DHR oxidation in postischemic venules of HCh animals suggests
that superoxide or a superoxide-derived oxidant is generated by venular endothelial cells. This contrasts with results obtained from venules exposed to inhibition of NO synthase (N^\text{G-}\text{nitro-}L\text{-arginine methyl ester induced}), wherein SOD has no effect on the increased DHR oxidation, but H_2O_2-directed interventions do have a profound effect on modifying the N^\text{G-}\text{nitro-}L\text{-arginine methyl ester–induced oxidant stress.}^{10,19} Hence, SOD does not appear to exert this inhibitory action on DHR oxidation in other models of venular oxidant stress.

The biochemical processes that contribute to the elevated oxidant production in HCh remain poorly defined. However, the work on arterial vessels has focused on NO-superoxide interactions and the potential influence of an imbalance between these 2 free-radical species on the redox status of the vessel. Indeed, this imbalance has been invoked as an explanation for the diminished NO-dependent vasodilator response to acetylcholine observed in HCh animals and human subjects. Because superoxide is known to avidly react with (and inactivate) NO, it is conceivable that an impaired ability of endothelial cells to produce NO (both under basal conditions and after I/R) could account for the excessive generation of superoxide in HCh microvessels. Inasmuch as arterial vessels are exposed to higher shear stresses than are their venous counterparts and shear is a major stimulus for NO generation by endothelial cells, one might expect the oxidant stress to be more readily manifested in arterial vessels.

An alternative explanation for the exaggerated oxidant production observed in postischemic venules of HCh rats is activation of an oxygen radical–producing enzyme that is localized in endothelial cells. The enzyme XO represents a viable candidate in this regard, because endothelial cells are a rich source of the enzyme and it is activated in endothelial cells by I/R (hypoxia/reoxygenation).^{20,21} Our findings confirm the role of this enzyme in the generation of oxidants by postcapillary venules after I/R in otherwise normal animals, because the enhanced oxidant stress (detected by DHR oxidation) elicited by I/R was largely abolished by treatment with oxypurinol. Of potentially greater significance is the novel observation that oxypurinol treatment was also effective in preventing the exaggerated oxidant stress induced by I/R in HCh rats. The latter observation suggests that activation of XO-mediated production of reactive oxygen metabolites represents the principal mechanism for the oxidant stress detected in postischemic venules of HCh animals.

XO activation has been previously invoked to explain the elevated basal production of superoxide in arterial vessels derived from HCh animals. White and associates\textsuperscript{22} have shown that the enhanced superoxide production in arterial rings from HCh rabbits is blunted by treatment of the vessels with either allopurinol or heparin, which competes with XO for binding to sulfated glycosaminoglycans on endothelial cells. These authors also noted that the circulating level of XO is elevated 2-fold in plasma of HCh animals, and they proposed that HCh induces the release of XO into the circulation, where it binds to endothelial cell glycosaminoglycans and produces superoxide. The functional significance of XO activation in HCh arterial vessels is exemplified by the observation that the blunted NO-dependent vasodilator re-

\textbf{Response to acetylcholine observed in HCh animals and human subjects.\textsuperscript{15,18,19} Because superoxide is known to avidly react with (and inactivate) NO,\textsuperscript{20} it is conceivable that an impaired ability of endothelial cells to produce NO (both under basal conditions and after I/R) could account for the excessive generation of superoxide in HCh microvessels. Inasmuch as arterial vessels are exposed to higher shear stresses than are their venous counterparts and shear is a major stimulus for NO generation by endothelial cells, one might expect the oxidant stress to be more readily manifested in arterial vessels.

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\textbf{References}

1. Osborne JA, Mentley RK, Lefer AM. Increased severity of acute myo-
2. Golino P, Maroko PR, Carew TE. The effect of acute hypercholesterol-
4. Henninger DD, Gerritsen ME, Granger DN. Low-density lipoprotein
11. Kurose I, Anderson DC, Miyasaka T, Tamatani T, Paulson JC, Todd RF, Rusche JR, Granger DN. Molecular determinants of reperfusion-induced oxidant production in arterial vessels after I/R.

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