Ischemia-reperfusion induced microvascular responses in LDL-receptor \(-/-\) mice

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Mori, Naoharu, Yoshinori Horie, Mary E. Gerritsen, and D. Neil Granger. Ischemia-reperfusion induced microvascular responses in LDL-receptor \(-/-\) mice. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1647–H1654, 1999.—The objective of this study was to determine whether the microvascular responses to ischemia and reperfusion (I/R) are altered in an animal model of atherosclerosis, the low-density lipoprotein-receptor knockout (LDLr \(-/-\)) mouse. Intravital video microscopy was used to monitor venular wall shear rate, leukocytes rolling velocity, the number of rolling, adherent and emigrated leukocytes, and albumin leakage in cremasteric postcapillary venules of wild-type (B6129) and LDLr \(-/-\) mice exposed to 60 min of ischemia and 60 min of reperfusion. The postcapillary venules of LDLr \(-/-\) mice exhibited two- to threefold larger increments in the number of adherent leukocytes and a more profound albumin leakage response to I/R than venules in wild-type mice. The exaggerated inflammatory responses noted in LDLr \(-/-\) mice placed on a normal diet were not exacerbated by a high-cholesterol diet. Treatment of LDLr \(-/-\) mice with either a platelet-activating factor (PAF) receptor antagonist (WEB-2086) or a monoclonal antibody (YN-1) against the endothelial cell adhesion molecule, intercellular adhesion molecule 1 (ICAM-1), markedly attenuated the I/R-induced leukocyte adherence and albumin leakage. These findings indicate that atherosclerotic mice are more vulnerable to the detrimental microvascular effects of I/R and that PAF-mediated, ICAM-1-dependent leukocyte adherence contributes to this exaggerated response to I/R.

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Atherosclerosis is often associated with coronary artery disease and other ischemic disorders. Hypercholesterolemia is one of the major risk factors for development of atherosclerosis. High plasma levels of low-density lipoprotein (LDL) and decreased high-density lipoprotein are correlated closely with accelerated atherogenesis. Because atherosclerotic lesions in major arteries typically manifest inflammatory cell infiltrates, enhanced cytokine production, and an increased expression of endothelial cell adhesion molecules, it has been proposed that atherosclerosis is a chronic inflammatory disease (7, 22, 23, 30). This contention is also supported by evidence derived from studies of the microcirculation. It has been shown that postcapillary venules of atherogenic mice (e.g., LDL-receptor knockouts) and hypercholesterolemic rats respond more intensely to acute inflammatory stimuli, such as lipid mediators [e.g., platelet-activating factor (PAF) and leukotriene B4 (LTB4)] and cytokines (e.g., tumor necrosis factor), with an exaggerated recruitment and activation of inflammatory cells (8, 11, 14, 15, 18).

Inflammatory cell recruitment is a cardinal histological feature of another circulatory disorder that can be precipitated by chronically elevated plasma cholesterol levels, i.e., ischemia and reperfusion (I/R). The sudden restoration of blood flow to a previously ischemic tissue, which occurs following organ transplantation, fibrinolytic therapy for myocardial or cerebral ischemia, and resuscitation after hemorrhagic shock, can elicit a cascade of events that results in endothelial cell dysfunction that is manifested as a decrement in endothelium-dependent vasodilation in arterioles, an accelerated rate of fluid filtration across capillaries, as well as leukocyte-endothelial cell adhesion and enhanced albumin leakage in postcapillary venules (9, 10). A number of mechanisms have been invoked to explain the altered endothelium-dependent responses in postischememic tissues, including increased oxygen radical formation, mediator release from adherent and activated neutrophils, and a reduction in the bioavailability of nitric oxide (9, 21). Although the mechanisms that mediate I/R-induced microvascular dysfunction remain controversial, there is growing evidence that the known risk factors for ischemic vascular diseases, such as diabetes, hypertension and hypercholesterolemia, can profoundly influence the microvascular responses to I/R. Diabetes mellitus, for example, is associated with exaggerated leukocyte-endothelial cell adhesion and albumin leakage responses of postcapillary venules to I/R (27). Similarly, rats made acutely hypercholesterolemic by placement on a high-fat diet for 2 wk exhibit exaggerated inflammatory responses in mesenteric venules exposed to I/R (18). Although the relevance of the latter observation to the pathobiology of atherogenesis remains unclear, there is some evidence suggesting that the microcirculation of atherogenic mice is hyperresponsive to inflammatory stimuli. We have previously demonstrated that the microcirculation of atherogenic mice is hyperresponsive to inflammatory stimuli. We have previously demonstrated that the numbers of rolling, adherent, and emigrating leukocytes in postcapillary venules of LDL-receptor knockout (LDLr \(-/-\)) mice are greater than in their wild-type counterparts after stimulation with either LTB4, PAF, or tumor necrosis factor-\(\alpha\) (11).

In the present study, the LDL-receptor knockout (LDLr \(-/-\)) mouse was employed to assess the influence of hypercholesterolemia on the inflammatory and microvascular responses to I/R. The LDLr \(-/-\) mouse closely resembles familial hypercholesterolemia in humans, and it is frequently used for studies of this naturally occurring genetic mutation that leads to atherosclerosis (13). Iasmuch as previous studies have
revealed that postcapillary venules of LDLr−/− mice are hyperresponsive to lipid mediators, such as PAF and LTB₄, as manifested by an increased intercellular adhesion molecule 1 (ICAM-1)-dependent leukocyte adhesion (11), we also assessed the contributions of PAF, leukotrienes, and ICAM-1 to the exaggerated inflammatory responses to I/R in LDLr−/− mice.

**MATERIALS AND METHODS**

Surgical procedure. Age-matched male B6129 (background strain for the LDLr−/− mice, n = 11) and LDLr−/− (n = 42) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained on one of two dietary regimens: 1) normal rodent chow (ND) for 4 wk or 2) 4 wk of high-cholesterol diet (HCD; Teklad 90221 containing 1.25% cholesterol and 15.8% fat, Harlan Teklad). The mice were anesthetized with ketamine hydrochloride (150 mg/kg body wt im) and xylazine (7.5 mg/kg body wt im). Anesthesia was maintained with supplemental doses of ketamine (15 mg/kg) as needed. The left jugular vein was cannulated for the administration of FITC-albumin. Systemic arterial pressure was measured using a pressure transducer (model P23A, Statham, Oxnard, CA) attached to a cannula inserted into the right carotid artery. Systemic arterial pressure was continuously monitored on a physiological recorder (Grass Instruments). Core body temperature was monitored with an intrarectal thermometer and maintained at 35.5 ± 0.5°C using an infrared lamp. Animal handling procedures were approved by the Louisiana State University Medical Center Institutional Animal Care and Use Committee and were in accordance with the guidelines of the American Physiological Society.

After placement of the mouse in the supine position on an adjustable Plexiglas microscope stage, the cremaster muscle was prepared as previously described (1, 2, 11) with minor modifications. Briefly, the right cremaster muscle was carefully dissected free of associated fascia, incised on its ventral surface, and spread over the viewing pedestal using peripherally attached sutures. The cremaster tissue was constantly perfused with PBS (8 ml/min, 35°C, pH 7.4).

Intravital microscopy. The cremaster muscle microvasculature was visualized using an intravital microscope (Optiphot, Nikon), ×20 objective lens (E plan 20/0.4, Nikon), and a multi-image module (Nikon). Transillumination of the tissue was provided with a 12-V, 50-W tungsten light source. A color video camera (VK-C150, Hitachi) mounted on the multi-image module projected the acquired images onto a color monitor (PVM-2030, Sony), whereas an interposed videocassette recorder (BR-s601MU, JVC) captured the images for off-line analysis. A videotime-date generator (WJ B10, Panasonic) projected the time, date, and clock function onto the on-line images.

Single-branched venules of 25 to 33 µm in diameter with a wall shear rate of >600 s⁻¹ were chosen for study. Venular diameter (Dᵥ) was measured on-line using a video caliper (Microcirculation Research Institute, Texas A & M University, College Station). An optical Doppler velocimeter (Microcirculation Research Institute) was also used on-line to determine centerline red blood cell velocity (V_rbc, mm/s). Calibration of the velocimeter was performed using a rotating glass disk coated with red blood cells. V_mean was calculated as V_mean equals V_rbc/Dᵥ. Shear rate was calculated as shear rate equals 8V_rbc/Dᵥ.

Rolling leukocyte flux was determined on- and off-line as the number of leukocytes per minute rolling past a specified point within the venule (over a 10-min period). Leukocyte rolling velocity (V_rol) was determined as the average time required for a leukocyte to transverse a 100-µm length of venule. A leukocyte was considered adherent to venular endothelium if it remained stationary ≥30 s (11). Adherent cells were expressed as the number per 100-µm length of venule. Emigrated leukocytes were determined on-line as the number of interstitial leukocytes in the field of view adjacent (within 30 µm) to the venule at the end of each recording period.

To quantify albumin leakage across venules, 50 mg/kg of FITC-labeled albumin (Sigma Chemical, St. Louis, MO) was administered intravenously to the animals 15 min before the baseline observation period (17). Fluorescence intensity (excitation wavelength 420–490 nm, emission wavelength 520 nm) was detected using a silicon-intensified target camera (C-2400-08, Hamamatsu Photonics, Shizuoka, Japan). The fluorescence intensities within a specified segment of the venule (Iᵥ) under study and in a contiguous area of perivenular interstitium (Iᵢ) were measured at various times after administration of FITC-albumin using a computer-assisted digital imaging processor (NIH Image 1.61 on a Macintosh computer). Vascular albumin leakage was given as the difference in fluorescence intensity between the outside and inside of the venular segment (1, 17, 19). An index of albumin leakage was determined from the Iᵢ/Iᵥ ratio at specific intervals during the course of the experiment.

Experimental protocols for intravital microscopy. After completion of the surgical preparation, the cremaster muscle was allowed to stabilize for 30 min. Preparations were considered acceptable when an appropriate-sized vessel maintained a shear rate of ≥600 s⁻¹ throughout the experiment. After recordings of all variables measured on-line (arterial pressure, V_rbc, and Dᵥ) were obtained under steady-state conditions, images from the cremaster muscle preparation were recorded for 10 min on videotape for subsequent analysis of leukocyte adherence, emigration, and rolling velocity. The cremaster muscles were then subjected to 60-min ischemia followed by 60-min reperfusion. Ischemia was achieved by occluding the primary artery and vein of the muscle with a small, atraumatic vascular clamp. Blood flow was restored by carefully removing the clamp. Video recordings and measurements of all parameters were repeated during minutes 20–30 and 50–60 of the reperfusion period. In some experiment, the animals were treated with either a monoclonal antibody (MAb) directed against ICAM-1 (YN-1; a rat IgG₂a, directed against mouse ICAM-1) (11), the PAF-receptor antagonist WEB-2086 (Boehringer) (19), or an orally active inhibitor of leukotriene synthesis, MK-886 (Merck Frosst, Pointe-Claire, Quebec, Canada) (5). The anti-ICAM-1 MAb (2 mg/kg) and WEB-2086 (10 mg/kg) were administered as a continuous infusion via the jugular vein from 15 min before release of ischemia until 30 min after reperfusion. MK-886 (10 mg/kg po) was given 2 h before the experiment.

Serum total cholesterol level. Blood samples were collected from the inferior vena cava after the 60-min reperfusion measurements were obtained. Total serum cholesterol concentration was enzymatically measured using a commercial kit (Sigma Chemical).

Statistical analysis. The data were analyzed using standard statistical analyses; i.e., one-way ANOVA and Scheffé's (post hoc) test. All values are reported as means ± SE, with at least 5 mice/group. Statistical significance was set at P < 0.05.

**RESULTS**

Plasma cholesterol levels obtained in each strain of mouse that was either maintained on ND or placed on
HCD for 4 wk are shown in Fig. 1. The LDLr−/− mice had threefold higher plasma cholesterol levels than the background (B6129) mice when placed on ND. However, profound hypercholesterolemia (>2,000 mg/dl) was noted in LDLr−/− mice on HCD for 4 wk.

In Fig. 2 the changes in shear rate and the numbers of adherent and emigrated leukocytes observed after I/R in cremaster venules of wild-type and LDLr−/− mice are shown. In all experimental groups, shear rate in the cremasteric venules were approximately 1,000 s⁻¹ under baseline conditions. In sham-operated (control) B6129 and LDLr−/− mice, shear rate showed no remarkable changes throughout the experimental period. Although shear rate was not significantly altered by I/R in B6129 mice, LDLr−/− mice placed on either ND or HCD exhibited a significant decline in shear rate after I/R. From previous studies on the influence of reduced shear rate on leukocyte adhesion in venules of cat and rat mesentery (21), it appears unlikely that the decline in shear rate after I/R noted in LDLr−/− mice accounts for the more profound recruitment of adherent leukocytes.

Ischemia followed by reperfusion resulted in an increased number of adherent leukocytes in postcapillary venules of both B6129 and LDLr−/− mice. Comparisons between groups revealed a significantly larger number of adherent leukocytes in LDLr−/− mice placed on either ND or HCD, compared with B6129 mice (Fig. 2B). Placement of LDLr−/− mice on HCD did not result in a further elevation in adherent leukocytes. However, a significant elevation in emigrated leukocytes was noted at 60 min in LDLr−/− mice placed on either ND or HCD (Fig. 2C).

In Fig. 3 the changes in leukocyte rolling velocity, flux of rolling leukocytes, and number of rolling leukocytes observed after I/R in cremaster venules of wild-type and LDLr−/− mice are summarized. Leukocyte rolling velocity and the number of rolling leukocytes were most notably affected by I/R in the two LDLr−/−
dietary (ND and HCD) groups, although statistical significance was not achieved for the number of rolling leukocytes at the 60-min observation period in LDLr−/− mice on HCD.

Figure 4 demonstrates that while 60 min of ischemia followed by 60 min of reperfusion did not significantly elevate albumin leakage from cremaster venules of B6129 mice, marked increases were noted in LDLr−/− mice placed on either ND or HCD. There was no difference in albumin leakage between the two groups (ND and HCD) of LDLr−/− mice exposed to I/R.

Figure 5 summarizes the responses of shear rate, adherent leukocytes, and emigrated leukocytes in cremaster venules of LDLr−/− mice fed HCD, which were treated with an anti-ICAM-1 MAb (YN-1), PAF receptor antagonist, or 5-lipoxygenase inhibitor. None of the pretreatment regimens altered either the decline in venular shear rate or enhanced leukocyte emigration normally observed after I/R. However, treatment with either MAb YN-1 or the PAF receptor antagonist, but not the 5-lipoxygenase inhibitor, dramatically attenuated the I/R-induced increase in adherent leukocytes.

Figure 6 summarizes the responses of leukocyte rolling velocity, flux of rolling leukocytes, and number of rolling leukocytes in cremaster venules of LDLr−/− mice fed HCD that were treated with an anti-ICAM-1 MAb (YN-1), PAF receptor antagonist, or 5-lipoxygenase inhibitor. None of the pretreatment regimens significantly altered the different indexes of leukocyte rolling in LDLr−/− mice fed HCD.

In Fig. 7 the actions of MAb YN-1, WEB-2086, and MK-886 on the increased albumin leakage elicited by I/R in cremaster venules of LDLr−/− mice fed HCD were summarized. The I/R-induced albumin extravasation was significantly attenuated in mice pretreated with either MAb YN-1 or the PAF receptor antagonist WEB-2086 but not MK-886.
DISCUSSION

Several murine models of hypercholesterolemia and atherosclerosis have been developed through targeted disruption, deletion, or insertion of specific genes related to lipoprotein metabolism (4). These models are now widely employed for mechanistic studies of arterial...
lesion development and the altered arterial reactivity associated with atherogenesis. Although much emphasis has been given to the inflammatory changes that occur in major arterial vessels during the progression of atherosclerosis, recent studies indicate that postcapillary venules become dysfunctional before the development of arterial lesions (11, 14). In LDLr mice, these abnormal responses are manifested as an exaggerated leukocyte-endothelial cell adhesion in venules exposed to lipid mediators or cytokines (11). These findings indicate that chronic hypercholesterolemia may render the microvasculature more vulnerable to the deleterious effects of inflammation. Inasmuch as atherosclerosis also renders different regional vascular beds more vulnerable to ischemic episodes (6) and since ischemia followed by reperfusion has been shown to elicit acute inflammatory responses in these same vascular beds (9), it is conceivable that I/R-induced inflammatory reactions occur more frequently and are more robust during atherogenesis. These events would help explain why hypercholesterolemia is a major risk factor for the development of regional ischemic disorders.

The results of the present study demonstrate that after I/R, postcapillary venules of LDLr mice exhibit an exaggerated recruitment of firmly adherent and emigrating leukocytes, with a correspondingly higher level of albumin leakage, compared with wild-type mice. These differences were clearly evident with the use of an I/R protocol of 60-min complete ischemia and 60-min reperfusion, which typically elicits few or no changes in the various inflammatory and microvascular parameters monitored in cremaster muscle venules of wild-type mice. Previous studies using wild-type mice indicate that at least 2 h of ischemia (plus 60 min of reperfusion) are needed to produce the responses observed in LDLr mice placed on a normal diet (unpublished observations). Our study also provides evidence that implicates the lipid mediator PAF, but not leukotrienes, and the endothelial cell adhesion molecule ICAM-1 in both the exaggerated leukocyte recruitment and endothelial barrier dysfunction observed in venules of LDLr mice after I/R. Overall, these findings indicate that LDLr mice, an animal model that closely resembles familial hypercholesterolemia in humans, are highly sensitive to the deleterious microvascular effects of I/R.

An interesting and potentially important observation in this study is that the exaggerated inflammatory responses elicited by I/R in LDLr mice were not profoundly affected by placement of the animals on HCD for 4 wk does not further enhance the LTB4-induced recruitment of adherent leukocytes in cremaster venules, compared with LDLr mice placed on normal chow. Similarly, our findings are consistent with reports showing that when rats, which do not develop atherogenic lesions, are placed on HCD for 2–4 wk, a comparable exaggeration of inflammatory responses is noted (8, 15, 18).

In normocholesterolemic animals, the recruitment of leukocytes into postischemic tissues has been linked to the production of lipid mediators that occurs secondary to phospholipase A2 activation (9, 26). The lipid mediators PAF and LTB4 have received much attention in this regard. Receptor antagonists for PAF or LTB4 as well as 5-lipoxygenase inhibitors have been shown to significantly blunt the adherence and emigration of leukocytes in postcapillary venules exposed to I/R (16, 19). In a recent study (19), it was shown that while both PAF and LTB4 receptor antagonists are individually effective in blunting reperfusion-induced leukocyte adherence, pretreatment with the combination of antagonists completely prevented the adhesion response. The present study suggests that PAF exerts a dominant influence over leukotrienes in mediating the inflammatory responses elicited in postcapillary venules by I/R in LDLr mice. It is possible that a greater production of PAF may occur in venular endothelial cells of LDLr mice after I/R because 1) hypercholesterolemia has been shown to exacerbate the oxidant stress elicited in venules exposed to I/R (20) and 2) oxidants are known to promote the generation of PAF by endothelial cells (24).

Although the positive results obtained with the PAF receptor antagonist strongly support a role for this lipid mediator in the I/R-induced responses, it is possible...
that oxidized phospholipids mediate these responses by engaging with and activating the PAF receptor. It has been shown that peroxide-treated endothelial cells generate biologically active phospholipids that efficiently recognize the PAF receptor (28) and PAF acetylhydrolase (29). These observations, coupled to reports demonstrating that peroxide-treated endothelial cell monolayers, sustain more adherent neutrophils via a PAF-receptor-dependent mechanism (28), support the possibility that oxidized phospholipids may mediate the PAF-receptor-dependent leukocyte adhesion that is observed in postischemic venules of LDLr-/- mice.

The ability of an anti-ICAM-1 MAb to blunt the I/R-induced inflammatory responses in LDLr-/- mice is consistent with a role for PAF in this model. PAF is known to act on leukocytes to increase the expression of CD11b/CD18, a counter receptor for endothelial cell ICAM-1 (26). Henninger et al. (11) have shown that ICAM-1 expression in the cremaster vasculature of LDLr-/- mice placed on either a ND or HCD is no different from ICAM-1 expression measured in corresponding wild-type mice. This observation, coupled to the fact that ICAM-1 is not likely to be significantly upregulated in cremaster venules at 60 min after reperfusion, suggests that it is the constitutively expressed ICAM-1 that contributes to the I/R-induced, PAF-mediated inflammatory responses.

Previous studies of the inflammatory responses in venules of LDLr-/- mice have focused exclusively on leukocyte-endothelial cell interactions, without consideration of potential changes in endothelial barrier function (11, 14). The present study demonstrates that the exaggerated recruitment of adherent leukocytes in venules of LDLr-/- mice is accompanied by a more profound increase in albumin extravasation. The observation that agents that interfere with I/R-induced leukocyte-endothelial cell adhesion (PAF antagonist, ICAM-1-specific MAb) in this model also attenuate the establishment of endothelial barrier dysfunction observed in venules of LDLr-/- mice. This dependence of endothelial barrier function on leukocyte adhesion is not unique to venules in LDLr-/- mice. It has previously been shown in normcholesterolemic animals that I/R-induced increases in vascular permeability can be blunted or prevented by antibodies that interfere with leukocyte adhesion (3, 17, 12, 25). Hence, it appears likely that hypercholesterolemia results in an exaggerated albumin leakage response by virtue of its ability to increase the intensity of leukocyte recruitment and activation. Irrespective of the mechanisms underlying the enhanced endothelial barrier dysfunction in LDLr-/- mice, this observation suggests that hypercholesterolemia not only makes a tissue more likely to experience an ischemic insult but also renders its microvasculature more vulnerable to the deleterious effects of I/R.

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