NAD(P)H Oxidase–Derived Superoxide Mediates Hypercholesterolemia-Induced Leukocyte–Endothelial Cell Adhesion


Abstract—Experimental animals placed on a high-cholesterol diet for 2 or more weeks exhibit an inflammatory response in postcapillary venules. The aims of this study were to determine (1) whether superoxide mediates the hypercholesterolemia-induced inflammatory response and (2) whether leukocyte and/or vessel wall NAD(P)H oxidase contributes to this response. Intravital videomicroscopy was used to quantify leukocyte–endothelial cell adhesion in cremasteric postcapillary venules of wild-type (WT) mice, CuZn-superoxide dismutase transgenic (SOD TgN) mice, and mice heterozygous (p47phox+/−) or homozygous (p47phox−/−) for NAD(P)H oxidase placed on either a normal diet or high-cholesterol diet (HCD) for 2 weeks. The number of adherent and emigrated leukocytes in postcapillary venules of WT HCD mice was significantly higher than that detected in venules of their normal-diet counterparts. However, the HCD-induced recruitment of adherent and emigrated leukocytes was not observed in SOD TgN mice. Whereas hypercholesterolemic p47phox+/− and WT mice exhibited similar inflammatory responses, p47phox−/− mice did not. Bone marrow chimeras were developed to selectively delete p47phox from either the vessel wall or circulating leukocytes. Whereas WT marrow transplanted into WT mice produced a normal inflammatory response of venules to HCD, chimeric mice with p47phox deficiency in either the vessel wall or leukocytes exhibited an attenuated inflammatory response to HCD that was comparable with that observed in p47phox−/− HCD mice. Our findings indicate that enhanced superoxide production is a critical event that initiates the leukocyte–endothelial cell adhesion in postcapillary venules of HCD mice. NAD(P)H oxidase appears to be an important source of this superoxide. (Circ Res. 2001;88:499-505.)

Key Words: superoxide dismutase • p47phox • microvasculature • leukocyte emigration • chimeras

Hypercholesterolemia is a major risk factor for the development of atherosclerosis, a primary cause of mortality in the United States.1 A chronic inflammatory state occurs during atherosclerosis that is characterized by endothelial cell injury, increased endothelial cell adhesion molecule (CAM) expression, and leukocyte infiltration of the vessel wall.2–6 Cytokines such as tumor necrosis factor-α and γ-interferon7,8 released from both the normal cellular components of the vessel wall and infiltrating leukocytes allow this response to continue, culminating in plaque formation in the vessel wall. Although it is well established that prolonged hypercholesterolemia can cause these adverse changes in large arteries such as the aorta and coronary arteries, there is growing evidence that similar inflammatory changes occur in the microvasculature during short-term hypercholesterolemia.9,10 The microvascular responses to hypercholesterolemia are associated with activation of endothelial cells and leukocytes and manifest as an impairment of endothelium-dependent relaxation of arterioles, increased CAM expression, and leukocyte–endothelial cell adhesion in venules.9–11 Furthermore, acute hypercholesterolemia appears to exacerbate the microvascular injury response to inflammatory stimuli such as ischemia-reperfusion.12–14

The mechanism by which hypercholesterolemia initiates atherosclerotic lesion development is not completely understood, although cholesterol oxidation products such as those found in oxidized LDL cholesterol have been implicated.15 Oxidized LDL promotes endothelial dysfunction and leukocyte chemotraction in both large vessels and the microcirculation.16–18 Increased oxidant stress, resulting from both increased oxygen free radical (OFR) production and decreased nitric oxide (NO) generation, appears to play an important role in the chronic inflammatory responses to hypercholesterolemia and atherosclerosis.19 The proinflammatory effects of OFRs may result from the ability of these reactive species to (1) increase the expression of CAMs on vascular endothelium20,21; (2) enhance the production of leukocyte-activating substances (e.g., platelet-activating factor, leukotriene B4, and complement 5a); and (3) promote the rolling, firm adhesion and emigration of leukocytes in the vasculature.22 The recognition that OFRs may contribute...
to the vascular pathology associated with hypercholesterolemia and atherosclerosis has led to an interest in defining the sources of OFRs in these conditions. Potential sources of OFRs include mitochondrial oxidases, xanthine oxidase, lipooxygenase, and NAD(P)H-dependent oxidases.23–24 Although some studies suggest that xanthine oxidase–mediated superoxide production may be one of the earliest vascular responses to hypercholesterolemia,25,26 more recent work has focused on the potential contribution of NAD(P)H oxidase to the enhanced production of OFRs in blood vessels of hypercholesterolemic human subjects and experimental animals.27–32

The enzyme NAD(P)H oxidase has been well characterized in phagocytes and, although less is known about its structure and activity in other cells, similarities exist between the phagocytic, endothelial, and vascular smooth muscle NAD(P)H oxidases.33,34 Phagocytic NAD(P)H oxidase consists of both membrane-bound and cytosolic components. The membrane-associated complex consists of 2 subunits, gp91phox and p22phox, whereas at least 2 other subunits (p67phox and p47phox) constitute the cytosolic component. Under basal conditions, these components are kept apart, rendering the enzyme inactive. However, on stimulation, p47phox becomes heavily phosphorylated and, along with p67phox, moves to the cell membrane where it binds the membrane complex, allowing for enzyme activation. The membrane portion of the enzyme then transfers an electron from NADH or, in the case of neutrophils, NADPH to molecular oxygen, forming superoxide.35 Targeted disruption or deletion of the enzyme (or 1 of its 4 subunits) has allowed for an assessment of the contribution of NAD(P)H oxidase to production of superoxide by neutrophils, endothelial cells, and vascular smooth muscle cells.

To date, evidence supporting a role for NAD(P)H oxidase in the hypercholesterolemia–induced vascular dysfunction and in the development of atherosclerosis is conflicting, and this work has largely focused on large arterial vessels.27,29–31 A recent study by Guzik et al29 demonstrated an association between vascular superoxide production by NAD(P)H oxidase and the endothelial dysfunction that accompanies hypercholesterolemia in human blood vessels. Whether such an association exists for the microvascular inflammatory changes that occur during hypercholesterolemia has not yet been addressed. Hence, the major aims of this study were (1) to define the role of superoxide, using transgenic (TgN) mice that overexpress the superoxide scavenging enzyme CuZn–superoxide dismutase (SOD), in mediating the leukocyte–endothelial cell adhesion observed in postcapillary venules of acutely hypercholesterolemic mice; (2) to assess the contribution of NAD(P)H oxidase to this hypercholesterolemia–induced inflammatory response using mice that are genetically deficient in the cytosolic p47phox subunit of NAD(P)H oxidase; and (3) to distinguish between the contributions of leukocyte versus vessel wall NAD(P)H oxidase to this response using chimeric mice that are selectively deficient in either leukocyte or vascular wall p47phox.

### Materials and Methods

#### Animals

Wild-type C57BL/6 mice (WT), CD45 congenic B6.SJL-PTPRCPEP/BOY mice (which express CD45.1), and breeder stocks for C57BL/6-TgN(SOD1)3Cje (SOD TgN) mice on a C57BL/6 background were obtained from The Jackson Laboratories (Bar Harbor, ME). The TgN mice were identified by qualitative demonstration of CuZn-SOD using nondenaturing gel electrophoresis followed by nitroblue tetrazolium staining. Breeder stocks for p47phox knockout mice were obtained from Dr Steven Holland (Laboratory of Host Defenses, NIH). Heterozygote/homozygote matings were used to produce approximately equal numbers of either heterozygous (p47phox+/−) or homozygous (p47phox−/−) offspring for this study. The genetic identity of animals was determined by PCR analysis of tail clip DNA.

At 9 to 10 weeks of age the mice were placed on either a normal diet (ND) or a high-cholesterol diet (HCD) (Teklad 90221 containing 1.25% cholesterol, 15.8% fat, and 0.125% choline chloride, Harlan Teklad) for 2 weeks (n=5 to 6 per group). Two separate groups of WT mice were used for the SOD and the p47phox sections of the study.

#### Chimeras

Three combinations of chimeras were used. WT→WT chimeras were WT animals that received bone marrow cells from CD45 congenic mice, reserving NAD(P)H oxidase function. This resulted in a significant increase of splenocytes expressing CD45.1 (of donor origin), from <5% in WT to >90% in the WT→WT chimeras, allowing verification of proper chimera reconstitution, as previously described.37 CD45 congenic bone marrow was transplanted into p47phox−/− mice, yielding WT→p47phox−/− chimeras with normal blood cell NAD(P)H oxidase function but a p47phox−/−-deficient vessel wall. The p47phox−/−→WT chimeras with NAD(P)H oxidase–deficient blood cells but normal vascular wall enzyme activity were produced by transplanting bone marrow from a p47phox−/− mouse into a CD45 congenic mouse.

Bone marrow transfer was performed to create chimeric mice. Briefly, bone marrow cells were isolated from the femurs and tibias of donors and resuspended at 4 × 106 cells/mL PBS. Recipient mice were irradiated with 2 doses of 500 to 525 rad, 3 hours apart, after which 8 × 106 donor bone marrow cells in 200 μL of PBS were injected into the femoral vein. The chimeras were kept in autoclaved cages, with 0.2% neomycin drinking water for 2 weeks, after which normal drinking water was used. Six weeks after reconstitution, chimeric mice were placed on HCD for 2 weeks and the cremaster preparation was performed. Flow cytometry was used to verify chimera reconstitution by staining for CD45.1 expression on splenocytes with an FITC-labeled anti-CD45.1 antibody (PharMingen Inc).

#### Surgical Protocol

The mice were anesthetized with ketamine hydrochloride (150 mg/kg body weight, IP) and xylazine (7.5 mg/kg body weight, IP). The right jugular vein was cannulated for administration of heparinized saline, and the left carotid artery was cannulated for measurement of systemic arterial pressure. Core body temperature was maintained at 35 ± 0.5°C. Animal handling procedures were approved by the Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee and were in accordance with the guidelines of the American Physiological Society.

#### Intravital Microscopy

The cremaster muscle microcirculation was evaluated in this study because (1) the microvascular and inflammatory responses to hypercholesterolemia previously described for this tissue are similar to those reported for other tissues, including rat mesentery,9,10,12–14,38 and (2) unlike the rat, the mouse stores large quantities of fat in the mesentery, making it technically difficult to locate and visualize mesenteric venules in both WT and mutant mice in the present study. Each mouse was placed in a semi-upright position on a plexiglas microscope stage. The cremaster muscle was isolated and placed across a viewing pedestal as previously described.14,38,39 The microcirculation was transilluminated and postcapillary venules with a wall shear rate (WSR) of ≤500 per second and diameters between 20 and 40 μm were observed. The venule with the smallest number of adherent and emigrated leukocytes at the end of a 30-minute observation period was microscopically imaged using an inverted microscope.
stabilization period was chosen and 5-minute recordings were made of the first 100 μm of every 300 μm along the length of the unstimulated vessel. Venular WSR and leukocyte emigration (number per field) were measured online at the end of each 5-minute observation period. Leukocyte rolling velocity ($V_{rol}$) and number of rolling (number per minute) and adherent (number per 100 μm) leukocytes were quantified in the cremaster muscle during playback of videotaped images. The mean value of each parameter in a single venule was calculated, and comparisons were made between the experimental groups.

**Serum Cholesterol Levels**

Serum was frozen for subsequent measurement of cholesterol levels using a spectrophotometric assay (Sigma Chemical Co).

**White Blood Cell Counts**

At the end of each experiment, 25 μL of blood was mixed with 465 μL of 3% acetic acid and 10 μL of 1% crystal violet for a white blood cell count using a hemocytometer.

**Statistical Analysis**

All values are reported as mean±SEM. ANOVA with the Scheffé post hoc test was used to compare between groups with statistical significance set at $P<0.05$.

**Results**

**Role of Superoxide in Hypercholesterolemia-Induced Leukocyte–Endothelial Interactions**

WT HCD mice had 3-fold higher serum cholesterol levels compared with WT ND mice (Table 1). Both SOD non-TgN and SOD TgN mice placed on HCD for 2 weeks exhibited similar increases in serum cholesterol versus their ND counterparts. Hypercholesterolemia did not significantly alter white blood cell count (6080±1130.8 versus 5000±1834.4 cells/μL), leukocyte rolling flux (55.0±22.20 versus 83.0±11.66 leukocytes/min), or $V_{rol}$ (36.2±11.43 versus 64.2±12.83 μm/second) in the WT mice (ND versus HCD, respectively) or in the SOD non-TgN or SOD TgN mice (data not shown), although there was more than a doubling of the number of adherent leukocytes in postcapillary venules of both WT and SOD non-TgN mice (Figure 1). A significant (nearly 4-fold) increase in the number of emigrated leukocytes was also observed in the WT HCD and SOD non-TgN HCD groups (Figure 2) when compared with data from ND mice. However, TgN mice that overexpress CuZn-SOD (SOD TgN) exhibited a significantly reduced number of non-TgN mice. Indeed, leukocyte adherence and emigration in the SOD TgN HCD mice were similar to values obtained in the corresponding ND group, suggesting that superoxide plays a major role in the enhancement of baseline leukocyte–endothelial cell adhesion associated with hypercholesterolemia. The observed changes in leukocyte adhesion and emigration were independent of any change in WSR (Table 1).

**Role of NAD(P)H Oxidase in Hypercholesterolemia-Induced Leukocyte Adhesion and Emigration**

Serum cholesterol levels in p47$^{phox}$ heterozygous and knockout mice maintained on ND were comparable (Table 2). However, placement of p47$^{phox}+/−$ and p47$^{phox}−/−$ mice on HCD for 2 weeks resulted in a significantly larger increment in serum cholesterol concentration compared with their WT counterparts. Nonetheless, the more profound elevation in serum cholesterol was not accompanied by a more intense inflammatory response in cremasteric venules. Heterozygous p47$^{phox}$ mice maintained on HCD for 2 weeks exhibited comparable elevations in basal leukocyte adherence (Figure 3) and emigration (Figure 4) relative to WT HCD mice. However, the HCD-induced in-

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**TABLE 1. Venular WSR and Serum Cholesterol Concentration in WT, SOD Non-TgN, and SOD TgN Mice Maintained on ND or HCD for 2 Weeks**

<table>
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<tr>
<th></th>
<th>WSR, /Second</th>
<th>Serum Cholesterol, mg/dL</th>
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<tbody>
<tr>
<td>WT ND</td>
<td>616.5±45.58</td>
<td>93.8±5.92</td>
</tr>
<tr>
<td>WT HCD</td>
<td>853.1±14.42</td>
<td>279.7±12.38*</td>
</tr>
<tr>
<td>SOD non-TgN ND</td>
<td>754.0±41.19</td>
<td>108.0±2.85</td>
</tr>
<tr>
<td>SOD non-TgN HCD</td>
<td>759.9±33.72</td>
<td>253.3±9.09*</td>
</tr>
<tr>
<td>SOD TgN ND</td>
<td>727.2±55.27</td>
<td>116.0±7.87</td>
</tr>
<tr>
<td>SOD TgN HCD</td>
<td>601.5±52.57</td>
<td>244.9±15.03*</td>
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*P<0.0001 vs all ND groups.

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**Figure 1.** Mean baseline leukocyte adhesion in postcapillary venules of cremaster muscle in WT, SOD non-TgN, and SOD TgN mice maintained on ND or HCD for 2 weeks. *P<0.0001 vs all ND groups; ^#P<0.0001 vs WT HCD mice; #P<0.0001 vs SOD non-TgN HCD mice.

**Figure 2.** Mean baseline leukocyte emigration in postcapillary venules of cremaster muscle in WT, SOD non-TgN, and SOD TgN mice maintained on a ND or HCD for 2 weeks. *P<0.005 vs all ND groups; #P<0.01 vs WT HCD mice; ^#P<0.01 vs SOD non-TgN HCD mice.
creases in leukocyte adherence and emigration did not occur in p47\(^{phox}\)\(^{-/-}\) mice (Figures 3 and 4). In fact, the values of leukocyte adherence and emigration observed in p47\(^{phox}\)\(^{-/-}\) HCD mice were similar to those measured in mice placed on ND. There were no significant differences in WSR (Table 2), white blood cell count, leukocyte rolling flux, or V\(_{bc}\) (data not shown) among any of these experimental groups.

**Hypercholesterolemia-Induced Inflammatory Responses in p47\(^{phox}\)\(^{-/-}\) Chimeric Mice**

After 2 weeks of HCD, all 3 groups of chimeric mice, WT→WT, WT→p47\(^{phox}\)\(^{-/-}\), and p47\(^{phox}\)\(^{-/-}\)→WT, had serum cholesterol levels that were comparable with those of WT HCD animals but significantly lower than levels found in the p47\(^{phox}\)\(^{+/+}\) and p47\(^{phox}\)\(^{-/-}\)→HCD groups (Table 2). When venules were studied in these groups, no differences were noted for WSR (Table 2), white blood cell count, leukocyte flux, or V\(_{bc}\) (data not shown). WT→WT chimeras placed on HCD exhibited high baseline leukocyte adherence (Figure 5) and emigration (Figure 6) analogous to levels observed in WT HCD and p47\(^{phox}\)\(^{-/-}\)→HCD groups, which suggests that the process per se of creating the chimeras did not alter the microvascular inflammatory responses to hypercholesterolemia. However, WT→p47\(^{phox}\)\(^{-/-}\) mice [NAD(P)H oxidase deficiency in the vessel wall but not in the leukocytes] exhibited significantly lower leukocyte adhesion (Figure 5) and emigration (Figure 6) relative to WT→WT HCD mice but similar to those observed in all ND groups and p47\(^{phox}\)\(^{-/-}\)→HCD mice. A very similar pattern was noted for the p47\(^{phox}\)\(^{-/-}\)→WT chimeras [NAD(P)H oxidase deficiency in leukocytes but not in the vessel wall]; i.e., leukocyte adhesion (Figure 5) and emigration (Figure 6) were comparable with values in p47\(^{phox}\)\(^{-/-}\)→HCD and p47\(^{phox}\)\(^{-/-}\)→WT HCD and ND mice but significantly lower than the responses observed in the WT→WT HCD group. These findings suggest that the presence of NAD(P)H oxidase in both the vessel wall and circulating leukocytes is essential for the

<table>
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<th>Groups</th>
<th>WSR, /Second</th>
<th>Serum Cholesterol, mg/dL</th>
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<tbody>
<tr>
<td>WT ND</td>
<td>665.6±11.62</td>
<td>102.1±4.53</td>
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<tr>
<td>WT HCD</td>
<td>721.5±53.46</td>
<td>218.3±8.98*</td>
</tr>
<tr>
<td>p47(^{phox})(^{+/+}) ND</td>
<td>715.9±134.11</td>
<td>163.5±12.69</td>
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<td>p47(^{phox})(^{+/+}) HCD</td>
<td>616.5±50.40</td>
<td>354.6±22.34†</td>
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<tr>
<td>p47(^{phox})(^{-/-})→ND</td>
<td>813.3±80.56</td>
<td>152.2±20.53</td>
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<tr>
<td>p47(^{phox})(^{-/-})→HCD</td>
<td>1009.5±162.19</td>
<td>372.9±23.05‡</td>
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<tr>
<td>WT→WT HCD</td>
<td>691.0±100.86</td>
<td>199.7±9.83*§</td>
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<tr>
<td>WT→p47(^{phox})(^{-/-})→HCD</td>
<td>963.9±88.96</td>
<td>240.3±13.84§</td>
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<tr>
<td>p47(^{phox})(^{-/-})→WT HCD</td>
<td>868.7±143.22</td>
<td>218.4±12.47§</td>
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*P<0.05 vs WT ND. †P<0.0005 vs WT HCD. ‡P<0.0001 vs WT HCD. §P<0.01 vs WT HCD and p47\(^{phox}\)\(^{-/-}\)→HCD.

**Figure 3.** Mean baseline leukocyte adhesion in postcapillary venules of cremaster muscle in WT, p47\(^{phox}\)\(^{-/-}\) heterozygous (p47\(^{phox}\)\(^{+/+}\)), and homozygous (p47\(^{phox}\)\(^{-/-}\)) mice maintained on ND or HCD for 2 weeks. The process per se of creating the chimeras did not alter the

**Figure 4.** Mean baseline leukocyte emigration in postcapillary venules of cremaster muscle in WT, p47\(^{phox}\)\(^{-/-}\) heterozygous (p47\(^{phox}\)\(^{+/+}\)), and homozygous (p47\(^{phox}\)\(^{-/-}\)) mice maintained on ND or HCD for 2 weeks. The process per se of creating the chimeras did not alter the

**Figure 5.** Mean baseline leukocyte adhesion in postcapillary venules of cremaster muscle in WT, p47\(^{phox}\)\(^{-/-}\) heterozygous (p47\(^{phox}\)\(^{+/+}\)), and homozygous (p47\(^{phox}\)\(^{-/-}\)) mice, as well as chimeras with WT marrow transplanted into WT mice (WT→WT), WT marrow transplanted into p47\(^{phox}\)\(^{-/-}\)→HCD mice (WT→p47\(^{phox}\)\(^{-/-}\)), or p47\(^{phox}\)\(^{-/-}\)→WT HCD mice maintained on ND or HCD for 2 weeks. The process per se of creating the chimeras did not alter the
E-selectin were largely unchanged by HCD for 2 weeks, as molecule-1 were upregulated in many major organs such as adhesion molecule-1 and vascular cellular adhesion associated with hypercholesterolemia. We observed role for superoxide in mediating the leukocyte– endothelial cell adhesion.9–11 We have previ-}

Discussion

There is a growing body of evidence that hypercholesterolemia can exert detrimental effects on the cardiovascular system via mechanisms that extend beyond the ability of this lipid to promote the formation of atherosclerotic plaques. The component of the vasculature that bears the brunt of the effects of hypercholesterolemia is the endo-

-Thelial lining of both large and microscopic branches of the arteries and veins. The endothelial cell activation and/or dysfunction in hypercholesterolemic human subjects and experimental animals is manifested as increased production of OFRs, impaired endothelium-dependent vasodila-

tion that accompanies experimental hypercholesterolemia.42,47 Another superoxide-generating enzyme that has received recent attention as a mediator of the vascular dysfunction associated with hypercholesterolemia is NAD(P)H oxidase.

There are several potential enzymatic sources of superoxide within the vasculature, including xanthine oxidase, NAD(P)H-dependent oxidases, mitochondrial oxidases, and lipoygenase. Xanthine oxidase has been implicated as a source of superoxide in hypercholesterolemia on the basis of the following 2 observations: (1) plasma xanthine oxidase levels are elevated during hypercholesterolemia,25 and (2) the xanthine oxidase inhibitor oxypurinol appears to blunt the enhanced vessel wall production of superoxide that accompanies experimental hypercholesterolemia.42,47 Another superoxide-generating enzyme that has received recent attention as a mediator of the vascular dysfunction associated with hypercholesterolemia is NAD(P)H oxidase.

There are several studies that support this possibility. For example, a strong association between vascular superoxide production by NAD(P)H oxidase and the endothelial dysfunction that accompanies hypercholesterolemia in human blood vessels was recently demonstrated.29 It has also been shown that rabbits placed on HCD for 8 weeks exhibit early aortic lesion development associated with an increased production of superoxide that is only partially reduced by oxypurinol but completely inhibited by diphenyleneiodonium, a potent inhibitor of flavin-containing enzymes such as NAD(P)H oxidase.48 There are also reports that do not support a role for NAD(P)H oxidase in hypercholesterolemia-induced vascular pathology, including atherosclerotic lesion development. For example, when apolipo-

protein E−/− mice (which spontaneously develop hypercholesterolemia and atherosclerotic lesions) were crossbred with p47phox−/− mice30 or gp91phox−/− mice,31 deleting the NAD(P)H oxidase enzyme did not alter the macrophage infiltration or lesion development in arteries that was normally observed in apolipoprotein E−/− mice.

To address the potential contribution of NAD(P)H oxidase to the superoxide-mediated leukocyte– endothelial cell adhesion associated with hypercholesterolemia, we placed p47phox−/− mice on HCD, like their WT counterparts and heterozygous (p47phox+−/−) siblings (with fully functional NAD(P)H oxidase activity), and compared the microvascular responses. The results

Figure 6. Mean baseline leukocyte emigration in postcapillary venules of cremaster muscle in WT, p47phox heterozygous (p47phox+−/−), and homozygous (p47phox−/−) mice, as well as chimera mice with WT marrow transplanted into WT mice (WT→WT), WT marrow transplanted into p47phox−/− mice (WT→p47phox−/−), or p47phox−/− marrow transplanted into WT mice (p47phox−/−→WT) maintained on ND or HCD for 2 weeks.

P<0.001 vs WT ND mice; #P<0.01 vs WT HCD and p47phox+−/− HCD mice; ^P<0.005 vs p47phox−/− HCD mice; †P<0.05 vs WT→WT HCD mice.

Our findings in CuZn-SOD TgN mice, which express 2 to 4 times the normal SOD activity in different tissues,41 support a role for superoxide in mediating the leukocyte–endothelial cell adhesion associated with hypercholesterolemia. We observed that the hypercholesterolemia-induced increments in basal leukocyte adhesion and emigration that are normally observed in postcapillary venules of WT mice (or in SOD non-TgN mice) are largely absent in CuZn-SOD TgN mice. The involvement of superoxide in this hypercholesterolemia-induced inflammatory response is consistent with reports describing (1) enhanced production of superoxide by blood vessels of hypercholesterolemic human subjects and experimental animals,25,26,42,43 (2) the ability of superoxide to induce endothelial CAM expres-

sion20,21 and promote leukocyte–endothelial cell adhesion in postcapillary venules,13,16,22 and (3) a protective effect of exog-

enously administered SOD against impaired endothelium-dependent vasodilation associated with hypercholesterolemia.44

Because superoxide reacts rapidly with NO, the increased superoxide generation by endothelial cells in hypercholesterol-

emia has been proposed to account for at least a portion of the NO deficit observed in this condition.42 Inasmuch as NO has been shown to serve as an endogenous inhibitor of leukocyte–endothelial cell adhesion,45,46 superoxide-mediated inactivation of NO may represent a major underlying cause of the hypercholesterolemia-induced leukocyte–endothelial cell adhesion.

There are several potential enzymatic sources of superoxide that accompanies experimental hypercholesterol-

emia.29,47 It has also been shown that rabbits placed on HCD for 8 weeks exhibit early aortic lesion development associated with an increased production of superoxide that is only partially reduced by oxypurinol but completely inhibited by diphenyleneiodonium, a potent inhibitor of flavin-containing enzymes such as NAD(P)H oxidase.48 There are also reports that do not support a role for NAD(P)H oxidase in hypercholesterolemia-induced vascular pathology, including atherosclerotic lesion development. For example, when apolipo-

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of these experiments clearly demonstrate an ablation of the hypercholesterolemia-induced inflammatory responses in p47<sup>phox</sup>−/− mice. The fact that the attenuated inflammatory responses observed in p47<sup>phox</sup>−/− mice were of comparable magnitude to the attenuation noted in CuZn-SOD TgN mice suggests that NAD(P)H oxidase is the major source of superoxide that mediates these hypercholesterolemia-induced microvascular alterations.

NAD(P)H oxidase is present in many cell types, including phagocytes, B lymphocytes, endothelial cells, and vascular smooth muscle cells. This raises the possibility that the cellular source of the NAD(P)H oxidase that is generating superoxide in response to hypercholesterolemia is either the venular endothelial cell, circulating leukocyte, or both. We attempted to determine the relative contributions of leukocyte-versus-vascular wall–associated NAD(P)H oxidase to the hypercholesterolemia-induced inflammatory responses using bone marrow chimeric mice. By transplanting marrow harvested from p47<sup>phox</sup>−/− mice into WT mice or vice versa, we produced mice with NAD(P)H oxidase deficiency in leukocytes but not in the vessel wall or vice versa, i.e., NAD(P)H oxidase deficiency in the vessel wall but not in the leukocyte. Control chimeric animals (WT marrow transplanted into WT mice) exhibited an inflammatory response to HCD comparable with that observed in WT mice, indicating that the irradiation and marrow transfer procedures per se do not interfere with the leukocyte adhesion and emigration responses elicited by HCD. However, the data obtained with WT → p47<sup>phox</sup>−/− → WT and p47<sup>phox</sup>−/− → WT chimeras indicate that deficiency of NAD(P)H oxidase in either the circulating leukocytes or the vessel wall attenuates the HCD-induced leukocyte adhesion and emigration to an extent comparable with that observed in p47<sup>phox</sup>−/− mice. This observation suggests that both the leukocyte- and vessel wall–associated forms of NAD(P)H oxidase are necessary for the superoxide-mediated, HCD-induced leukocyte–endothelial cell adhesion. Although a definitive explanation for this dependence of the inflammatory response on both sources of NAD(P)H oxidase is not readily available, it may reflect a HCD-initiated process wherein NAD(P)H oxidase–derived superoxide from one cell type (e.g., leukocytes) is required to activate the same enzyme in the other cell population (e.g., endothelial cells). Hence, if NAD(P)H oxidase–mediated formation of superoxide in endothelial cells is necessary to induce the leukocyte–endothelial cell adhesion and superoxide produced by the leukocyte is responsible for activating the endothelial enzyme, then inhibition or deletion of either source of NAD(P)H oxidase should afford a comparable level of protection against HCD. This does not exclude the likely possibility of other inflammatory mediators such as cytokines or CAMs from participating in this response, nor does it exclude the possibility that the superoxide derived from either cell type influences the expression of important inflammatory proteins other than NAD(P)H oxidase.

An interesting and potentially important observation in this study was the significantly greater increments in plasma cholesterol levels that were observed in the p47<sup>phox</sup>−/+ and p47<sup>phox</sup>−/− mice placed on HCD versus WT animals. Because the further elevation in plasma cholesterol levels in the p47<sup>phox</sup>−/+ HCD group did not result in a correspondingly larger increment in leukocyte adhesion/emigration and the p47<sup>phox</sup>−/− deficiency was associated with inhibition of the inflammatory response, the data suggest that the further increments in plasma cholesterol exerted little if any direct influence on the HCD-induced responses. This is consistent with other reports that demonstrate no further amplification of an inflammatory response in microvessels once a threshold serum cholesterol level is reached. Nonetheless, the exaggerated elevations in plasma cholesterol noted in p47<sup>phox</sup>−/+ and p47<sup>phox</sup>−/− mice placed on HCD suggest that manipulation of this gene may affect cholesterol metabolism and/or its distribution.

Although much of the work in the area of atherosclerosis research has focused on plaque formation in and dysfunction of large arteries, relatively little attention has been devoted to the potentially important and rather profound inflammatory phenotype in venules that precedes the appearance of large artery plaques and lesions. Nonetheless, there appear to be several shared mechanistic features between the early venular responses and the later large vessel dysfunction, including increased superoxide formation/bioavailability. Although the relevance of an early, superoxide-dependent inflammatory phenotype in venules to the later plaque formation and vessel dysfunction in large arteries remains unclear, the venular response may be equally important if it predisposes the microvasculature to the injurious effects of disease processes that are associated with an acute inflammatory response, such as ischemia/reperfusion. Hence, hypercholesterolemia may increase the risk for cardiovascular disease not only by rendering tissues more likely to experience an ischemic episode (due to vessel obstruction with plaques) but also by exacerbating the inflammatory and tissue injury responses to a given ischemic insult.

In conclusion, the results of this study suggest that hypercholesterolemia induces a superoxide-dependent adhesion and emigration of leukocytes in postcapillary venules. The major source of the superoxide that mediates the HCD-induced inflammatory response is the enzyme NAD(P)H oxidase. Both leukocyte- and vessel wall–associated forms of NAD(P)H oxidase appear to be necessary for the superoxide-mediated, HCD-induced leukocyte–endothelial cell adhesion.

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