CD4+ T lymphocytes mediate hypercholesterolemia-induced endothelial dysfunction via a NAD(P)H oxidase-dependent mechanism


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Submitted 27 August 2007; accepted in final form 7 April 2008

Wolfort RM, Stokes KY, Granger DN. CD4+ T lymphocytes mediate hypercholesterolemia-induced endothelial dysfunction via a NAD(P)H oxidase-dependent mechanism. Am J Physiol Heart Circ Physiol 294: H2619–H2626, 2008. First published April 11, 2008; doi:10.1152/ajpheart.00989.2007.—Although hypercholesterolemia is known to impair endothelium-dependent vasodilation (EDV) long before the appearance of atherosclerotic plaques, it remains unclear whether the immune mechanisms that have been implicated in atherogenesis also contribute to the early oxidative stress and endothelial cell dysfunction elicited by hypercholesterolemia. EDV (wire myography), superoxide generation (cytochrome c reduction), and NAD(P)H oxidase mRNA expression were monitored in aortic rings from wild-type (WT) and mutant mice placed on either a normal diet or a cholesterol-enriched diet (HC) for 2 wk. WT mice on HC exhibited impaired EDV, enhanced superoxide generation, and increased expression of NAD(P)H oxidase subunit Nox-2 mRNA. The impaired EDV and increased superoxide generation induced by HC were significantly blunted in severe combined immunodeficient (SCID) mice and CD4+ T lymphocyte-deficient mice. These responses were also attenuated in HC mice genetically deficient in IFN-γ; however, adoptive transfer of WT-HC CD4+ T lymphocytes to IFN-γ-deficient recipients restored HC-induced responses. The HC-induced impaired EDV and oxidative stress were also attenuated in HC mice genetically deficient in Nox-2 (gp91phox−/−) and in WT→gp91phox−/−-HC chimeras. HC-induced gp91phox mRNA expression was significantly blunted in mice deficient in CD4+ T cells or IFN-γ and was restored with adoptive transfer of WT-HC CD4+ T cells to IFN-γ-deficient recipients. These findings implicate the immune system in the early endothelial cell dysfunction associated with hypercholesterolemia and are consistent with a mechanism of impaired EDV that is mediated by CD4+ T cells and IFN-γ, acting through the generation of superoxide from vascular NAD(P)H oxidase.

1 T cells; interferon-γ; vasodilation

CARDIOVASCULAR RISK FACTORS, such as hypercholesterolemia, exert several deleterious effects on the circulatory system, including inflammation (27), thrombosis (8), and endothelial cell dysfunction (28). A variety of mechanisms have been implicated in the pathogenesis of these disease processes, including immune cell activation and increased production/secretion of inflammatory mediators. Recent evidence suggests that lymphocytes exert a significant regulatory influence on the development of atherosclerotic lesions (1, 19), as evidenced by reports describing significant delays in the onset and progression of atherosclerotic lesions in immunodeficient mice (31). Similar compelling evidence exists to support the involvement of immune cell-derived cytokines in atherogenesis. For example, apolipoprotein E knockout mice crossed with IFN-γ-deficient mice exhibit a substantial reduction in atherosclerotic lesion size (compared with apolipoprotein E knockout mice) (11), and IL-12 vaccination has been shown to attenuate atherosclerosis in LDL receptor-deficient mice (16). T lymphocytes and cytokines (e.g., IFN-γ and IL-10) have also been implicated in the proliferation of vascular smooth muscle following endothelial cell injury (14, 15, 29). However, despite mounting evidence that T lymphocytes and the cytokines produced by these immune cells contribute to atherogenesis and smooth muscle proliferation in large arteries, there are no published reports that address the role of the immune system in the impaired endothelium-dependent vasodilation (EDV) that occurs in large arteries early after the induction of hypercholesterolemia.

There is a large body of evidence that implicates superoxide in the endothelium-dependent vasomotor dysfunction induced by hypercholesterolemia, with endothelial cell-associated NAD(P)H oxidase and xanthine oxidase considered the most likely sources of oxygen radicals in this condition (4, 6, 10, 13, 40). Although it is generally accepted that superoxide production by the vasculature is greatly enhanced during hypercholesterolemia, the cellular/chemical mediators of the superoxide production remain poorly understood. However, there are several lines of circumstantial evidence that lend support to a potential role for immune cell-derived cytokines as mediators of the increased superoxide production by endothelial cells and the consequent endothelium-dependent vasomotor dysfunction. For example, immune cell-derived cytokines such as IFN-γ have been shown to 1) activate and increase the expression of NAD(P)H oxidase (22), 2) contribute to the oxidative stress in postcapillary venules of mice placed on a cholesterol-enriched diet for 2 wk (36), 3) directly induce an impairment of EDV after an incubation with isolated arterial vessels (5), and 4) mediate the impaired endothelium-dependent dilation in cremaster arterioles of hypercholesterolemic mice (37). Despite this evidence, no effort has been previously undertaken to assess the contribution of enhanced superoxide production to the impaired vasomotor function mediated by immune cell-derived cytokines during hypercholesterolemia.

The overall objectives of this study were to determine whether lymphocytes contribute to the impaired vasomotor function and enhanced superoxide production that are observed in large arteries in the early stage of hypercholesterolemia and whether the immune cell-mediated responses are related to NAD(P)H oxidase-dependent superoxide production. We also...
assessed the contributions of B lymphocytes, CD4+ and CD8+ T lymphocytes, and IFN-γ to the hypercholesterolemia-induced oxidative stress and vasomotor dysfunction. Our findings are consistent with a mechanism that involves the activation of CD4+ T lymphocytes and the IFN-γ-dependent production of NAD(P)H oxidase-derived superoxide, which leads to the impaired endothelium-dependent vasomotor function in hypercholesterolemia.

MATERIALS AND METHODS

Animals and experimental groups. Wild-type (WT) mice, lymphocyte-deficient severe combined immunodeficient (SCID) mice (homozygous for the spontaneous mutation Prkdc<sup>−/−</sup>), B lymphocyte-deficient mice (homozygous for the Igκ<sup>−/−</sup> mutation), CD8<sup>−/−</sup> T lymphocyte-deficient mice (homozygous for the Cd8a<sup>−/−</sup> mutation), CD4<sup>−/−</sup> T lymphocyte-deficient mice (homozygous for the Cd4<sup>−/−</sup> mutation), IFN-γ-deficient mice (homozygous for the Ifng<sup>−/−</sup> mutation), IFN-γ receptor deficients mice (homozygous for the Cd4tm1Mak<sup>−/−</sup> mutation), and CD4<sup>+</sup> T cells (CD4<sup>+</sup>/H11002<sup>+/+</sup>/H11001<sup>+/+</sup>) mice, all on a C57Bl/6 background, were obtained from Jackson Laboratories (Bar Harbor, ME). Mice (5–6 wk old) were placed on a normal diet (ND), cholesterol-enriched diet (HC; Teklad 94059, Harlan Teklad), or cholate-free HC (Teklad 94059, Harlan Teklad) for 60 min before the generation of dose-response curves.

Splenocyte transfer. The procedures used for the adoptive transfer of splenocytes into SCID mice have been previously described (34, 35). Briefly, spleens harvested from WT donor mice on ND or 9 days of HC were scraped through a screen (E-C Apparatus) and suspended in cold PBS. Red blood cells were lysed, and the remaining splenocytes were resuspended in PBS (2.5 × 10<sup>6</sup> cells/ml). Recipient SCID mice on the ND received intraperitoneally 0.2 ml of the splenocyte suspension from WT-ND donor mice. Similarly, recipient SCID-ND mice received (intraperitoneally) 0.2 ml of the splenocyte suspension at 9 days of HC. Five days after reconstitution, recipient SCID-ND mice were used for experimentation (SCID-ND mice received a total of 14 daily doses of HC). In another series of experiments, splenocytes from gp91<sup>phox</sup><sup>−/−</sup>/SCID mice were isolated at 9 days of HC and intraperitoneally injected into recipient SCID-ND mice also at 9 days of HC (gp91<sup>phox</sup>−/−/<sub>−</sub>/SCID). In a third series of adoptive transfer experiments, CD4+ T cells were isolated from spleens of WT-ND and WT-SCID mice using the MACs system (Miltenyi Biotec) with negative selection for CD8<sup>+</sup> T cells, B cells (B220<sup>+</sup>), and macrophage/monocyte/neutrophils (CD11b<sup>+</sup>). This procedure yielded a cell population of >90% CD4<sup>+</sup> T lymphocytes, as verified by flow cytometry. We (34, 35) have previously reported that this method of transfer does not restore circulating populations of lymphocytes, despite being sufficient to restore HC-induced inflammation. This lack of lymphocytes in the blood of mice receiving splenocytes supports a role for soluble mediators from the injected immune cells in the HC-phenotype. Recipient mice received 3 × 10<sup>6</sup> T cells in 200 µl intraperitoneally at 9 days of HC and were allowed to recover for 5 days (2 wk of HC total) before experimentation. These mice were designated as CD4+ T cells/IFN-γ<sup>−/−</sup> mice.

Surgical protocols. Mice were anesthetized with xylazine (7.5 mg/kg body wt ip) and ketamine chloride (150 mg/kg body wt ip). After the mouse had been placed in the supine position, the right carotid artery was cannulated for systemic arterial pressure measurement. The thoracic and abdominal cavities of the mouse were opened through a midline incision. After hemostasis was achieved, the thoracoabdominal aorta was carefully dissected, removed, and placed in an oxygenated petri dish filled with ice-cold physiological salt solution (PSS). The composition of PSS was (in mM) 119 NaCl, 4.5 KCl, 25 NaHCO<sub>3</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>, 11 t-glucose, and 2.5 CaCl<sub>2</sub>. Using a dissecting microscope, the attached fat and adventitia were meticulously removed by sharp dissection. Clotted blood was removed from the vessel lumen with sharp forceps. The Louisiana State University Health Sciences Center (Shreveport, LA) Institutional Animal Care and Use Committee approved all animal procedures employed in this study.

Wire myography. The aorta was cut into 2-mm-long segments and mounted on an eight-channel wire myograph (Radeon Glass, Monrovia, CA). Vessel rings were maintained in 30-ml organ baths with oxygenated PSS (95% O2 and 5% CO2) at 37.1°C. The PSS in each organ bath was changed every 15 min throughout the experiment; 1,000-mg pretension was placed on each aortic ring (appropriate starting tension for optimal vasomotor function as determined in previous experiments). An eight-channel octal bridge (Powerlab) and data-acquisition software (Chart version 5.2.2) were used to record all force measurements. After equilibration for 1 h, aortic rings were rinsed with a 120 mM KCl solution for vascular smooth muscle activation and to determine the maximal contractile response. This was repeated twice with 200-mg increments in tension. Vessel contractility to KCl at each tension setting was analyzed (DMT normalization module, AB Instruments), and the optimal resting tension was determined (23). Following the final KCl rinse, aortic rings were adjusted to the optimal tension and allowed to equilibrate for a further 60 min before the generation of dose-response curves.

Experimental protocols. Each aortic ring was used to generate a dose-response curve to test for 1) endothelium-dependent contraction to phenylephrine (PE), 2) endothelium-independent dilation to sodium nitroprusside (SNP), and 3) endothelium-dependent dilation to acetylcholine (ACh). The vasoactive agent under study was added to the organ bath in increasing concentration from 10<sup>−9</sup> M to 10<sup>−4</sup> M. For the determination of SNP and ACh dose-response relationships, aortic rings were precontracted with 10<sup>−6</sup> M PE, and SNP or ACh was then added in increasing concentrations from 10<sup>−9</sup> M to 10<sup>−4</sup> M. Complete ACh concentration-vasodilation response relationships are presented for some experimental groups (Fig. 1A), with subsequent histograms depicting the dilation responses noted at 10<sup>−4</sup> M ACh. Endothelium-dependent dilation was expressed as the percent dilation from the precontraction (10<sup>−6</sup> M PE).

Cytochrome c reduction assay. Superoxide production by aortic tissue was quantified using the cytochrome c reduction assay (7). The specificity of the assay for superoxide was verified by measuring the difference in absorbance (at 550 nm) in the presence and absence of superoxide dismutase.

Real-time PCR measurements of NADPH oxidase subunits Nox-1, Nox-2, and Nox-4. Quantification of mRNA expression of NADPH oxidase subunits Nox-1, Nox-2 (anti<sub>M14</sub>), and Nox-4 was performed on aortic tissue derived from WT-ND, WT-HC, CD4<sup>−/−</sup>/HC, IFN-γ<sup>−/−</sup>/HC, IFN-γ<sup>−/−</sup>/HC, CD4+ T cells/IFN-γ<sup>−/−</sup>/HC (n = 5 mice/group) using a predeveloped assay for real-time PCR (RT-PCR, Applied Biosystems). Samples of cDNA (10 ng) derived from the aorta of each animal were assayed in duplicate using an ABI PRISM 7500 bioanalyzer, and gene expression was quantified using a comparative critical threshold (Ct) method according to the manufacturer’s suggestions. The Ct value reflects the cycle number at which the DNA amplification is first detected. For each sample, a Ct value was obtained by subtracting GAPDH Ct values from those of each target gene, thereby allowing the expression of each target gene to be normalized to GAPDH content.
Chimeras. Three different of bone marrow chimeras were produced. WT → WT chimeras were C57B/6 mice (CD45.2-positive leukocytes) that received bone marrow cells from CD45 congenic mice (CD45.1-positive leukocytes). This resulted in a significant increase of leukocytes expressing CD45.1 (of donor origin), from \( \frac{5}{H^{11021}} \) % in WT mice to \( \frac{90}{H^{11022}} \) % in WT → WT chimeras, allowing the verification of chimerization, as previously described (33). gp91phox+/−/− → WT chimeras were produced by transplanting bone marrow from gp91phox+/−/− mice (CD45.2-positive leukocytes) into congenic WT recipients (CD45.1-positive leukocytes). Similarly, WT → gp91phox+/−/− chimeras were produced by transplanting bone marrow from congenic WT mice (CD45.1-positive leukocytes) into gp91phox+/−/− recipients (CD45.2-positive leukocytes). Flow cytometry was used to verify the degree of chimerization in all animals by staining for CD45.1 and CD45.2 expression on leukocytes with a FITC-labeled anti-CD45.1 antibody and a biotinylated anti-CD45.2 antibody with a Streptavidin-PerCP secondary antibody (PharMingen). Only chimeras demonstrating 90% or greater conversion of leukocyte antigen to donor phenotype were used in the experiments.

Statistical analysis. All values are reported as means ± SE. ANOVA (Scheffé) was used for statistical comparison of the experimental groups with statistical significance set at \( P < 0.05 \).

RESULTS

While the Paigen diet, which is supplemented with cholate to facilitate fat assimilation, has been widely used to cause hypercholesterolemia and promote atherogenesis (25, 26), evidence linking dietary cholate to the activation of inflammatory and fibrotic genes in the liver (39) has raised concerns about whether the vascular responses elicited by cholate-containing, cholesterol-enriched diets may be elicited by cholate per se rather than the resultant hypercholesterolemia (9). To directly address this issue, we compared the changes in EDV and superoxide production in aortic rings between mice placed on the Paigen diet versus a diet enriched in only cholate (at the same concentration as the Paigen diet). We noted a significant difference in ACh-mediated vasodilation between the two diet groups, demonstrating impairment of endothelium-dependent vasomotor function in mice on cholesterol-enriched chow, whereas WT mice placed on a cholate-enriched diet exhibited ACh-mediated vasodilation similar to ND controls (Fig. 1A) without any increase in plasma cholesterol concentration (61 ± 8.9 mg/dl). Aortic rings from WT mice on a cholate-enriched diet also showed no increase in superoxide formation compared with the WT-ND group (Fig. 2A). Our findings indicate
that the responses of aortic tissue receiving the cholate-containing, cholesterol-enriched chow cannot be reproduced by dietary cholate alone, suggesting that cholate does not account for the HC-dependent responses that were evaluated in this study, which is consistent with the results of a previous report (18) describing no effect of dietary cholate on ischemiaperfusion-induced leukocyte adhesion in the mouse brain. However, to further address the role of hypercholesterolemia in the impaired EDV and increased superoxide in aortic rings, we placed WT mice on cholate-free HC. In this instance, the 2-wk dietary treatment was associated with a smaller increment in plasma cholesterol (126 ± 9.9 vs. 179 ± 21.2 mg/dl) and a comparable attenuation of diet-enhanced superoxide production (52 ± 11.6 pmol/ring) compared with the Paigen diet response (88 ± 6.7 pmol/ring). The blunted responses were accompanied by a diminished EDV response (17–19% reduction) that occurred only over an ACh concentration range of 10⁻⁶–10⁻⁸. These findings, coupled with our data with the cholate-enriched diet, indicate that the primary contribution of cholate may be to produce a plasma cholesterol concentration within a 2-wk period that is sufficient to elicit a large superoxide flux and the consequent impairment of EDV.

The serum cholesterol concentration in WT mice placed on the cholesterol-enriched diet (180.94 ± 6.76 mg/dl) was significantly (P < 0.05) higher than that detected in WT mice placed on normal chow (61.26 ± 8.12 mg/dl). No significant differences in serum cholesterol concentration were noted between any of the mutant mouse groups placed on HC and their WT counterparts placed on the same diet (data not shown).

A comparison of the dose-response relationships for endothelium-independent contraction (PE) and dilation (SNP) between aortic rings derived from mice placed on ND versus HC revealed no statistically significant differences (data not shown). However, ACh-mediated vasodilation (EDV) was significantly attenuated in aortic rings from WT-HC mice compared with their normocholesterolemic (WT-ND) counterparts (Fig. 1B). The attenuated vasodilatory response in WT-HC mice was accompanied by increased superoxide formation by aortic rings (Fig. 2B). The inhibitory effect of HC on EDV was not evident in SCID mice placed on HC (Fig. 1B), nor was the elevated superoxide generation by aortic tissue (Fig. 2B). Adoptive transfer of splenocytes isolated from WT-HC mice into SCID-HC recipients restored the defective vasodilatory phenotype (Fig. 1B) as well as the increased superoxide production (Fig. 2B), suggesting that lymphocyte deficiency protects against the impaired EDV and oxidative stress that were noted in SCID-HC mice.

Since the results obtained from SCID-HC mice implicate lymphocytes as potential mediators of the impaired endothelium-dependent vasomotor response and enhanced superoxide production associated with HC, we next chose to identify the specific lymphocyte population that mediates these HC-induced responses (Fig. 3, A and B). Mice deficient in either B lymphocytes or CD8⁺ T lymphocytes placed on HC exhibited vasodilatory responses (Fig. 3A) and changes in superoxide production (Fig. 3B) that were no different from those observed in WT-HC mice. However, CD4⁺ T cell-deficient mice placed on HC (like SCID-HC mice) did not exhibit the impaired EDV (Fig. 3A) or enhanced superoxide production (Fig. 3B) that were noted in aortic tissue derived from WT-HC mice, suggesting that CD4⁺ T lymphocytes mediate these vascular responses to HC.

Since IFN-γ is a major cytokine produced by CD4⁺ T lymphocytes, the plasma concentration of IFN-γ is elevated in HC mice (37), and the cytokine is known to upregulate the superoxide-producing enzyme NAD(P)H oxidase (22), we examined whether IFN-γ deficiency alters the vasomotor dysfunction and oxidative stress observed in the murine aorta during hypercholesterolemia. Figure 4 shows that both the impairment of EDV (A) and increased superoxide production (B) elicited by HC in WT mice were not observed in IFN-γ⁻/⁻ mice placed on the same HC. Reconstitution of IFN-γ⁻/⁻-HC mice with WT-HC CD4⁺ T cells restored the enhanced superoxide production and EDV dysfunction normally elicited by HC (Fig. 5, A and B). These findings suggest that CD4⁺ T cell-derived IFN-γ contributes to the increased superoxide production and vasomotor dysfunction induced by HC.

Another objective of this study was to determine whether NAD(P)H oxidase is a source of the superoxide that is produced at an accelerated rate in aortic tissue from HC mice and whether the enzyme contributes to the accompanying endothelium-dependent contraction (PE) and dilation (SNP) between aortic rings derived from mice placed on ND versus HC revealed no statistically significant differences (data not shown). However, ACh-mediated vasodilation (EDV) was significantly attenuated in aortic rings from WT-HC mice compared with their normocholesterolemic (WT-ND) counterparts (Fig. 1B). The attenuated vasodilatory response in WT-HC mice was accompanied by increased superoxide formation by aortic rings (Fig. 2B). The inhibitory effect of HC on EDV was not evident in SCID mice placed on HC (Fig. 1B), nor was the elevated superoxide generation by aortic tissue (Fig. 2B). Adoptive transfer of splenocytes isolated from WT-HC mice into SCID-HC recipients restored the defective vasodilatory phenotype (Fig. 1B) as well as the increased superoxide production (Fig. 2B), suggesting that lymphocyte deficiency protects against the impaired EDV and oxidative stress that were noted in SCID-HC mice. Since the results observed from SCID-HC mice implicate lymphocytes as potential mediators of the impaired endothelium-dependent vasomotor response and enhanced superoxide production associated with HC, we next chose to identify the specific lymphocyte population that mediates these HC-induced responses (Fig. 3, A and B). Mice deficient in either B lymphocytes or CD8⁺ T lymphocytes placed on HC exhibited vasodilatory responses (Fig. 3A) and changes in superoxide production (Fig. 3B) that were no different from those observed in WT-HC mice. However, CD4⁺ T cell-deficient mice placed on HC (like SCID-HC mice) did not exhibit the impaired EDV (Fig. 3A) or enhanced superoxide production (Fig. 3B) that were noted in aortic tissue derived from WT-HC mice, suggesting that CD4⁺ T lymphocytes mediate these vascular responses to HC.

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lium-dependent vasomotor dysfunction observed in our model. We found that neither the impaired vasodilation response to ACh (Fig. 6A) nor the increased superoxide production (Fig. 6B) normally elicited by HC in WT mice was noted in HC mice that were genetically deficient in the gp91phox subunit of NAD(P)H oxidase, suggesting a major role for this enzyme in the HC-induced vascular alterations. To address the possibility that lymphocyte-derived Nox-2 is a source of HC-induced ROS and the impaired EDV function, we adoptively transferred gp91phox−/− splenocytes into SCID recipients (gp91phox−/−SCID mice). There were no differences between the vascular responses of gp91phox−/−SCID-HC mice and WT-HC controls (Fig. 6), suggesting that Nox-2 in either T or B lymphocytes is not involved in HC-induced ROS production or EDV dysfunction.

In a separate series of experiments, we used gp91phox−/− bone marrow chimeras to determine the relative contributions of blood cell versus vessel wall Nox-2 to the HC-induced superoxide production and EDV dysfunction (Fig. 7, A and B). These experiments revealed normal HC-induced vascular responses in WT→WT and gp91phox−/−→WT chimeras, whereas attenuated ROS production and restored vasomotor function were detected in WT→gp91phox−/− chimeras (similar to what we already noted with gp91phox−/−HC mice). These findings suggest that Nox-2 in the vascular wall, rather than in circulating blood cells (including lymphocytes), mediates the vascular responses (enhance superoxide production and impaired EDV) elicited by HC.

Table 1 summarizes the changes in gp91phox mRNA expression observed in WT-ND, WT-HC, CD4−/−-ND, CD4−/−-HC, IFN-γ−/−-ND, IFN-γ−/−-HC, CD4+ T cells/IFN-γ−/−-ND, and CD4+ T cells/IFN-γ−/−-HC mice. We found a significant increase in Nox-2 (gp91phox) mRNA expression in the WT-HC group compared with WT-ND controls. The expression of gp91phox mRNA was significantly lower in aortic tissues of all ND groups (WT and mutant mice) compared with WT-HC mice. Deficiency of either CD4+ T cells or IFN-γ significantly abrogated HC-induced Nox-2 mRNA expression. However, adoptive transfer of WT-HC CD4+ T cells into IFN-γ−/−HC mice resulted in increased Nox-2 mRNA expression similar to WT-HC controls. Nox-1 and Nox-4 mRNA were detected in all samples; however, their expression did not differ between groups (data not shown).

**DISCUSSION**

There is a substantial and growing body of evidence that implicates immune cell activation and oxidative stress in the...
atherosclerotic lesion development associated with prolonged hypercholesterolemia (1, 19, 20, 31). However, there is relatively little information in the literature that bears on the contribution of immune cells to the endothelium-dependent vasomotor dysfunction that is manifested even in the early stages of hypercholesterolemia and on whether immune cell activation underlies the accelerated production of ROS by blood vessels exposed to elevated cholesterol levels. The findings of the present study strongly implicate CD4+ T lymphocytes and CD4+ T cell-derived IFN-γ in the impaired EDV induced by HC and provide novel insights into the causal relationship between lymphocyte activation, enhanced production of ROS, and impaired EDV.

The results of this study indicate that T lymphocytes, but not B lymphocytes, are important mediators of the HC-induced vasomotor dysfunction. This conclusion is supported by the observations that hypercholesterolemic SCID, but not B cell-deficient, mice exhibit a vasodilator response to ACh that is similar to that observed in WT mice on normal chow. Further support is provided by the observation that adoptive transfer of splenocytes from WT-HC mice into SCID-HC recipients restored the defective vasodilation phenotype. Our finding that T lymphocytes mediate the early vasomotor alterations associated with HC are consistent with reports describing a major role for T cells in driving the vessel inflammation in, and

Table 1. mRNA expression of gp91phox in aortic tissue of WT and mutant mice on ND and HD

<table>
<thead>
<tr>
<th>Groups</th>
<th>Relative gp91phox mRNA Expression</th>
</tr>
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<tbody>
<tr>
<td>WT-ND</td>
<td>1.00±0.070</td>
</tr>
<tr>
<td>WT-HC</td>
<td>2.97±0.230*</td>
</tr>
<tr>
<td>CD4+/−-ND</td>
<td>1.28±0.110*</td>
</tr>
<tr>
<td>CD4+/−-HC</td>
<td>1.19±0.190*</td>
</tr>
<tr>
<td>IFN-γ+/−-ND</td>
<td>1.21±0.171*</td>
</tr>
<tr>
<td>IFN-γ+/−-HC</td>
<td>1.30±0.226*</td>
</tr>
<tr>
<td>CD4+ T cells/IFN-γ−/−-ND</td>
<td>0.94±0.065*</td>
</tr>
<tr>
<td>CD4+ T cells/IFN-γ−/−-HC</td>
<td>2.26±0.263*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 mice/group. mRNA expression of gp91phox was determined in aortic tissue derived from wild-type (WT) mice, CD4+ T lymphocyte-deficient (CD4+/−) mice, mice deficient in the cytokine IFN-γ (IFN-γ−/− mice), and IFN-γ−/− mice that received adoptive transfer of WT CD4+ T cells (CD4+/+ T cells/IFN-γ+/− mice). Each group was placed on either a normal diet (ND) or a high-cholesterol diet (HC) for 2 wk. gp91phox levels were normalized to GAPDH and expressed relative to the gp91phox mRNA expression of the WT-ND group. *P < 0.05 vs. the WT-ND group; †P < 0.05 vs. the WT-HC group.
progression of, atherosclerotic lesions (2, 17) and in mediating the impaired EDV in cremaster arterioles of mice placed on a cholesterol-enriched diet for 2 wk and in the genesis of angiotensin II-induced hypertension and vascular dysfunction (12).

We also provide evidence that the T lymphocyte-dependent vasomotor dysfunction induced by HC is mediated by CD4+, but not CD8+, T cells (Fig. 3). These findings are consistent with a report (41) describing a major role for CD4+ T cells in the development of atherosclerotic plaques in apolipoprotein E-deficient mice as well as a study (32) that implicates CD4+ T lymphocytes in the formation of human atherosclerotic plaques. Although it has been reported that patients with coronary artery disease exhibit endothelium-dependent vascular dysfunction in the forearm vasculature that is significantly correlated with CD8+ T cells expressing ICAM-1 (30), the relative contributions of CD4+ and CD8+ T cells to vasomotor dysfunction have not been previously addressed in hypercholesterolemia.

A likely mediator of CD4+ T cell-dependent vasomotor responses in HC mice is IFN-γ. This cytokine is produced by activated CD4+ T lymphocytes (24), the plasma IFN-γ concentration is significantly elevated in WT-HC mice (37), and it has been implicated in the development of atherosclerotic plaques (3) as well as the early inflammatory phenotype assumed by the microvasculature in mice placed on a cholesterol-enriched diet (35). Although it has previously been shown that incubation of isolated bovine mesenteric arterial rings with IFN-γ results in a significantly blunted dilation response to ACh (5), our previous findings in arterioles suggest an indirect role for IFN-γ (37). Our finding that IFN-γ-mice placed on HC do not exhibit the impaired EDV seen in WT-HC mice is consistent with a role for IFN-γ in HC-induced vasomotor dysfunction (Fig. 4). This possibility is supported by our observation that adoptive transfer of WT-HC CD4+ T cells into HC IFN-γ-deficient recipients restored the HC-induced EDV dysfunction.

The contribution of enhanced superoxide production to the impaired endothelium-dependent vascular responses of large arteries and microvessels induced by hypercholesterolemia has been extensively characterized (4, 6, 38, 40). Superoxide is believed to mediate its effects on endothelial-dependent dilation by inactivating endothelial cell-derived nitric oxide, which relaxes the underlying smooth muscle cells via a guanylate cyclase-dependent mechanism (3, 21). Vascular NAD(P)H oxidase is generally considered to be a major source of the enhanced superoxide fluxes produced by large arteries and microvessels in hypercholesterolemic animals (3, 6, 10) and in humans with coronary artery disease (13). The results of our study provide additional support for a major role of NAD(P)H oxidase-derived superoxide in the impaired endothelium-dependent vasomotor response to hypercholesterolemia (Fig. 6). We confirmed with RT-PCR that mRNA expression of Nox-2 (gp91phox), but not Nox-1 or Nox-4, is significantly elevated in aortic tissue from WT-HC mice (Table 1). In addition, using a cytochrome c reduction assay, we demonstrated greatly enhanced production of superoxide in aortic rings from HC mice compared with their ND counterparts (Fig. 2). This HC-induced enhancement of superoxide production was not observed in mice genetically deficient in gp91phox (Fig. 6). Furthermore, gp91phox−/− mice did not exhibit the HC-induced vasomotor impairment seen in WT mice (Fig. 6). Bone marrow chimeras developed using gp91phox−/− mice as either marrow donors or recipients revealed that vascular wall (rather than circulating cell) NAD(P)H oxidase is the source of the HC-induced superoxide production and subsequent EDV dysfunction (Fig. 7).

The results of our study also demonstrate an important link between immune cells and the enhancement of superoxide production in hypercholesterolemia. SCID mice as well as CD4−/− mice did not exhibit the HC-induced enhancement of superoxide production that is manifested in WT mice as well as in B cell−/− and CD8+ T cell−/− mice (Fig. 3). While it remains unclear how this subset of T lymphocytes enhances superoxide production by the vasculature, our finding that IFN-γ−/− mice placed on HC exhibit attenuated superoxide production similar to that detected in hypercholesterolemic SCID and CD4−/− mice suggests a role for CD4+ T cell-derived IFN-γ (Figs. 2–4). This possibility is supported by our observation that adoptive transfer of WT-HC CD4+ T cells into IFN-γ−/−HC recipients restored the enhanced superoxide production normally seen in WT-HC mice (Fig. 5). Further support is provided by our gp91phox mRNA expression data as well as a recent report (22) that describes the ability of IFN-γ to induce the expression of p22phox mRNA and stimulate superoxide production by human mesangial cells. Moreover, we (35) have previously reported that the prooxidative and proinflammatory phenotype that is assumed by postcapillary venules in WT-HC mice is not manifested in IFN-γ−/−HC mice, but the phenotype can be restored by adoptive transfer of WT-HC splenocytes into IFN-γ−/−HC mice.

Overall, the findings of this study suggest a major role for CD4+ T lymphocytes, IFN-γ, and NAD(P)H oxidase-derived superoxide in the impaired endothelium-dependent vasomotor responses that accompany hypercholesterolemia. Our findings support the concept that hypercholesterolemia results in the activation of CD4+ T cells, which results in an increased production/release of IFN-γ. This cytokine then acts, either directly or indirectly, on the vascular wall (possibly endothelial cells) to increase the production of superoxide by NAD(P)H oxidase. The elevated superoxide levels result in the inactivation of nitric oxide that is generated by nitric oxide synthase in response to the engagement of ACh with its receptor on endothelial cells, leading to impaired relaxation of the underlying smooth muscle. Additional work is needed to better understand the contribution of the immune system to the vasomotor dysfunction that accompanies cardiovascular risk factors such as hypercholesterolemia.

ACKNOWLEDGMENTS

The authors thank Dr. Chantal Rivera and Monique Allman for the help and expertise in RT-PCR.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant HL-26441.

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