Hypercholesterolemia Promotes Endothelial Dysfunction in Vitamin E- and Selenium-Deficient Rats

Leopoldo Raij, Judit Nagy, Karen Coffee, Eugene G. DeMaster

Abnormal regulation of local vascular tone occurs early in human and experimental atherosclerosis. Impaired endothelium-dependent vascular relaxations mediated by endothelium-derived relaxing factor are an important contributor to these abnormalities. Endothelium-derived relaxing factor is nitric oxide released as such or attached to a carrier molecule. Oxidized lipoproteins impede endothelium-derived relaxing factor-mediated responses in vitro. We designed in vivo experiments to determine whether hypercholesterolemia with and without deficiency of two endogenous lipid antioxidants, vitamin E and selenium, would result in endothelial dysfunction. Vitamin E and selenium deficiencies were induced in a group of hypertension-prone Dahl salt-sensitive rats fed a diet high in cholesterol (4%) but low in NaCl (0.5%) for 18 weeks. Two other groups of Dahl salt-sensitive rats received diets sufficient in vitamin E and selenium but containing either high or normal cholesterol levels (control group). Serum cholesterol levels increased approximately 10-fold in the two groups of rats fed high-cholesterol diets. Systolic blood pressure was 143±3 mm Hg in high-cholesterol/vitamin E- and selenium-sufficient rats and 142±5 mm Hg in high-cholesterol/vitamin E- and selenium-deficient rats (NS). Mild intimal thickening and occasional mononuclear cell infiltration were observed in both of these groups. Serum vitamin E levels were decreased, whereas serum thiobarbituric acid-reactive substances and exhaled pentane (two indicators of endogenous lipid oxidation) were significantly increased in high-cholesterol/vitamin E- and selenium-deficient rats compared with high-cholesterol/vitamin E- and selenium-sufficient rats. Vascular relaxations to acetylcholine and adenosine diphosphate, two agonists of endothelium-dependent relaxations, were significantly impaired in aortic rings from only the high-cholesterol/vitamin E- and selenium-deficient rats compared with high-cholesterol/vitamin E- and selenium-sufficient rats. Neither indomethacin nor the scavenger of superoxide anion superoxide dismutase normalized relaxations in the impaired aortic rings. Relaxations in response to the endothelium-independent vasodilator sodium nitroprusside were normal in all three rat groups. Our findings indicate that hypercholesterolemia coexisting with increased levels of endogenous oxidants or deficient levels of antioxidants results in impaired endothelium-dependent vasodilation mediated by endothelium-derived relaxing factor. (Hypertension 1993;22:56-61)

KEY WORDS • lipoproteins • endothelium • vitamin E • atherosclerosis • hypercholesterolemia • selenium

The association between atherogenesis and hypercholesterolemia has been documented in animals and humans. However, the mechanisms involved in the initiation and progression of the vascular lesions are not completely understood. The normal physiology of the vascular wall depends on the presence of a functionally intact endothelium capable of modulating local vascular tone and preventing thrombosis. Endothelial dysfunction occurs early in atherosclerosis and is manifested by impaired endothelium-dependent vascular relaxations mediated by endothelium-derived relaxing factor (EDRF). EDRF is nitric oxide (NO) released as such or attached to a carrier molecule. However, an increase in endothelium-derived contracting factor or factors may also contribute to the abnormal vascular tone of atherosclerotic vessels.

Studies in vitro have shown that exposure of intact vessels to oxidized low-density lipoprotein (LDL) results in impaired endothelium-mediated relaxations. This impairment has been attributed to decreased synthesis and/or release as well as to increased inactivation of EDRF. More recent in vitro studies have shown that not only oxidized LDL but also oxidized high-density lipoprotein (HDL) can inactivate NO and that this inactivation is due to the lipid component of the lipoproteins. Indeed, on a molar basis, oxidized LDL and HDL are equally effective in blocking the NO enhancement of guanylate cyclase activity and cyclic guanosine monophosphate production. Experimentally, probucol, which has antioxidant properties, ameliorates atherosclerotic changes in hypercholesterolemic rabbits.

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Both vitamin E and selenium are important factors in the protection of lipids from oxidation. Vitamin E is structurally incorporated in lipoproteins and acts as an intrinsic antioxidant that blocks electron transfers involved in the initiation and propagation of lipid peroxidation.\(^{16-18}\) Selenium is critical for both the synthesis and activity of glutathione peroxidase, an enzyme that detoxifies organic peroxides, which in turn can oxidize lipoproteins, particularly when vitamin E is deficient.\(^{19,20}\)

In early preliminary experiments, we found that deficiency in vitamin E and selenium does not result in abnormal endothelium-dependent or endothelium-independent relaxations in normocholesterolemic rats. The present experiments were designed to determine (1) whether hypercholesterolemia in the presence and absence of vitamin E and selenium deficiency would result in endothelial dysfunction and (2) whether endothelial dysfunction would lead to hypertension in salt-sensitive, hypertension-prone rats maintained on a low-salt diet.

**Methods**

**Materials**

Bovine erythrocyte superoxide dismutase (No. S-2515) and all drugs used were purchased from Sigma Chemical Co, St Louis, Mo. A radioimmunoassay kit for the analysis of plasma endothelin-1 was obtained from Amersham Corp, Arlington Heights, Ill.

**Experimental Animals**

Male, 6-week-old (190-210 g), Dahl salt-sensitive rats from the Brookhaven strain were purchased from Harlan Sprague Dawley, Indianapolis, Ind. All rats were housed five to a cage and had free access to water. The animals were housed in facilities accredited by the American Association for Accreditation of Laboratory Animal Care, and the animal studies were approved by the Institutional Animal Care and Use Committee.

Three groups were studied: a normal-cholesterol (NChol) control group fed standard rat chow containing 0.5% NaCl, 0.3 mg/kg selenium, and 50 mg/kg vitamin E (certified rodent chow No. 5002, Purina Mills, St Louis, Mo); a high-cholesterol (HChol) group fed a diet containing 4% cholesterol and 1% cholic acid,\(^{21}\) with a selenium content of 0.3 mg/kg and a vitamin E content of 50 mg/kg; and a high-cholesterol and deficient (HChol-Def) group fed a high-cholesterol diet containing no vitamin E or selenium. Both experimental diets (diets 91026 and 91027, Teklad Premier Laboratory Diets, Madison, Wis) had sodium, mineral, and protein contents similar to the standard diet and were given for 18 weeks.

Systolic blood pressure was measured in conscious, unanesthetized rats by the tail-cuff method using a Physiograph MK IV (Narco Biosystems, Houston, Tex) as described previously.\(^{22}\) After an overnight fast, rats were given sodium pentobarbital (50 mg/kg ip); after a midline incision, blood was collected from the abdominal aorta for biochemical analysis. The rats were killed by exsanguination, after which thoracic aortas were excised and processed for studies in organ chambers according to techniques previously described.\(^{22,23}\)

**Biochemical Analysis**

Total plasma cholesterol was analyzed with an Ektachem 700XR (Eastman Kodak Co, Rochester, NY). Levels of serum thiobarbituric acid–reactive substances (TBARS) in units of nanomoles of malondialdehyde equivalents per milliliter of serum were determined as previously described.\(^{24}\) Exhaled pentane was determined as described elsewhere for measurement of ethane production. Pentane was quantified by gas chromatographic analysis using a Porapak Q column (Alltech Associates Inc, Deerfield, Ill) maintained at 150°C (carrier gas, nitrogen; carrier gas flow rate, 40 mL/min). Limitations and specificity of these measures of lipid peroxidation have been reviewed.\(^{25,26}\) Serum vitamin E levels were measured with a high-performance liquid chromatographic method.\(^{27}\)

**Organ Chamber Experiments**

Aortic rings were suspended between two stirrups in organ chambers filled with oxygenated modified Krebs-Ringer bicarbonate (25 mL) at 37°C.\(^{21,22}\) In some rings, the endothelium was removed.\(^{21,22}\) The rings were contrated with 1-norepinephrine bitartrate (NE; 10⁻³ to 10⁻⁸ M), and the contractions were expressed in absolute tension in grams. In some experiments (n = 4), 100 U superoxide dismutase per milliliter was added to the organ baths containing aortic rings from HChol-Def rats; superoxide dismutase scavenges superoxide anion (O₂⁻), which is a powerful inactivator of NO.\(^{4,5}\)

Relaxations to acetylcholine (10⁻⁴ to 10⁻⁸ M) and to adenosine diphosphate (ADP; 10⁻⁶ M) were studied in rings precontracted 60% to 70% of maximal contraction with NE. Relaxations to sodium nitroprusside (10⁻⁴ to 10⁻⁸ M) were studied in endothelium-denuded rings precontracted maximally. For acetylcholine, relaxations are expressed as percent decrease in tension (Figure). In addition, for acetylcholine and sodium nitroprusside, the concentration causing 50% relaxation in contracted rings (EC₅₀) is expressed as the negative log M.\(^{21,22}\) For
ADP, relaxations are expressed as percent decrease in tension (Table 1).

Morphological Examination

Processing of aortic rings for light and transmission electron microscopy was performed using techniques previously described. For light microscopy, aortic sections (1-μm thickness, toluidine staining) were prepared and examined from five rats in each group in a blinded fashion by one of us (J.N.). The average thickness of the media and intima was measured with a calibrated ocular micrometer in three aortic sections from each rat, with four measurements per section.

Statistics

Results are expressed as mean±SEM. Statistical analysis was performed by analysis of variance or unpaired Student's t test, as indicated. A value of P<.05 was considered significant.

Results

Weight

A decrease in body weight in HChol-Def rats (Table 2) was observed similar to that previously reported. Therefore, NChol rats were selected by body weight to match the HChol-Def rats.

Cholesterol Levels

Similar levels of hypercholesterolemia developed in the two experimental rat groups. Serum vitamin E levels were significantly lower in the HChol-Def rats (Table 2). The high levels of vitamin E in HChol rats are due to the fact that vitamin E is lipoprotein bound; therefore, absolute values of vitamin E are increased in hypercholesterolemia. This becomes evident when average vitamin E serum levels are expressed as a ratio of vitamin E (in micrograms per milliliter) to total cholesterol (in milligrams); these ratios were 2.7, 0.18, and 10.9 for the HChol, HChol-Def, and NChol groups, respectively. Two indicators of endogenous lipid oxidation, exhaled pentane and TBARS, were significantly increased in HChol-Def rats (Table 2).

Vascular Relaxations

In all the rats, acetylcholine (10⁻⁹ to 10⁻⁴ M) and ADP (10⁻⁶ M), two receptor-dependent agonists of EDRF, induced relaxation of NE-contracted aortic rings in those with endothelium but not in those without endothelium. Relaxations in response to acetylcholine were significantly diminished in aortic rings from HChol-Def versus NChol and HChol rats over a concentration range of 10⁻⁹ to 10⁻⁴ M (Figure and Table 1). Moreover, maximal relaxations to acetylcholine were significantly reduced in HChol-Def rats (65±10%) versus NChol rats (98±2%) and HChol rats (95±3%) (P<.05, Figure). Vascular relaxations in response to ADP (10⁻⁵ M) were, like maximal relaxations to acetylcholine, significantly impaired only in HChol-Def rats (Table 1). Maximal contractions in response to NE were similar in all rat groups (Table 1). In aortic rings denuded of endothelium and constricted maximally with NE, vascular relaxations to sodium nitroprusside were similar in all three rat groups (Table 1).

No evidence was found to support an involvement of the cyclooxygenase pathway, endothelin-1, or acute exposure to O₂⁻ in the observed endothelial dysfunction in the HChol-Def rats. Addition of indomethacin (10⁻⁵

<table>
<thead>
<tr>
<th>Rat group</th>
<th>Body weight (g)*</th>
<th>Total cholesterol (mg/dL)*</th>
<th>Serum vitamin E (μg/mL)*</th>
<th>Serum TBARS (nmol/mL†)</th>
<th>Exhaled pentane (pmol/30 min)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>HChol</td>
<td>466±5</td>
<td>650±56†</td>
<td>16.0±12</td>
<td>1.38±0.02</td>
<td>0.5±0.4</td>
</tr>
<tr>
<td></td>
<td>(n=8)</td>
<td>(n=8)</td>
<td>(n=5)</td>
<td>(n=8)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>HChol-Def</td>
<td>355±20</td>
<td>542±53†</td>
<td>1.0±0.2§</td>
<td>3.45±0.08§</td>
<td>9.1±4.7§</td>
</tr>
<tr>
<td></td>
<td>(n=6)</td>
<td>(n=6)</td>
<td>(n=5)</td>
<td>(n=8)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>NChol</td>
<td>338±9</td>
<td>55±15†</td>
<td>0.6±0.3</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>(n=6)</td>
<td>(n=6)</td>
<td>(n=5)</td>
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</tbody>
</table>

TBARS, thiobarbituric acid-reactive substances; HChol, high-cholesterol diet with vitamin E and selenium; HChol-Def, high-cholesterol diet with no vitamin E or selenium; NChol, normal-cholesterol control diet with vitamin E and selenium.

*Statistical comparison between groups performed by analysis of variance.
†Statistical comparison between groups performed by Student's t test.
‡P<.05 vs NChol.
§P<.05 vs HChol.
M) to the organ bath buffer did not affect the dose response to acetylcholine of aortic rings of HChol-Def rats (EC_50, \(-\log M\), without indomethacin, 6.02±0.56; with indomethacin, 6.01±0.58, P=NS). Plasma endothelin-1 levels were similar in all rats (NChol, 13±1.2 fmol/mL, n=6; HChol, 13±0.5 fmol/mL, n=8; HChol-Def, 13±1.6 fmol/mL, n=6, P=NS). Superoxide dismutase failed to augment the endothelium-dependent vascular relaxations of aortas from HChol-Def rats, therefore suggesting that NO released from the endothelium was not destroyed by \(O_2^-\) generated within the organ chamber. However, an increased level of \(O_2^-\) within the endothelial cells cannot be completely excluded, because not enough superoxide dismutase may have diffused into the cells from the bath.

**Morphology**

Light and transmission electron microscopic studies revealed an intact endothelium and occasional macrophage-like cells in the subendothelium. There were no significant differences in medial thickness in HChol and HChol-Def rats compared with NChol rats (116±0.3, 117±0.4, and 113±0.3 \(\mu\)m, respectively). Mean intimal thickness in the NChol, HChol, and HChol-Def groups was 0.98±0.11, 3.87±0.12, and 3.99±0.16 \(\mu\)m, respectively (P=0.01 for HChol and HChol-Def versus NChol; P=NS for HChol-Def versus HChol). With the morphometric technique used, an increase of less than 5 \(\mu\)m in intimal thickness is considered minimal.

**Hemodynamics**

The magnitude of endothelial dysfunction in the HChol-Def rats was not sufficient to cause hypertension. Even in this hypertension-prone rat strain, the systolic blood pressure of the HChol-Def rats (142±5 mm Hg) was not significantly different from that observed in the HChol rats (143±3 mm Hg).

**Discussion**

Oxidized lipoproteins are believed to play an important role in atherogenesis. The rat is a species resistant to the development of atherosclerotic changes in response to hypercholesterolemia. Other species, such as the rabbit and pig, readily develop functional and morphological vascular abnormalities in response to hypercholesterolemia. Therefore, it could be speculated that the susceptibility of other species, as well as the increased resistance of rats, to develop marked vascular pathology due to hypercholesterolemia may be linked in part to their particular levels of endogenous oxidants or antioxidants or both.

The Dahl rat is genetically predisposed to development of hypertension when given a diet high in NaCl content but remains normotensive when maintained on a low-sodium diet, as in this study. This rat strain was chosen because we wanted to determine whether impaired endothelium-dependent relaxations, were they to occur, could be sufficient for the development of hypertension even in the absence of high-salt diet. Recent animal studies have shown that near-complete inhibition of NO (an EDRF) synthesis with \(l\)-arginine analogues results in sustained elevations of blood pressure.

Our studies show that hypercholesterolemia induces endothelial dysfunction but not vascular smooth muscle dysfunction in rats deficient in endogenous antioxidants. Vascular relaxations in response to receptor-dependent agonists of EDRF were significantly impaired in HChol-Def rats, whereas vascular responses to agonists of endothelium-independent relaxations were unaffected (Table 1, Figure). These changes in endothelium-dependent response mimic those observed in arteries from hypercholesterolemic humans.

Our results are consistent with the mediation of the observed endothelial dysfunction by oxidized lipoprotein. Vitamin E and selenium deficiency did cause the anticipated rise in serum TBARS and exhaled pentane levels (Table 2), classic markers for lipid peroxidation. The increased levels of serum TBARS in the HChol-Def rats suggest that oxidation did occur in the lipoprotein compartment of serum; however, a direct quantitation of the oxidized LDL and HDL serum fraction was not performed. In addition, lysolipids, substances known to produce a selective unresponsiveness to receptor-mediated endothelium-dependent relaxations, may also have contributed to the observed endothelial dysfunction in the HChol-Def animals. Formation of LDL-derived lysolipids occurs in an oxidative environment and is reduced in the presence of various antioxidants, including vitamin E. In previous unpublished studies, we found that endothelium-dependent relaxations in response to acetylcholine (10^(-9) to 10^(-4) M) were normal in a group of Sprague-Dawley rats fed for 13 weeks a diet similarly deficient in vitamin E and selenium but with normal cholesterol content. The EC_50 values for these normocholesterolemic, vitamin E-deficient and vitamin E-sufficient rats were 7.1 al±0.4 and 7.01±0.1, respectively (P=NS). This would suggest that (1) hypercholesterolemia is required to induce endothelial dysfunction in the setting of vitamin E and selenium deficiency and (2) vitamin E, selenium, or both do not have a direct effect on EDRF synthesis or release.

Endothelial dysfunction did not appear to be associated with increased cyclooxygenase-derived vasoconstrictor substances or with endothelin-1. Studies in hypercholesterolemic pigs have suggested that the impairment in endothelium-dependent relaxations may be due in part to increased synthesis of vasocostrictive prostanoids synthesized through the cyclooxygenase pathway. However, we found that indomethacin did not restore the impaired vascular response to acetylcholine of aortas from HChol-Def rats. Furthermore, plasma endothelin-1 levels, which have been reported to be increased in some patients with atherosclerosis, were similar in all three rat groups.

Light and transmission electron microscopic examination revealed an intact aortic endothelium and only a mild increase in intimal thickness with few macrophage-like cells present in this location in both HChol and HChol-Def rats. The lack of formation of atherosclerotic plaque is of interest. It can be speculated that our studies suggest that, in addition to lipid peroxidation, the participation of other coadjuvant factors is necessary for plaque development. For instance, modifications of the apoprotein in oxidized lipoproteins are important for their recognition by macrophage acetyl LDL receptors. However, the present studies were not designed to address the question of whether or not...
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qualitative or quantitative changes in apoproteins or in other critical factors required for plaque formation occurred.

The morphological and functional changes observed in vessels of the HChol-Def rats more closely mimic early atherosclerotic changes. Indeed, our findings agree with those of previous clinical14 and experimental13,14 studies, which suggest that endothelial dysfunction may precede the development of classic atherosclerotic changes. Moreover, the paucity of morphological changes in both groups of hypercholesterolemic rats and the severity of endothelial dysfunction confined to HChol-Def rats support the notion introduced by previous studies which suggested that oxidized lipoproteins may interfere with EDRF synthesis, release, or both via biochemical mechanisms.13,14

Functional impairment of the aortic endothelium of the magnitude observed in vitamin E– and selenium-deficient rats was not enough to foster development of significant hypertension even in hypertension-prone rats.21,22 On the other hand, it is important to note that this degree of endothelial dysfunction may be of clinical pathophysiological importance in the coronary and cerebral circulation, particularly when associated with increased thrombogenesis.39-41 Indeed, epidemiological studies have suggested a negative correlation between plasma levels of vitamin E and mortality from ischemic heart disease in humans.30

In conclusion, these studies provide in vivo evidence supporting the suggestions of earlier in vitro studies that oxidized lipoproteins, both LDL and HDL, can promote endothelial dysfunction characterized by impaired endothelium-dependent relaxations.11,13 Moreover, to the extent that our model approximates certain aspects of the atherogenic process,42 the results suggest that increased levels of endogenous oxidants or deficient levels of antioxidants may be pathophysiologically important in the initiation, progression, or both of vascular disease in hypercholesterolemic humans.15,43

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