Circulating and Tissue Endothelin Immunoreactivity in Hypercholesterolemic Pigs

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Background. Hypercholesterolemia is characterized by a coronary vasoconstrictive response to the endothelium-dependent vasodilator acetylcholine. This abnormality may be due to reduced synthesis of endothelium-derived relaxing factor and/or enhanced synthesis and release of an endothelium-derived contracting factor. Endothelin is an endothelium-derived vasoconstrictor and mitogenic peptide that is present in normal plasma, and its circulating concentrations are elevated in disease states that are characterized by abnormal endothelium-dependent relaxation to acetylcholine. The current studies were designed to test the hypotheses that experimental hypercholesterolemia results in elevation of plasma and tissue endothelin immunoreactivity and that the abnormal acetylcholine-evoked coronary vasoconstriction in the hypercholesterolemic animals is associated with further elevation of plasma endothelin.

Methods and Results. Plasma concentrations and molecular forms of endothelin immunoreactivity were determined following 2% cholesterol diet for 4 months in pigs and during intracoronary acetylcholine administration. Second, we assessed the presence of endothelin in the coronary vascular wall by using immunohistochemistry. Hypercholesterolemia elevated plasma endothelin concentration and enhanced coronary artery tissue endothelin immunoreactivity. The endothelium-dependent vasodilator acetylcholine further increases plasma endothelin in hypercholesterolemia in association with coronary vasoconstriction. The predominant molecular form of endothelin in hypercholesterolemia is the biological active endothelin-1.

Conclusions. This study suggests a role for endothelins as an early participant and a marker for the endothelial dysfunction in hypercholesterolemia as well as a participant in the atherogenic process. (Circulation. 1993;88:2923-2928.)

Key Words • endothelin • cholesterol • relaxing factors • coronary

Hypercholesterolemia in animal models and humans is characterized by an endothelial dysfunction. This is manifested by a coronary vasoconstrictive response to the endothelium-dependent vasodilator acetylcholine in the absence of gross morphological findings of atherosclerosis.1-5 This abnormality may be due to reduced synthesis of endothelium-derived relaxing factor (EDRF), altered membrane receptor coupling mechanisms affecting EDRF synthesis, and impaired diffusion or augmented destruction of EDRF in the vascular wall.3,6-8 Alternatively, the vasoconstrictive response could be mediated by enhanced synthesis and/or release of an endothelium-derived contracting factor.8,9 Endothelin is a 21-amino-acid endothelial-derived vasoconstrictor and mitogenic peptide that may function in a paracrine fashion to regulate vascular tone.10,11 In addition, endothelin is present in normal plasma, and its circulating concentrations are elevated in disease states that are characterized by abnormal endothelium-dependent relaxation to acetylcholine such as atherosclerosis and congestive heart failure,12-14 reflecting an imbalance between these two endothelium-derived factors. Indeed, studies have demonstrated that such pathophysiological concentrations of endothelin have functional importance in the control of regional and systemic vascular resistance.15

The current study was designed to test the hypotheses that experimental hypercholesterolemia results in elevation of plasma and tissue endothelin immunoreactivity and that the abnormal acetylcholine-evoked coronary vasoconstriction in the hypercholesterolemic animals is associated with further elevation of plasma endothelin. We therefore determined concentrations and molecular forms of plasma endothelin immunoreactivity following a 2% cholesterol diet for 4 months in pigs and during intracoronary acetylcholine administration. Second, we assessed the presence of endothelin in the coronary vascular wall by using an immunohistochemistry.

Methods

Fourteen 2-month-old male Yucatan minipigs weighing approximately 15 kg were randomly allocated to receive a normal diet (group 1, n=6) or a 2% cholesterol atherogenic diet including 20% tallow and 1% hog

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bile extract (group 2, n=8). Serum cholesterol and plasma endothelin concentrations were determined monthly. On the day of the experiment, the pigs were premedicated with 1 g ketamine IM, intubated, and mechanically ventilated with room air. Anesthesia was maintained with a titrated IV infusion (3 to 5 mL/min) of a mixture of 1 g ketamine, 10 mg fentanyl citrate, and 40 mg etamidate. An 8.0F Judkins-type guiding (Cordis, Fla) catheter was introduced via a femoral artery cut down and advanced to the ostia of the left anterior descending coronary artery (LAD) at which time a 2F, coronary infusion catheter was advanced through the guiding catheter into the proximal LAD. A 2.5-mm metal-ringed angiographic catheter (used for both baseline angiography and in situ calibration for quantitative angiography) was inserted via arterial puncture at the carotid artery cut down site and advanced into the ostia of the LAD in position for injection of contrast media. A bolus of 100 US units/kg heparin was given intravenously, and ECG and arterial pressure were monitored continuously. Coronary angiography was performed in the left anterior oblique projection at a standardized radiograph tube height using 6 mL of nonionic contrast media (Omnipaque) injected by hand. A 2.5F intracoronary infusion catheter (Cook, Ind) was positioned over a guide wire in the proximal LAD.

After a 30-minute recovery period, baseline hemodynamic parameters and arterial blood samples for endothelin were obtained. Serial 3-minute intracoronary infusions of acetycholine into the LAD were administered in the following sequence using a Harvard infusion pump: control infusion (5% dextrose in water) and graded concentrations of acetycholine to achieve estimated final blood concentrations in the coronary bed of 10-8, 10-7, and 10-6 mol/L followed by a control infusion. The final coronary blood concentrations of acetycholine were estimated with the assumptions that blood flow in the coronary artery is approximately 80 mL/min and all infusions with the Harvard pump were at 0.8 mL/min to keep the infusion rate under 1% of the estimated coronary flow. Separate syringes of acetycholine were prepared, and the infusion lines were cleared and primed with the new concentration. All pigs received stepwise increases in acetycholine infusion that were terminated either when vessel occlusion occurred or when the maximal dose (10-6 mol/L estimated, which is an infusion of a 10-4 mol/L solution) was reached. After each dose, arterial blood samples for endothelin were obtained, and a selective injection of contrast media and a spot film were performed. At the end of the procedure, the animals were immediately killed with a lethal dose of sodium penobarbital, and the coronary arteries were harvested and placed into 10% formalin.

Plasma endothelin was determined by endothelin-1,2,32 assay system from Amersham (Amersham, UK). Blood was drawn from pigs into tubes containing chilled potassium EDTA and immediately placed on ice until centrifugation at 4°C. Plasma was separated and frozen at -20°C until assay. Before the radioimmunoassay, plasma was acidified with 0.5% trifluoroacetic acid (TFA). C8 Bond Elut cartridges were washed with 4 mL methanol and 4 mL water to extract the plasma. After the plasma was applied, cartridges were washed with 2 mL normal saline and 6 mL water. Endothelin was eluted from the cartridges with 2 mL 90% methanol in 1% TFA and then dried and reconstituted for the radioimmunoassay. The recovery of the extraction procedure was 81% as determined by addition of synthetic endothelin to plasma. Interassay and intra-assay variations were 9% and 5%, respectively. The minimal level of detection is 0.5 pg per tube. The cross-reactivity of endothelin-2 and -3 and proendothelin in this assay was <5%, <3%, and <37%, respectively.

For immunohistochemistry, the LAD was embedded in paraffin, and 6-μm-thick sections were cut and mounted on silanized slides as previously described.12 The slides were incubated overnight at 60°C and deparaffinized with graded concentrations of xylene and ethanol. The slides were washed with 0.6% H2O2 in ethanol for 20 minutes at room temperature to block endogenous peroxidase activity. Tissue was then incubated with 5% normal goat serum for 10 minutes (Dako Corp, Santa Barbara, Calif) at room temperature to reduce nonspecific background staining and then with rabbit polyclonal endothelin-1 antiserum diluted 1:1600 (Peninsula, Belmont, Calif) in humidified chambers for 24 hours at room temperature. Control slides were treated with dilute normal rabbit serum (Dako Corp). All treated slides were exposed for 30 minutes to goat anti-rabbit antiserum diluted 1:100 (Tago Inc, Burlingame, Calif) to which peroxidase had been covalently linked. Peroxidase activity was visualized with 3-amino-9-ethylcarbazole (Sigma Chemical, St Louis, Mo) dissolved in dimethylformamide and sodium acetate. The sections were counterstained with hematoxylin, mounted, and reviewed with an Olympus microscope.

Gel permeation chromatography (GPC) was used to characterize the molecular form of endothelin in plasma. Endothelin was characterized from nonextracted plasma by a P-6 (Bio-Rad Laboratories, Richmond, Calif) gel filtration column (1×13 cm). Five hundred microliters of plasma was applied to the column and eluted with 0.5 mol/L acetic acid buffer. Fractions of 0.5 mL were collected and dried by Speedvac. The concentrations of endothelin were determined by radioimmunoassay as described above. The P-6 column was calibrated with synthetic 125I endothelin-1 and 125I big endothelin (Peninsula). Big endothelin was eluted from fractions 5 to 9 (peak fraction at 7), and endothelin-1 was eluted from fraction 7 to 17 (peak fraction at 11). Total endothelin recovery was determined by adding the concentrations of each fraction (5 through 17) after the subtraction of background (5 pg per fraction). The mean recovery of total endothelin was 86%.

Data Analysis

All results are presented as mean±SD. Statistical analysis was performed by repeated-measures ANOVA and by t test for paired and unpaired observations. Statistical significance was accepted for P<.05.

Results

The Table summarizes the lipid profiles and plasma endothelin concentrations in the two experimental groups at baseline and after 4 months of diet. There was no difference in baseline values between the experimental groups. High cholesterol diet for 4 months resulted in a significant increase in plasma cholesterol levels [64±3,
Lipid Profile and Plasma Endothelin in the Experimental Groups

<table>
<thead>
<tr>
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<th>Group 1: Normal Diet</th>
<th>Group 2: 2% Cholesterol Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cholesterol, mg/dL</strong></td>
<td><strong>Triglycerides, mg/dL</strong></td>
<td><strong>Endothelin, pg/mL</strong></td>
</tr>
<tr>
<td>Baseline</td>
<td>64±3</td>
<td>23±2</td>
</tr>
<tr>
<td>4-Month diet</td>
<td>48±5</td>
<td>26±5</td>
</tr>
</tbody>
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Values are mean±SEM.
*P<.05 vs baseline; †P<.05 vs normal diet.

240±45, 280±30, and 244±38 mg/dL, respectively) in group 2 compared with group 1 and compared with baseline. Plasma endothelin concentrations increased gradually over the 4 months in the group that received the high cholesterol diet (7.9±1.0, 9.2±1.5, 12.6±3.2, and 14±1.5, pg/mL, respectively) and reached statistical significance by the fourth month compared with baseline levels and group 1. Angiography revealed no structural abnormalities in either the hypercholesterolemic or control groups at baseline. Furthermore, luminal diameters were comparable at baseline in both groups. In response to intracoronary administration of acetylcholine, a further and significant increase in plasma endothelin concentrations was observed in the hypercholesterolemic group (Fig 1) with no change in the control group. This increase was associated with coronary vasoconstriction (58±8%) in group 2 and an increase in mean arterial pressure (60±3 to 84±7 mm Hg, P<.05). The increase in plasma endothelin was observed over a period time of approximately 20 minutes. The increase in endothelin with acetylcholine was not maintained and decreased to a level not significantly different from baseline at approximately 10 minutes after discontinuation of acetylccholine. This was also associated with the reversal of coronary vasoconstriction. There was a significant correlation (r=9, P<.01) between peak plasma endothelin concentrations and maximal coronary vasoconstriction achieved (Fig 2). There was no significant change in plasma endothelin concentrations, coronary diameter (12±8%), or mean arterial pressure in the group on normal diet (group 1). An additional group of pigs (n=3) underwent a similar protocol to investigate the effect of contrast media alone on plasma endothelin concentrations. Administration of similar doses of intracoronary contrast media did not change plasma endothelin concentrations.

Specific immunohistochemical staining of endothelin-1-like immunoreactivity (Fig 3) in the LAD demonstrated that endothelin was present in the endothelial cells as well as in the subendothelial myointimal cells and/or macrophages in coronary arteries taken from the hypercholesterolemic pigs. There was no endothelin immunoreactivity in the LADs from the pigs that were on a normal diet. Sections of coronary arteries from both groups treated with nonimmune rabbit serum showed an absence of immunoreactivity.

GPC studies revealed that the dominant molecular form of endothelin in both groups at baseline and after acetylccholine administration is endothelin-1 (Fig 4).

**Discussion**

This study establishes that hypercholesterolemia induced by a 2% cholesterol diet for 4 months in pigs elevates plasma endothelin concentration and enhances coronary artery tissue endothelin immunoreactivity. Second, the endothelium-dependent vasodilator acetylcholine further increased plasma endothelin in hypercholesterolemia in association with coronary vasoconstriction. The predominant molecular form of endothelin in hypercholesterolemia is the biologically active endothelin-1.

We have previously reported the elevation of circulating endothelin in humans with advanced atherosclerosis.
FIG 2. Scatterplot of correlation between peak plasma endothelin concentrations and peak coronary vasoconstriction during administration of intracoronary $10^{-4}$ mol/L acetylcholine. Each data point represents individual pigs at the acetylcholine concentration that achieve maximal coronary vasoconstriction.

in association with its presence in atherosclerotic aortic tissue.\textsuperscript{12} These studies extend our previous observations and demonstrate that plasma and tissue endothelin immunoreactivity are enhanced early in the evolution of atherosclerosis induced by hypercholesterolemia and co-exist with abnormal endothelial function. The elevation of plasma and tissue endothelin immunoreactivity could represent one of the major events in the development of the early atherosclerotic lesion, which is characterized by functional alteration of endothelial cells without significant morphological changes.\textsuperscript{16} Previous reports have demonstrated that there is a progressive impairment in endothelium-mediated modulation of coronary vasomotor tone with different stages of early atherosclerosis.\textsuperscript{3} Vita and colleagues\textsuperscript{17} have suggested that the loss of endothelium-dependent vasodilatory response to acetyl-

FIG 3. A representative photomicrograph showing the distribution of endothelin immunoreactivity in 6-μm sections of the left anterior descending coronary artery from a hypercholesterolemic pig. Immunoreactivity can be seen in the endothelial cells as well as in the subendothelial myointimal cells and/or macrophages (A; left, ×400). Sections of the left anterior descending artery from a pig on normal diet show an absence of immunoreactivity (B; right, ×200). There was no immunoreactivity detected in the sections that were treated with nonimmune rabbit serum (not shown).
acetylcholine may be an early marker of atherosclerosis in the absence of angiographically demonstrable lesions. Based on the current findings, we conclude that plasma endothelin concentrations may also serve as a marker for endothelial dysfunction and early atherosclerosis.

Previous in vitro studies have demonstrated that hypercholesterolemia and oxidized low-density lipoprotein interfere with endothelium-dependent relaxation by attenuating EDRF activity and/or stimulating the expression of endothelin mRNA as well as the release of endothelin. Our current study is in accordance with these previous observations and suggest that the functional balance between endothelium-mediated vasodilation and vasoconstriction may be disrupted in the early stage of atherosclerosis. This may lead to attenuated release of endothelium-derived vasodilator and antimitogenic factor EDRF and augmented release of the vasoconstrictor and mitogenic peptide endothelin. In response to intracoronary acetylcholine, a further and brief increase in circulating endothelin concentration occurred. This increase in circulating endothelin may suggest a regulated mechanism that releases endothelin from secretory granules. The elevation of plasma endothelin concentrations correlated with the degree of coronary vasoconstriction, implying an association between these two observations.

The mechanism for the increase in plasma endothelin concentrations in response to intracoronary acetylcholine administration may be multifactorial. Endothelin may be released secondary to coronary vasoconstriction induced by acetylcholine and be related to tissue hypoxia, decreased shear stress, and myocardial ischemia and reperfusion, all of which stimulate endothelin release. While endothelin-like immunoreactivity was localized to the coronary circulation, both within endo-

therial cells and in subendothelial myointimal cells and/or macrophages, atherosclerosis is a generalized process, and the increase in plasma endothelin may have also involve sites other than the coronary circulation. Another alternative mechanism is that in pathophysiological states with functional alteration of the endothelium, acetylcholine may release endothelin rather than EDRF. This latter hypothesis is supported by studies that demonstrated that several agonists such as thrombin stimulate simultaneous release of EDRF and endothelin.

The enhanced endothelin immunoreactivity in the circulation and in the vascular wall of the coronary arteries may mediate vasoconstriction and also sensitize the vascular smooth muscle to other vasoconstrictive factors such as angiotensin II and catecholamines. Moreover, recent in vitro studies suggest that hypercholesterolemia in the absence of atherosclerotic lesions increases vascular reactivity to endothelin. The coronary vasoconstriction and the increase in mean arterial pressure in association with further increase with plasma endothelin during intracoronary acetylcholine administration in the hypercholesterolemic pigs may reflect biological activity of endothelin. This hypothesis is underscored by recent studies that demonstrated that inhibition of EDRF enhances endothelin-mediated vasoconstriction and by the observation in the current study that the dominant molecular form of circulating endothelin is endothelin-1.

The presence of endothelin-like immunoreactivity in the vascular wall of the coronary artery of the hypercholesterolemic pigs could reflect an internalization of endothelin produced in endothelial cells or the active production of endothelin. The specific cell types that are positive for endothelin-like immunoreactivity could include vascular smooth muscle cells, macrophages, and fibroblasts. The localization of endothelin in the hypercholesterolemic vascular wall may reflect the site of atheroma progression and neointimal proliferation since endothelin is a mitogenic peptide. One may thus speculate that endothelin participates as a mitogen or as a comitogen in the presence of other growth factor release by macrophages in the atherogenic process to stimulate smooth muscle cell proliferation.

In summary, the present study demonstrates that a high cholesterol diet for 4 months in pigs results in elevated plasma endothelin concentrations and enhances endothelin immunoreactivity in the coronary vascular wall. In response to intracoronary administration of acetylcholine, further elevation of plasma endothelin concentrations occurs that correlates with the degree of coronary vasoconstriction. Moreover, the current study demonstrates that this plasma increase is that of the molecular biologically active endothelin-1. This study supports a role for endothelin as an early participant and/or a marker for the endothelial dysfunction in hypercholesterolemia and in the atherogenic process.

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