The Role of Nitric Oxide in Endothelium-Dependent Vasodilation of Hypercholesterolemic Patients

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Background. Patients with hypercholesterolemia have a reduced response to endothelium-dependent vasodilators. However, the regulatory function of the endothelium on vascular tone is mediated through the release of several vasoactive substances; therefore, a reduced response to endothelium-dependent agents does not identify which of the factors released by the endothelium is involved in this abnormality.

Methods and Results. To investigate the role of nitric oxide in the endothelium-dependent vasodilation in hypercholesterolemia, we studied the effect of Nω-monomethyl-L-arginine (L-NMMA), an inhibitor of endothelial nitric oxide synthesis, on basal vascular tone and on the responses to acetylcholine, an endothelium-dependent vasodilator, and to sodium nitroprusside, a direct smooth muscle dilator. The study included 33 hypercholesterolemic patients (17 men; 51±8 years; plasma cholesterol, ≥240 mg/dL) and 23 normal controls (12 men; 48±7 years; plasma cholesterol, <210 mg/dL). Drugs were infused into the brachial artery, and the response of the forearm vasculature was measured by strain-gauge plethysmography. Basal blood flow and vascular resistance were similar in hypercholesterolemic patients and normal controls (3.1±1 versus 2.6±0.8 mL/min per 100 mL and 32.1±13 versus 36.1±12 mm Hg/mL⋅min−1⋅100 mL−1, respectively). The reduction in basal blood flow and increase in vascular resistance produced by L-NMMA were not significantly different between the two groups. L-NMMA markedly blunted the response to acetylcholine in normals (maximum flow decreased from 16.4±8 to 7.0±3; P<.005); however, the analogue arginine did not significantly modify the response to acetylcholine in the hypercholesterolemic patients (maximum flow, 11.1±8 versus 10.0±8). L-NMMA did not modify the vasodilator response to sodium nitroprusside in either controls or patients.

Conclusions. These findings indicate that hypercholesterolemic patients have a defect in the bioactivity of nitric oxide that may explain their impaired endothelium-dependent vascular relaxation. (Circulation. 1993;88:2541-2547.)

Key Words • cholesterol • endothelium • acetylcholine • arginine • plethysmography

The endothelium plays a major role in determining vascular tone through the production and release of different vasodilator and vasoconstrictor substances that control the activity of the underlying smooth muscle.1-2 One of the factors that mediate the effect of the endothelium on vascular tone is endothelium-derived relaxing factor (EDRF). Although the endothelium may produce several EDRFs, at least one of them has been identified as nitric oxide,6 which is synthesized by endothelial cells from the amino acid L-arginine7 in a process that can be competitively antagonized by arginine analogues.8,9

Previous investigations have shown that the regulatory function of the endothelium on vascular tone may be abnormal in certain cardiovascular conditions. Thus, several studies in both animal10-14 and human15-17 models demonstrated that endothelium-dependent vasodilator responses are impaired in hypercholesterolemia, even in the absence of atherosclerosis.17-21 However, because endothelial modulation of vascular smooth muscle is mediated by several factors, the finding of reduced endothelium-dependent vasodilation does not identify the precise mediators involved in the abnormal endothelial function observed in hypercholesterolemia.

The purpose of this investigation, therefore, was to determine the role of endothelium-derived nitric oxide in the abnormal vascular responses of hypercholesterolemic patients. We achieved this by measuring the effect of inhibition of nitric oxide synthesis on basal vascular tone and on the vascular responses to endothelium-dependent and -independent agents.

Methods

Study Population

Thirty-three patients with hypercholesterolemia and without any other apparent medical condition were recruited into the study. Each subject was screened by clinical history, physical examination, routine chemical analyses, ECG, and chest radiography. Patients were admitted into the study if their plasma cholesterol level, measured at the time of initial screening after a 12-hour
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fasting period, was more than 240 mg/dL and they had no history or evidence of present or past hypertension, cardiac disease, diabetes mellitus, peripheral vascular disease, coagulopathy, or any other disease predisposing them to vasculitis or Raynaud's phenomenon. There were 17 men and 16 women. Mean age was 51±8 years (range, 37 to 67 years). Thirty-two of the 33 patients had never been treated with antihyperlipidemic drugs. The remaining patient was instructed to discontinue his medication (lovastatin) 6 weeks before the study; during this period, his plasma cholesterol level increased from 178 to 249 mg/dL.

A population of 23 normal volunteers (12 men and 11 women), matched with the patients for approximate age and sex, was selected as a control group. Their mean age was 48±7 years (range, 37 to 63 years). Each subject underwent a screening identical to that described for the hypercholesterolemic patients and had no evidence of present or past hyperlipidemia (plasma cholesterol, ≤210 mg/dL), hypertension, cardiac disease, or any other systemic condition. None of these subjects were taking any medication at the time of the study.

All participants gave written informed consent for all procedures. This study was approved by the National Institutes of Health Investigational Review Board.

**Lipid Measurements**

Initial screenings for 12-hour fasting plasma cholesterol levels were performed on all participants using the enzymatic cholesterol oxidase and esterase technique (Boehringer Mannheim Diagnostics). In addition, on a separate day, blood samples were obtained after a 12-hour fast for measurement of a lipid profile using the Allain method with the Abbott VP Super System auto analyzer and Abbott reagents and Boehringer Mannheim standards (Abbott Labs Diagnostics, Irving, Tex). This profile included measurements of total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, very-low-density lipoprotein cholesterol, and triglycerides.

**Protocol**

All studies were performed in the morning in a quiet room with a temperature of approximately 22°C (72°F). Participants were asked to refrain from drinking alcohol or beverages containing caffeine and from smoking for at least 24 hours before their studies.

Each study consisted of the infusion of drugs into the brachial artery and the measurement of the response of the vasculature (changes in regional blood flow) by means of forearm plethysmography. While the participants were supine, a needle was inserted in the left brachial artery. This arm was slightly elevated above the level of the right atrium, and a mercury-filled Silastic strain-gauge was placed on the widest part of the forearm.22-23 The strain-gauge was connected to a plethysmograph (model EC-4, D.E. Hokanson, Issaquah, Wash)24 calibrated to measure the percent change in volume; the plethysmograph in turn was connected to a chart recorder to record the forearm blood flow. For each measurement, a cuff placed on the upper arm was inflated to 40 mm Hg with a rapid cuff inflator (model E-10, Hokanson) to occlude venous outflow from the extremity. A wrist cuff was inflated to suprasystolic pressures 1 minute before each measurement to exclude the hand circulation.25 Flow measurements were recorded for approximately 7 seconds every 15 seconds; seven readings were obtained for each mean value.

Basal measurements were obtained after a 3-minute infusion of 5% dextrose solution at 1 mL/min. Forearm flows were then measured after the infusion of acetylcholine and sodium nitroprusside. Acetylcholine was used as an endothelium-dependent agent that induces vasodilation by stimulating the release of relaxing factors from the vascular endothelium.1,2,26-28 Sodium nitroprusside was used as an endothelium-independent substance since its vasodilator effect is largely due to its direct action on smooth muscle cells.29,30

Acetylcholine chloride (Sigma Chemical, St Louis, Mo) was infused at a rate of 7.5, 15, and 30 μg/min and sodium nitroprusside at a rate of 0.8, 1.6, and 3.2 μg/min (infusion rates, 0.25, 0.5, and 1 mL/min, respectively, for each drug). Each dose was infused for 5 minutes, and forearm flow was measured during the last 2 minutes of each infusion. A 30-minute rest period was allowed, and basal measurements were repeated between the infusion of the two drugs.

After another 30-minute rest period, flow measurements were obtained to corroborate return to basal values. Then, the arginine analogue N⁶-monomethyl-L-arginine (L-NMMA; Calbiochem, La Jolla, Calif) was subsequently infused at a rate of 4 μmol/min (infusion rate, 1 mL/min) for 5 minutes, and forearm blood flow was measured during the last 2 minutes of the infusion. L-NMMA is an arginine analogue that competitively antagonizes the synthesis of nitric oxide from L-arginine,31,32 and thus provides a tool with which to investigate the rate of nitric oxide production by the vascular endothelium.26 L-NMMA was dissolved in a dextrose solution to achieve a concentration of 4 μmol/mL. The solution was then subjected to sterility and pyrogen testing and finally to high-performance liquid chromatography assay to ascertain its purity.

Subsequently, in 20 hypercholesterolemic patients and 10 normal controls, cumulative dose-response curves for acetylcholine and sodium nitroprusside were repeated using the same doses, infusion rates, and resting interval mentioned previously. The infusion of L-NMMA was discontinued during the rest period but reinstated before the second of these dose-response curves.

The sequence of administration of acetylcholine and sodium nitroprusside, both before and after infusion of the arginine analogue, was randomized to avoid any bias related to the order of drug infusion. During the study, the participants did not know which drug was being infused. All blood pressures were recorded directly from the intra-arterial catheter immediately before each measurement. Forearm vascular resistance was calculated as the mean arterial pressure divided by the forearm blood flow.

**Statistical Analysis**

Differences between two means were compared by paired or unpaired Student's t test, as appropriate. Differences between three means were compared by ANOVA. The responses to sodium nitroprusside and acetylcholine were compared by ANOVA for repeated measures using a multiple linear regression model that included dummy variables to correct for between-sub-
Plasma Lipoprotein Levels (mg/dL) Measured in the 33 Hypercholesterolemic Patients and 23 Normal Controls

<table>
<thead>
<tr>
<th>Lipoprotein Fraction</th>
<th>Hypercholesterolemic Patients</th>
<th>Normal Controls</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>292±45</td>
<td>170±29*</td>
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<td>Very-low-density lipoprotein cholesterol</td>
<td>46±39</td>
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<td>116±36</td>
<td>&lt;.0001</td>
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<tr>
<td>High-density lipoprotein cholesterol</td>
<td>44±10</td>
<td>48±13</td>
<td>.8</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>176±105</td>
<td>94±41</td>
<td>.002</td>
</tr>
</tbody>
</table>

*For total cholesterol, the values obtained at the time of initial screening are shown. All other values were obtained on a separate day.

Results

Plasma Lipid Measurements

By selection, the plasma cholesterol levels at the time of screening were significantly higher in the hypercholesterolemic patients than in the normal controls (292±45 versus 170±29 mg/dL; P<.0001). The different lipoprotein fractions subsequently measured in the hypercholesterolemic patients and controls are shown in the Table.

Effect of the Arginine Analogue L-NMMA on Basal Blood Flow and Vascular Resistance

Mean arterial blood pressure was similar in hypercholesterolemic patients and normal controls (87±8 and 86±8 mm Hg, respectively). Basal forearm blood flow (3.1±1 and 2.6±0.8 mL/min per 100 mL, respectively) and basal vascular resistance (32.1±13 and 36.1±12 mm Hg/mL-mL-1·min-1, 100 mL-1, respectively) were also similar in both groups.

The infusion of L-NMMA produced a significant decrease in forearm blood flow and increase in forearm vascular resistance in both groups. In hypercholesterolemic patients, blood flow decreased from 3.1±1 to 2.3±0.7 mL/min per 100 mL (P<.0005), and vascular resistance increased from 32.1±13 to 41.2±14 mm Hg/ mL-mL-1·min-1, 100 mL-1 (P<.002). In normal controls, blood flow decreased from 2.6±0.8 to 1.8±0.5 mL/min per 100 mL (P<.0003), and vascular resistance increased from 36.1±12 to 49.9±16 mm Hg/ mL-mL-1·min-1, 100 mL-1 (P<.003). L-NMMA did not increase systemic blood pressure in either hypercholesterolemic patients (mean blood pressure, 87±8 and 87±10 mm Hg before and after L-NMMA infusion, respectively) or normal controls (mean blood pressure, 86±8 and 83±8 mm Hg before and after L-NMMA infusion, respectively).

The magnitude of vasoconstriction observed with L-NMMA was not significantly different between normal controls and hypercholesterolemic patients (Fig 1).

<Fig 1. Bar graphs of decrease in forearm blood flow and increase in vascular resistance with N^6-monomethyl-L-arginine (L-NMMA) in 23 normal controls (solid bars) and 33 hypercholesterolemic patients (open bars). Values represent mean and SEM.>

The percent decrease in blood flow was 28±18% in normal controls and 21±20% in hypercholesterolemic patients (P=NS), and the percent increase in vascular resistance was 42±38% in normals and 36±41% in patients (P=NS).

Vascular Responses to Acetylcholine and Sodium Nitroprusside

Acetylcholine produced a substantial vasodilator effect in both patients and controls; however, the increase in blood flow and decrease in vascular resistance in response to acetylcholine were significantly attenuated in hypercholesterolemic patients compared with normal
controls (Fig 2). At the highest dose (30 μg/min), forearm blood flow was 18.1±9 mL/min per 100 mL in the controls and 11.2±8 mL/min per 100 mL in the hypercholesterolemic patients (P<.005).

For the overall group of normal controls and hypercholesterolemic patients included in our study, a weak but statistically significant correlation was found between the plasma cholesterol level and the maximum increase in blood flow achieved with acetylcholine (r = -.39, P<.03). When the study subjects were divided according to the total plasma cholesterol level, it was evident that patients with the highest levels (>275 mg/dL) were those with the least increase in blood flow with acetylcholine (Fig 3).

No significant differences were found between the two groups in the forearm blood flow and vascular resistance responses to sodium nitroprusside (Fig 4). At the highest dose (3.2 μg/min), forearm blood flow was 10.6±3 mL/min per 100 mL in the normal controls and 10.8±5 mL/min per 100 mL in the hypercholesterolemic patients.

Effect of L-NMMA on the Vascular Responses to Acetylcholine and Sodium Nitroprusside

In the 10 normal controls who received L-NMMA, the vasodilator response to acetylcholine was significantly blunted after infusion of the arginine analogue (Fig 5). At the highest dose of acetylcholine (30 μg/min), blood flow was 16.4±8 mL/min per 100 mL before and 7.01±3 mL/min per 100 mL after infusion of L-NMMA (P<.004). In contrast, in the 20 hypercholesterolemic patients studied, no difference was observed in the blood flow response to acetylcholine before or after the infusion of L-NMMA, although a change was found when the response was analyzed in terms of vascular resistance (Fig 6). At the maximum dose of acetylcholine, blood flow was 11.1±8 mL/min per 100 mL before and 10.0±8 mL/min per 100 mL after infusion of the arginine analogue, respectively. When the effect of L-NMMA on the vasodilator response to acetylcholine was compared between the two groups, it was evident that both the decrease in blood flow and the increase in vascular resistance produced by the arginine analogue were significantly reduced in patients with hypercholesterolemia compared with normal controls (Fig 7).

As a consequence of this attenuated effect of L-NMMA in patients with hypercholesterolemia, the vasodilator response to acetylcholine measured after administration of the arginine analogue was similar in both subject groups (maximum blood flow, 7.01±3 mL/min per 100 mL in controls and 10.0±8 mL/min per 100 mL in hypercholesterolemic patients [P=NS]).
Fig 6. Plots of forearm blood flow and vascular resistance responses to acetylcholine in 20 hypercholesterolemic patients before (open circles) and after (closed circles) infusion of N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA). Values represent mean and SEM.

note, in the 20 hypercholesterolemic patients, a significant correlation was found between the reduction in basal blood flow with L-NMMA and the vasodilator effect (increase in blood flow) with acetylcholine ($r = .47$, $P < .04$).

The infusion of L-NMMA did not modify the vasodilator response to sodium nitroprusside either in normal controls (maximum flow, 10.6±3 versus 9.9±3 mL/min per 100 mL before and after L-NMMA infusion, respectively) or in hypercholesterolemic patients (maximum blood flow, 11.1±6 versus 8.9±3 mL/min per 100 mL before and after L-NMMA infusion, respectively).

**Discussion**

Previous studies in animal models and patients with hypercholesterolemia have shown abnormally reduced endothelium-dependent vasodilator responses.\textsuperscript{10-21} The findings of the present investigation confirm those reports and expand previous observations by demonstrating that such impairment in endothelial function is largely related to a deficit in the nitric oxide system. This system contributes to the regulation of vascular tone through the production and release of endothelium-derived nitric oxide, which importantly modulates the contractile activity of the underlying smooth muscle.\textsuperscript{5,7,26}

To assess the status of the nitric oxide system, we used L-NMMA, an analogue of L-arginine that selectively antagonizes the synthesis of nitric oxide in a competitive manner.\textsuperscript{6,9} Thus, the vascular responses to L-NMMA are indexes of the rate of production and release of nitric oxide, and consequently the use of this substance permits the study of the physiology and pathophysiology of the endothelium-derived nitric oxide system.\textsuperscript{26}

The results of our investigation indicate that the basal release of nitric oxide is not substantially impaired in the peripheral vasculature of hypercholesterolemic patients since the vasoconstrictor effect of L-NMMA was not significantly different between our study patients and a group of age- and sex-matched normal controls. Because nitric oxide is normally an important determinant of peripheral vascular tone,\textsuperscript{26} these findings are consistent with our observation that the peripheral vascular resistance was similar in both subject groups. It must be emphasized, however, that patients with hypercholesterolemia who had elevated blood pressure were excluded from our study because essential hypertension has been shown to be associated with impaired endothelial function.\textsuperscript{27,28} It is therefore possible that, by design, we selected out those patients with hypercholesterolemia who have a more severe abnormality in the nitric oxide system, leading to reduced basal release of nitric oxide and consequent increase in systemic blood pressure.

In agreement with previous observations, our study shows that the endothelium-dependent vasodilator response to acetylcholine is blunted in patients with hypercholesterolemia compared with normal controls.\textsuperscript{15-17,21} Importantly, and in contrast to the findings in normals, the blood flow response to acetylcholine was not significantly modified by administration of L-NMMA in hypercholesterolemic patients. This observation indicates that the stimulated release of nitric oxide is substantially impaired in these patients and therefore is not significantly affected by inhibition of its synthesis.

It must be noted that the vasodilator effect of acetylcholine is only partially reduced by L-NMMA in normal controls,\textsuperscript{26} suggesting that other substances, such as endothelium-derived hyperpolarizing factor,\textsuperscript{32,33} must be released by the endothelium in response to acetylcholine and importantly contribute to its vasodilator action. Therefore, the fact that the response to acetylcholine after
infusion of L-NMMA was similar in patients and controls suggests that the defect in the nitric oxide system may account exclusively for the abnormal endothelium-dependent vascular relaxation observed in hypercholesterolemia since the other components of the endothelium-mediated vasodilator response to acetylcholine (those not affected by infusion of L-NMMA) do not appear to be reduced in hypercholesterolemic patients.

Alternatively, the release of certain vasoconstrictor factors by the endothelium, such as endothelin, 34,35 may be increased in hypercholesterolemia. This could potentially overcome the release of endothelium-derived vasodilators such as nitric oxide and thus diminish their role in the regulation of vascular tone, even if their absolute rate of synthesis and release is not reduced. However, based on the findings of our study, the possibility that an exaggerated activation of vasoconstrictor substances is the single cause of endothelial dysfunction in hypercholesterolemia is unlikely. If the impaired endothelium-dependent vasodilation of hypercholesterolemic patients was solely due to increased production of constrictor factors with normal nitric oxide action, the infusion of L-NMMA should have still produced a marked reduction in the vasodilator response to acetylcholine given the significant contribution of nitric oxide to endothelium-dependent vasodilation. Thus, although our study findings do not rule out a possible role for increased activation of vasoconstrictor factors in hypercholesterolemia, they clearly indicate that the role of endothelium-derived nitric oxide in the regulation of vascular tone is diminished in patients with this condition.

It is also possible that the inhibition of nitric oxide synthesis by L-NMMA may have been only partial. In this case, a reduced availability of L-arginine, the natural substrate for nitric oxide production, could explain the differential effect of the arginine analogue on the endothelium-dependent vascular responses of patients and controls. The design of the present investigation, however, does not allow us to determine the precise location of the abnormality in the nitric oxide system in hypercholesterolemic patients. The defect may lie in the availability of substrate for nitric oxide production within the endothelial cell, 36,37 in the intracellular signal transduction pathway that carries the message from the membrane receptors to the enzyme that synthesizes nitric oxide,38 in the enzyme itself, or even in the breakdown of nitric oxide by superoxide anions in the interstitium.39 In fact, the results of previous studies suggest that the production of nitric oxide by endothelial cells may actually be increased in hypercholesterolemia, supporting the concept of accelerated degradation of nitric oxide as the cause for impaired endothelium-mediated vasodilation in this condition.40,41 These possibilities therefore merit further investigation.

In our study patients, the reduction in blood flow with L-NMMA (an index of the release of nitric oxide) significantly correlated with the vasodilator response to acetylcholine, suggesting that patients with a lower rate of release of nitric oxide at baseline also have a greater impairment in endothelium-dependent vasodilation, and vice versa. This relation between the vascular effect of L-NMMA and acetylcholine, therefore, provides confirmatory evidence that the blunted response to acetylcholine in hypercholesterolemic patients is indeed related to reduced release of nitric oxide. Moreover, this relation suggests that in some of these patients, the basal production of nitric oxide may already be affected and thus determine a more severe impairment of endothelium-dependent vasodilation.

In our study, the vasodilator response to sodium nitroprusside was not different between hypercholesterolemic patients and normal controls. This indicates that the blunted response to acetylcholine observed in the patients was indeed an expression of impaired endothelium-dependent vascular relaxation and not a consequence of a reduced responsiveness of the vascular smooth muscle to nitrovasodilators. These findings are consistent with previous observations in animals16,17 and patients16,17 with hypercholesterolemia but are at variance with the results of a previous study in which the forearm vascular response to sodium nitroprusside was found to be reduced in hypercholesterolemic patients compared with a group of normal controls.15 In that study, however, the response to sodium nitroprusside was reported to be depressed at a dose of 10 μg/min,15 which is about three times higher than the maximum dose used in our study (3.2 μg/min). Although it is possible that we would have found a similar difference in our study had we infused higher doses of sodium nitroprusside, it is our experience that such doses, even when infused intra-arterially, invariably produce a fall in systemic blood pressure. This would lead to activation of compensatory mechanisms, which could obscure interpretation of the results. Finally, in more recent studies from the same56 and other23 groups of investigators, no significant differences between normal controls and hypercholesterolemic patients were reported in the response to identical doses of sodium nitroprusside.

Another important finding of the present investigation is the existence of a relation between plasma cholesterol levels and the endothelium-dependent response to acetylcholine. This observation is in agreement with previous reports42 and is consistent with the concept that the endothelial dysfunction in hypercholesterolemia is related to the magnitude of the lipid disorder and can even progress as the underlying disease process advances.11,12,17,37 Furthermore, because the vascular responses measured in our study reflect changes in the small resistance vessels of the forearm, which are unlikely to suffer from atherosclerosis, the relation between plasma cholesterol levels and the response to acetylcholine observed in our patients is unlikely to be due to the atherosclerotic changes that may occur in larger arteries.

The findings of the present study cannot be used to ascertain whether the impaired endothelium-dependent vascular relaxation of hypercholesterolemic patients is due to a primary endothelial abnormality or whether it is a consequence of the lipid abnormality itself. The observations that abnormal endothelium-dependent vascular responses can be elicited in animals fed a hypercholesterolemic diet10-14 and that such responses can be restored when animals are returned to a normal diet43 indicate that the endothelial dysfunction is indeed secondary to the lipid abnormality. However, even as a secondary phenomenon, the endothelial dysfunction can importantly contribute to the pathophysiology of the vascular complications associated with hypercholesterolemia.
In conclusion, our study shows that the impaired endothelium-dependent vascular relaxation characteristic of hypercholesterolemic patients is predominantly a consequence of a diminished role of nitric oxide in the endothelial regulation of vascular tone. These findings contribute to our understanding of the pathophysiology of the vascular system in hypercholesterolemia and provide a basis for future investigations designed to define the intimate mechanisms that lead to endothelial dysfunction in this condition.

References

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