Increased Activity of Endogenous Endothelin in Patients With Hypercholesterolemia

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OBJECTIVE We sought to assess the activity of endogenous endothelin-1 (ET-1) in hypercholesterolemic patients using antagonists of ET-1 receptors.

BACKGROUND Endothelial dysfunction in hypercholesterolemic patients may contribute to their risk of premature atherosclerosis. Endothelin, a peptide released by endothelial cells, may be involved in this process by activating smooth muscle cell mitogenesis and leukocyte adhesion. As increased nitric oxide (NO)–dependent vasodilator responsiveness to acetylcholine (2,3), endothelial dysfunction in hypercholesterolemic patients may contribute to their risk of premature atherosclerosis. The relevance of increased plasma levels as an evidence of activation of the ET-1 system in hypercholesterolemia, and of a potential role of ET-1 in atheromatous vascular disease, stems from studies performed both in experimental models and in humans. Thus, plasma immunoreactive ET-1 is increased in rats (14) and pigs (15) fed a high-cholesterol diet. Also, oxidized low density lipoproteins stimulate the expression of prepro-ET-1 messenger RNA and the release of ET-1 in cultured endothelial cells (16). In humans, the presence of immunoreactive ET-1 has been demonstrated in smooth muscle and endothelial cells at the sites of atherosclerotic lesions (17). Moreover, in keeping with the results of animal studies, plasma ET-1 levels are elevated in patients with hypercholesterolemia (18,19). The relevance of increased plasma levels as an evidence of the vascular activity of the ET-1 system is, however, questionable because the peptide acts predominantly in an autocrine and paracrine manner and its secretion by endothelial cells is polarized toward the underlying vascular smooth muscle (20). Consequently, plasma ET-1 levels may not necessarily reflect endothelial cell production or its biological effect on smooth muscle cells. Recently, selective and nonselective blockers of ET-1 receptors have become available for clinical studies and provide a more meaningful tool to assess the role of ET-1 in vascular homeostasis in vivo. The present study, therefore, was designed to determine whether the activity of the ET-1

initiation and/or the progression of the atherosclerotic process.

Evidence of activation of the ET-1 system in hypercholesterolemia, and of a potential role of ET-1 in atheroma-tous vascular disease, stems from studies performed both in experimental models and in humans. Thus, plasma immunoreactive ET-1 is increased in rats (14) and pigs (15) fed a high-cholesterol diet. Also, oxidized low density lipoproteins stimulate the expression of prepro-ET-1 messenger RNA and the release of ET-1 in cultured endothelial cells (16). In humans, the presence of immunoreactive ET-1 has been demonstrated in smooth muscle and endothelial cells at the sites of atherosclerotic lesions (17). Moreover, in keeping with the results of animal studies, plasma ET-1 levels are elevated in patients with hypercholesterolemia (18,19). The relevance of increased plasma levels as an evidence of the vascular activity of the ET-1 system is, however, questionable because the peptide acts predominantly in an autocrine and paracrine manner and its secretion by endothelial cells is polarized toward the underlying vascular smooth muscle (20). Consequently, plasma ET-1 levels may not necessarily reflect endothelial cell production or its biological effect on smooth muscle cells. Recently, selective and nonselective blockers of ET-1 receptors have become available for clinical studies and provide a more meaningful tool to assess the role of ET-1 in vascular homeostasis in vivo. The present study, therefore, was designed to determine whether the activity of the ET-1
system is increased in the forearm resistance vessels of hypercholesterolemic patients using specific blockers of ET-1 receptors.

METHODS

Study subjects. Twelve patients (Table 1) were recruited for this study. Patients were selected on the basis of elevated plasma cholesterol levels (>250 mg/dl) at the time of a screening visit at the outpatient clinic of the National Heart, Lung, and Blood Institute (NHLBI). Patients were excluded from this study if they had a history of diabetes, hypertension, coronary or peripheral vascular disease, myocardial infarction, stroke, coagulopathy or any disease predisposing them to vasculitis or Raynaud’s phenomenon. Eight patients had never been treated with lipid-lowering drugs; the remaining four patients discontinued their medications (statins in three patients and cholestyramine in one patient) for four weeks before enrollment into this study.

Twelve normal volunteers matched with the patients for approximate race, gender and age were selected as a control group (Table 1). Each subject was screened by clinical history, physical examination, electrocardiograph (ECG) chest X-ray and routine chemical analyses. None had evidence of present or past hypertension, hyperlipidemia, cardiovascular disease or any other systemic condition.

None of the study participants was taking any medication at the time of the study. In particular, all patients and control subjects were asked to refrain from taking vitamin supplements for four weeks, aspirin for two weeks and smoking and caffeine for at least 24 h before the study.

The study protocol was approved by the NHLBI Investigational Review Board, and all participants gave written informed consent.

Protocols. All studies were performed in the morning in a quiet room with a temperature of approximately 22°C. Each study consisted of infusion of drugs into the brachial artery and measurement of the response of the forearm vasculature by means of strain-gauge venous occlusion plethysmography. All drugs utilized in this study were approved for investigational use in humans by the Food and Drug Administration and were prepared by the Pharmaceutical Development Service of the National Institutes of Health following specific procedures to ensure accurate bioavailability and sterility of the solutions.

While the participants were in supine position, a 20-gauge Teflon catheter (Arrow Inc.; Reading, Pennsylvania) was inserted into the brachial artery of the nondominant arm (left in most cases). This arm was slightly elevated above the level of the right atrium, and a mercury-filled silicone rubber strain-gauge was placed in the widest part of the forearm (21). The strain-gauge was connected to a plethysmograph (model EC-4; D.E. Hokanson; Bellevue, Washington), calibrated to measure the percent change in volume and connected in turn to a chart recorder. For each measurement, a cuff placed around 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 min before each measurement to exclude the hand circulation (22). Flow measurements were recorded for approximately 7 s every 15 s; seven readings were obtained for each mean value. All blood pressures (BPs) were recorded directly from the intra-arterial catheter after each flow measurement. Heart rate was recorded from an ECG lead.

Because of the prolonged infusion time required to assess the hemodynamic effect of the different substances and their relatively long-lasting effects, in each patient different studies were performed on separate days at least one week apart in random sequence. Throughout all studies, volumes infused were matched by administration of variable amounts of saline.

Assessment of vascular responses to ETA receptor blockade in normal subjects and hypercholesterolemic patients. Basal measurements were obtained after a 15-min infusion of saline at 1 ml/min. Then, BQ-123 (Peninsula Laboratories; Belmont, California), a synthetic peptide with high potency of antagonism for the ETA receptor (23), was

<table>
<thead>
<tr>
<th>Normal Control Subjects</th>
<th>Hypercholesterolemic Patients</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (m/f)</td>
<td>8/4</td>
<td>9/3</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>51 ± 2</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>Smoking (y/n)</td>
<td>0/12</td>
<td>1/11</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>86 ± 2</td>
<td>91 ± 2</td>
</tr>
<tr>
<td>FBF (ml/min/dl)</td>
<td>2.9 ± 0.2</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>164 ± 8</td>
<td>292 ± 10</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>101 ± 10</td>
<td>223 ± 9</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>49 ± 7</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>92 ± 20</td>
<td>205 ± 42</td>
</tr>
</tbody>
</table>

*FBF = forearm blood flow; HDL = high density lipoprotein; LDL = low density lipoprotein; MAP = mean arterial pressure.
infused at 100 nmol/min (100 nmol/ml solution). This dose results in an intravascular concentration approximately 10-fold higher than the pA2 (negative logarithm of the molar concentration of antagonist that causes a twofold parallel shift to the right of the concentration-response curve) at the ETA receptor (23), and it has been previously shown to shift to the right of the concentration-response curve) at the concentration of antagonist that causes a twofold parallel shift to the right of the concentration-response curve) at the ETA receptor (23), and it has been previously shown to effectively counteract the vasoconstrictor effect of an ET-1 infusion in the human forearm (24). BQ-123 was given for 60 min (1 ml/min infusion rate), and forearm blood flow (FBF) was measured every 10 min.

Assessment of vascular responses to endothelin-1 in normal subjects and hypercholesterolemic patients. To ascertain whether there is a difference in vascular sensitivity to the hemodynamic effects of ET-1 between hypercholesterolemic patients and normal control subjects, experiments were performed to compare the vasomotor responses to exogenous ET-1 in the two groups. To this end, after basal measurements were obtained, normal subjects and hypercholesterolemic patients received an intra-arterial infusion of ET-1 (Bachem Inc.; Torrens, California; 5 pmol/ml solution) at 5 pmol/min (1 ml/min infusion rate) for 60 min. Forearm blood flow was measured at 10-min intervals.

Comparison of vascular responses to selective ETA receptor blockade and nonselective ETA/ETB blockade in hypercholesterolemic patients. In 6 of the 12 hypercholesterolemic patients, the infusion of BQ-123 was extended for another 60 min (total infusion time: 120 min) at the same dose and infusion rate as indicated above, with repeated measurements of FBF every 10 min. On a different occasion, these six patients, following the infusion of BQ-123 for 60 min, received co-infusion of BQ-123 and BQ-788 for another 60 min with measurements of FBF every 10 min. BQ-788 (Peninsula Laboratories; 50 nmol/ml solution) is a synthetic and highly selective antagonist of ETB receptors (25) and was given at 50 nmol/min (1 ml/min infusion rate). The dose of BQ-788 was selected to achieve a local concentration in the forearm more than 10-fold higher than the pA2 at the ETB receptor (25).

Statistical analysis. Two means were compared by paired or unpaired t test, as appropriate. Within each group, the effect of ET-1 receptor blockade on basal FBF was assessed by one-way analysis of variance for repeated measures. Comparison of the response to ET-1 and ET-1 receptor blockade between the two groups was performed with two-way analysis of variance for repeated measures. Comparison of the effect of selective ETA blockade vs. combined ETA/ETB blockade in hypercholesterolemic patients was performed using two-way analysis of variance for repeated measures. Multiple comparison was performed using the Dunnett’s test. All calculated p values are two-tailed, and a p value <0.05 was considered to indicate statistical significance. All group data are reported as mean ± SEM.

RESULTS

Mean arterial pressure and heart rate did not significantly change after infusion of any of the drugs used in the study, thus indicating that the drug effects were limited to the infused forearm and not extended to the systemic circulation. Baseline FBF was similar in hypercholesterolemic patients and healthy control subjects (Table 1).

Vascular responses to ETA receptor blockade in normal subjects and hypercholesterolemic patients. In normal subjects, BQ-123 did not significantly modify FBF (p = 0.78). By contrast, in hypercholesterolemic patients, BQ-123 infusion resulted in a significant vasodilator response (p < 0.001 vs. baseline). As a result, FBF values during selective ETA blockade were significantly higher in hypercholesterolemic patients than in normal subjects (Fig. 1).

Vascular responses to endothelin-1 in normal subjects and hypercholesterolemic patients. Endothelin-1 caused a significant vasoconstrictor response in both hypercholesterolemic patients (p < 0.001 vs. baseline) and normal control subjects (p < 0.001 vs. baseline). This effect was not significantly different between the two groups (Fig. 2).

Vascular responses to selective ETA and nonselective ETA/ETB blockade in hypercholesterolemic patients. In the six hypercholesterolemic patients who received BQ-123 on two different days, the magnitude of the vasodilator response during the initial 60 min of BQ-123 administration was not different between the two occasions (Fig. 3, left). Prolongation of BQ-123 infusion for 2 h did not result in any significant change in the degree of vasodilation compared with that observed after 60 min (p = 0.67). By contrast, addition of BQ-788 to BQ-123 progressively
blunted the vasodilation induced by BQ-123 alone, with return of FBF values to levels similar to those recorded at baseline (3.6 ± 0.7 ml/min/dl after combination of BQ-123 and BQ-788 vs. 3.2 ± 0.6 ml/min/dl at baseline; p = 0.28). As a result, FBF values were significantly higher during selective ETA than during nonselective ETA/ETβ blockade (Fig. 3, right).

**DISCUSSION**

The present study demonstrates that selective blockade of ETA receptors results in a vasodilator effect in patients with hypercholesterolemia but not in control subjects. These findings suggest that ETA-dependent vasoconstrictor tone is enhanced in hypercholesterolemic patients.

**Mechanisms of increased endothelin-1 activity in hypercholesterolemia.** Different possibilities may explain this increased ETA-dependent vasoconstriction in hypercholesterolemic patients, including increased availability of ET-1 at the ETA receptor level or enhanced susceptibility of blood vessels to the vasoconstrictor effect of ET-1 due to, for example, upregulation of ETA receptors. In order to identify which mechanism may be operative in hypercholesterolemia, we compared vascular responsiveness to administration of exogenous ET-1 in patients and control subjects. Our results indicate that the vasoconstrictor effect of ET-1 is not different between the two groups, thereby suggesting that the difference in the hemodynamic effect of ETA antagonism is not dependent on increased sensitivity of hypercholesterolemic vessels to the vasoconstrictor effect of ET-1. An alternative explanation to our findings is an enhanced endogenous production of ET-1 which, in turn, might be related to a stimulatory effect of low-density lipoproteins on endothelial synthesis and release of the peptide (16). Finally, we cannot exclude that upregulation of both ET-1 receptor subtypes is present in hypercholesterolemia, leading to a balanced hemodynamic effect, which could explain the similar vasomotor response to exogenous ET-1 in patients and control subjects.

**Role of ETβ-mediated vasodilation.** The vasodilator response to selective ETA blockade observed in hypercholesterolemic patients was reversed by superimposing ETβ receptor antagonism, suggesting the presence of an endogenous ETβ-mediated vasodilator tone that becomes predominant when ETA receptors are blocked. These results are in keeping with those previously reported by other investigators (9) who observed that, in human resistance arteries in vivo, ETβ receptor antagonism on a background of ETA antagonism blunts the vasodilator response induced by selective ETA blockade. At the same time, these findings differ from those previously observed in our laboratory in patients with essential hypertension (26) in whom nonselective ETA and ETβ receptor antagonism results in further enhancement of the vasodilator response to selective ETA blockade. These observations suggest that ETβ-mediated vasodilation is impaired in hypertensive patients, whereas in hypercholesterolemia, this mechanism is preserved and participates in determining the overall hemodynamic effect of endogenous ET-1. Although in this study we did not investigate the precise mechanism underlying ETβ-mediated vasodilation, previous reports have demonstrated
that in human peripheral resistance vessels, relaxation in response to stimulation of ET\(_A\) receptors is largely dependent on endothelial generation of NO as it is blunted by NO synthase inhibition with L-NMMA (9). Therefore, it is possible that ET\(_A\)-dependent NO production is impaired in endothelial cells of hypertensive, but not hypercholesterolemic, patients. Taken in conjunction with previous observations (27), these observations support the notion that different mechanisms may be involved in the pathophysiology of endothelial dysfunction in hypercholesterolemic compared with hypertensive patients.

**Role of Endothelin-1 in atherosclerosis.** In our study, the vasodilator response elicited by selective ET\(_A\) blockade in hypercholesterolemic patients, although statistically significant, was of relatively modest magnitude. Although this could suggest a limited role of ET-1 in the vascular pathophysiology of hypercholesterolemia, it is possible that ET-1 has an even greater role in vessels susceptible to inflammation and atherosclerosis. For example, previous studies have shown that, in atherosclerotic coronary vessels, increased ET-1 immunoreactivity is associated predominantly with macrophages and activated smooth muscle cells (28). These cells are unlikely to play a significant role in the forearm microcirculatory bed, which is spared from the atherosclerotic process. Thus, our study model could have underestimated the true significance of ET-1 in the pathophysiology of vascular damage in hypercholesterolemia. Similarly, it must be noted that hypercholesterolemic patients with associated risk factors, such as diabetes and hypertension, were excluded from the present study in order to investigate the effect of hypercholesterolemia per se on ET-1 activity. Because these other risk factors may also be associated with enhanced ET-1 activity, it is possible that our results underestimate the vascular activity of the peptide in the general population of hypercholesterolemic patients, who often have concomitant cardiovascular conditions.

**Endothelin-1 vasoconstrictor activity in normal subjects.** By contrast with the results obtained in hypercholesterolemic patients, selective blockade of ET\(_A\) receptors did not result in any significant change of FBF in normal control subjects. This finding is in keeping with those previously reported by our group (26), but is at odds with those observed by another group of investigators who infused BQ-123 in normal subjects (9,24,29). Because similar doses and infusion times of BQ-123 have been used in the studies of normal subjects (9,24,29), the discrepancy cannot be accounted for by differences in the study method. It is reasonable to hypothesize that subjects showing a vasodilator response to ET\(_A\) receptor antagonism have, for some reason, higher ET-1 production compared with those with no hemodynamic response to ET\(_A\) receptor blockade, leading in turn to greater contribution of the peptide to the regulation of vascular tone. This hypothesis seems supported by the results of previous studies in experimental models, showing that ET-1 receptor blockade is not associated with any BP-lowering effect in the absence of increased ET-1 expression in small resistance arteries (30,31).

**Implications.** The finding that, in hypercholesterolemic patients, selective ET\(_A\) receptor blockade counteracts the vasoconstrictor effects of increased endogenous ET-1 activity may have important pathophysiologic and therapeutic implications. Thus, because of the role of ET-1 in atherogenesis (12,13) and vascular remodeling (11), our demonstration of increased ET-1 generation in blood vessels of hypercholesterolemic humans supports the notion of an involvement of this peptide in the pathophysiology of vascular damage in these patients. At the same time, the present findings suggest that targeting the ET-1 system might be potentially beneficial in preventing cardiovascular damage in hypercholesterolemia.

**REFERENCES**


