Responses to neither exogenous nor endogenous endothelin-1 are altered in patients with hypercholesterolemia

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Abstract There is some controversy regarding whether vascular responses to endothelin are altered in hypercholesterolemia. Studies performed to date have been compromised by the use of endothelin antagonists at inappropriate concentrations. In the current study, we examine the role of endothelin-1 in hypercholesterolemic patients using lower, more selective doses of specific endothelin antagonists. Twenty-two patients with hypercholesterolemia (total plasma cholesterol > 6.0 mmol/l) and 17 healthy controls were recruited. Forearm vascular responses to endothelin-1 (5 pmol/min), the endothelin A antagonist BQ-123 (10 nmol/min), and the endothelin B antagonist BQ-788 (1 nmol/min) were obtained. Endothelin-1 caused a significant vasoconstriction in both hypercholesterolemic and control subjects, an effect that was not significantly different between the two groups ($P = 0.784$). BQ-123 caused a significant vasodilatation that was not significantly different between the two groups ($P = 0.899$). Similarly, responses to BQ-788 ($P = 0.774$) and mean plasma endothelin-1 levels were not different (control vs. hypercholesterolemia, 1.16 ± 0.18 vs. 1.06 ± 0.15 fmol/ml; $P = 0.64$). Responses to neither exogenous nor endogenous endothelin are influenced by plasma cholesterol levels in humans. It is thus unlikely that the endothelin system contributes to early vascular disease pathology in patients with hypercholesterolemia.—Boak, L. M., A. M. Dart, S. J. Duffy, and J. P. F. Chin-Dusting. **Responses to neither exogenous nor endogenous endothelin-1 are altered in patients with hypercholesterolemia. *J. Lipid Res.* 2005.** 46: **2667–2672.**

Supplementary key words cholesterol • endothelium • vascular

The endothelin system is well reported to be activated in several cardiovascular disease states (1, 2) and in some groups at high risk of cardiovascular disease (3, 4). In essential hypertension, for example, with the exception of a single report (5), the majority of studies demonstrate an increased vasoconstrictive response to endogenous endothelin (6–9). The role of endothelin in patients with high plasma concentrations of cholesterol, on the other hand, is less unequivocal. Despite reports of increased plasma endothelin-1 levels in both experimental hypercholesterolemia (10, 11) and in patients with increased plasma cholesterol levels (12, 13), vascular responses to endothelin have been examined in only two studies: one reported that these responses are unaltered (9), and the other that responses are enhanced (4). Both studies, however, have been compromised by the use of endothelin antagonists at concentrations that have been reported to be both nonselective and systemically active (14–16), either of which can interfere with data interpretation (17). In the current study, we examine the role of both exogenous and endogenous endothelin-1 responses in patients with hypercholesterolemia using lower, more selective doses of the specific endothelin antagonists to overcome this limitation.

METHODS

Twenty-two patients with high plasma cholesterol levels (>6 mmol/l) were recruited from the Lipid Clinic at the Heart Centre, Alfred Hospital, Melbourne. Those on lipid-lowering therapy were instructed to stop taking their medication for at least 4 weeks before study entry. These included three subjects in the endothelin-1 infusion protocol, one subject in the BQ-123 protocol, and no subjects in the BQ-788 protocol. Seventeen control subjects (total plasma cholesterol < 5.5 mmol/l) with a similar age and gender profile were recruited by advertisement. Initial screening of patients took place by phone, followed by a medical interview and physical examination. Inclusion criteria for both groups were systolic blood pressure ⩽ 140 mmHg and diastolic blood pressure ⩽ 90 mmHg. Exclusion criteria were age > 65 years; HDL cholesterol level < 1.0 mmol/l; current or a history of diabetes, overt cardiovascular disease, or chronic medical illness; pregnancy; long-term medication (including blood pressure-lowering drugs); and heavy alcohol consumption (more than three drinks per day). All participants gave written informed con-

Manuscript received 9 June 2005 and in revised form 16 August 2005 and in re-revised form 9 September 2005.

Published, JLR Papers in Press, September 21, 2005.
DOI 10.1194/jlr.M500236-JLR200

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sent and were given the option to take part in one, two, or all three infusion protocols described below; all protocols were performed on separate days. Those who opted to participate in more than one protocol had at least 2 week intervals between arterial punctures. In total, 6 of the 22 hypercholesterolemic subjects and 3 of the 17 control subjects underwent two of the infusion protocols; a different 2 control subjects consented to participate in all three protocols.

Study protocols

The study was approved by the Alfred Hospital Institutional Ethics Committee. All subjects were instructed to abstain from alcohol- and caffeine-containing beverages as well as to fast overnight before the study day. Each protocol was conducted in a clinical laboratory under quiet, thermoregulated (22°C) conditions. Subjects were rested in a comfortable supine position throughout the course of the study. The nondominant arm was supported at an angle of ~80° from the body and elevated 30° vertical above heart level. The brachial artery was cannulated under local anesthesia (1% lignocaine; Pharmacia and Upjohn, Bentley, Western Australia, Australia) and full aseptic conditions with the forearm antecubital area swabbed down with chlorhexidine (0.5% chlorhexidine gluconate and 70% ethanol; Pharmacia and Upjohn). Cannulation was with a 3.0 F catheter (5 cm; Cook, Eight Mile Plains, Queensland, Australia). Heparinized (2 U/ml 1% heparin sodium; David Bull Las, Mulgrave, Victoria, Australia) physiological saline (0.9% NaCl; Baxter Healthcare, Toogabbie, New South Wales, Australia) was delivered at a constant flow rate of 4 ml/h to maintain vessel patency. Blood was removed and collected in lithium heparin (0.5 ml) tubes before drug delivery for measurement of total, HDL, and LDL cholesterol, triglyceride, and glucose levels, which was performed on a Cholestech LDX lipid analyzer (Cholestech, Hayward, CA). Whole blood (5 ml) was also collected in EDTA tubes, which were subsequently centrifuged for 15 min at 3,000 rpm and 4°C (J6-HC centrifuge; Beckman Instruments, Palo Alto, CA). The plasma obtained was frozen and stored at −20°C until analyzed for plasma endothelin-1 levels. All samples were analyzed (in duplicate) within the same assay. This was performed using an ELISA system (Biotrak Cellular Communication Assay; Amersham Pharmacia Biotech) according to the manufacturer's instructions. The sensitivity of the assay was 0.5 fmol/well; within-assay precision (coefficient of variation) for duplicate determinations, generated by preparing replicates of each standard, ranged from 5% to 16.5%. In the range of our samples (i.e., between 1 and 2 fmol/ml), the coefficient of variation was between 12.2% and 16.5%.

All participants were allowed to acclimatize at rest for ⩾15 min after insertion of the brachial cannula. Forearm blood flow was monitored using forearm venous occlusion plethysmography as described previously (18). In essence, a cuff was placed around the wrist and inflated to ~200 mmHg. The second cuff was placed around the upper arm and inflated to between 40 and 50 mmHg periodically every 10 or 20 s during the period of analysis. A double-stranded alloy (gallium and indium)-filled strain gauge (DE Hokanson, Inc., Bellevue, WA) was applied around the widest part of the forearm. The Hokanson was connected to a MacLab data-acquisition system (ADInstruments, New South Wales, Australia), which was in turn connected to an Apple Macintosh Computer (Apple Computers, Inc., Cupertino, CA). One of three drug infusion protocols (described below) was followed on any one study day.

Drug protocols

After a stable, resting forearm blood flow had been obtained (i.e., after 15 min of rest postcannulation), endothelin-1 (Clinalfa, Basel, Switzerland) was infused at 5 pmol/min for 60 min (hypercholesterolemic patients, n = 12; controls, n = 9). In protocol 2, the endothelin A receptor antagonist BQ-123 (Clinalfa) was infused at 10 nmol/min again for 60 min (hypercholesterolemic patients, n = 11; controls, n = 7). In protocol 3, the endothelin B receptor antagonist BQ-788 (Clinalfa) was infused at 1 nmol/min over 60 min (hypercholesterolemic patients, n = 6; controls, n = 5). Forearm blood flow measurements were obtained every 5 min over the 60 min infusion period. Blood pressure was monitored in the noninfused arm every 3 min using the Dinamap (Critikon, North Ryde, New South Wales, Australia), a semiautomated, noninvasive method of measuring blood pressure by oscillometry.

Statistical analysis

Data are presented as means ± SEM. Two means were compared by Student's *t*-test (unpaired). Within each group, the effect of drug infusion was assessed by one-way ANOVA for repeated measures. Comparison of responses between groups was by two-way ANOVA for repeated measures. All tests were performed using SigmaStat (Jandel Scientific Software, San Rafael, CA). $P < 0.05$ was used as a measure of statistical significance. Sample size was determined based on mean differences (50%) and standard deviations (10–12%) published previously for responses to infusions of endothelin-1 (19) and likewise for responses to BQ-123 (4). The power desired was set at 0.80 and the α numeric at 0.05.

RESULTS

Subject characteristics

The clinical characteristics of the two groups for all three study protocols are listed in **Table 1**. In essence, both groups were well matched for age and gender in all three drug protocols. As well as the anticipated increase in plasma cholesterol levels, the hypercholesterolemic subjects participating in the endothelin-1 infusion protocol also had significantly increased resting blood pressure, triglyceride levels, and body mass index, although none of them individually exceeded the set exclusion criteria.

Plasma endothelin-1 levels

Mean plasma endothelin-1 levels were not different between the hypercholesterolemic subjects (1.06 ± 0.15 fmol/ml; n = 22) and the control subjects (1.16 ± 0.18 fmol/ml; n = 17; $P = 0.64$).

Effect of drug infusions on blood pressure

In all three drug protocols used, mean arterial pressure was not altered during the infusion period (repeated-measures ANOVA within group, all sets, $P < 0.01$).

Vascular responses to exogenous endothelin-1 infusion

Endothelin-1 caused a significant vasoconstrictor response in both hypercholesterolemic subjects (one-way repeated-measures ANOVA, $P < 0.001$) and control subjects ($P < 0.001$). The maximal constrictor response to endothelin-1 in hypercholesterolemic patients was 37.8 ± 5.4% compared with 32.3 ± 7.2% in controls. The entire time-response curve to endothelin-1 was not significantly different between the two groups (**Fig. 1**) (two-way repeated-measures ANOVA, $P = 0.784$). Analysis was also performed

TABLE 1. Basal characteristics of healthy control subjects and patients with hypercholesterolemia

Characteristics	Healthy Control Subjects	Hypercholesterolemic Subjects	*P*
Endothelin-1 study			
Number of subjects	9	12	
Gender (male/female)	7/2	9/3	
Age (years)	48.6 ± 5.2	48.3 ± 3.4	0.959
Smoking (yes/no)	1/8	2/10	
Body mass index	23.7 ± 0.4	25.2 ± 0.5	0.036
Systolic blood pressure (mmHg)	114.9 ± 2.8	133.1 ± 5.6	0.015
Diastolic blood pressure (mmHg)	69.5 ± 1.5	78.7 ± 2.0	0.003
Mean arterial pressure (mmHg)	88.8 ± 2.2	98.2 ± 3.1	<0.001
Heart rate (beats per min)	56.3 ± 1.9	59.1 ± 2.7	0.420
Total cholesterol (mmol/l)	4.9 ± 0.2	7.3 ± 0.4	<0.001
LDL cholesterol (mmol/l)	3.2 ± 0.1	4.4 ± 0.3	<0.001
HDL cholesterol (mmol/l)	1.2 ± 0.1	1.2 ± 0.1	0.899
Triglyceride (mmol/l)	1.1 ± 0.2	3.4 ± 0.5	0.002
VLDL (mmol/l)	0.5 ± 0.1	1.2 ± 0.2	0.006
Glucose (mmol/l)	5.6 ± 0.3	5.6 ± 0.2	0.970
BQ-123 study			
Number of subjects	7	11	
Gender (male/female)	6/1	7/4	
Age (years)	57.7 ± 3.5	51.3 ± 2.3	0.122
Smoking (yes/no)	0/7	0/11	
Body mass index	24.9 ± 0.9	24.6 ± 0.5	0.757
Systolic blood pressure (mmHg)	129.6 ± 7.5	123.1 ± 3.8	0.409
Diastolic blood pressure (mmHg)	75.9 ± 2.2	75.7 ± 2.4	0.971
Mean arterial pressure (mmHg)	95.3 ± 2.6	95.6 ± 3.2	0.955
Heart rate (beats per min)	56.0 ± 2.7	60.5 ± 2.6	0.271
Total cholesterol (mmol/l)	4.7 ± 0.4	7.0 ± 0.3	<0.001
LDL cholesterol (mmol/l)	2.6 ± 0.5	4.2 ± 0.3	0.009
HDL cholesterol (mmol/l)	1.6 ± 0.2	1.8 ± 0.2	0.061
Triglyceride (mmol/l)	1.5 ± 0.4	1.7 ± 0.2	0.668
VLDL (mmol/l)	0.7 ± 0.2	0.8 ± 0.1	0.704
Glucose (mmol/l)	5.5 ± 0.2	5.1 ± 0.1	0.054
BQ-788 study			
Number of subjects	6	5	
Gender (male/female)	6/0	2/3	
Age (years)	55.3 ± 3.7	55.0 ± 2.8	0.946
Smoking (yes/no)	0/6	0/5	
Body mass index	25.1 ± 1.1	23.8 ± 1.0	0.416
Systolic blood pressure (mmHg)	122.7 ± 8.7	118.0 ± 6.2	0.685
Diastolic blood pressure (mmHg)	73.2 ± 3.5	78.6 ± 3.1	0.286
Mean arterial pressure (mmHg)	92.3 ± 5.7	92.4 ± 3.8	0.993
Heart rate (beats per min)	59.8 ± 2.3	58.8 ± 1.6	0.734
Total cholesterol (mmol/l)	4.9 ± 0.3	6.5 ± 0.2	0.004
LDL cholesterol (mmol/l)	3.3 ± 0.3	4.3 ± 0.2	0.016
HDL cholesterol (mmol/l)	1.1 ± 0.1	1.5 ± 0.2	0.429
Triglyceride (mmol/l)	1.3 ± 0.3	1.5 ± 0.3	0.522
VLDL (mmol/l)	0.6 ± 0.1	0.8 ± 0.2	0.435
Glucose (mmol/l)	5.4 ± 0.2	5.2 ± 0.2	0.458

Where $P < 0.05$ indicates statistical significance.

on two subgroups of patients: those who had never received lipid-lowering therapy (n = 9) compared with controls, and those whose triglyceride levels were <3.5 mmol/l (bringing the average down to 2.1 mmol/l). The outcomes of these analyses were not altered (Fig. 1).

Vascular response to endothelin A receptor blockade

BQ-123 caused a significant vasodilatory response in both hypercholesterolemic subjects ($P = 0.002$) and control subjects ($P = 0.002$). The maximal vasodilatory effect of BQ-123 in hypercholesterolemic patients was 39.7 ± 8.7% compared with 34.90 ± 4.21% in controls. The entire time-response curve to BQ-123 was not significantly different between the two groups (**Fig. 2**) ($P = 0.899$). Analysis was also performed on the subgroup of patients who had never received therapy (n = 10) compared with controls. The outcome of this analysis was not different (Fig. 2).

Vascular responses to endothelin B receptor blockade

BQ-788 did not induce a clear effect on forearm blood flow in either hypercholesterolemic subjects ($P = 0.332$) or control subjects ($P = 0.239$). Analysis by two-way repeated-measures ANOVA did not reveal a difference in the response to this drug between the two groups (**Fig. 3**) ($P = 0.774$).

DISCUSSION

The current study demonstrates that responses to neither exogenous endothelin-1 nor endogenous endothelin-1, assessed by selective blockade of endothelin A receptors with BQ-123 and endothelin B receptors with BQ-788, are altered by high plasma concentrations of cholesterol. Previous studies examining the influence of plasma cho-

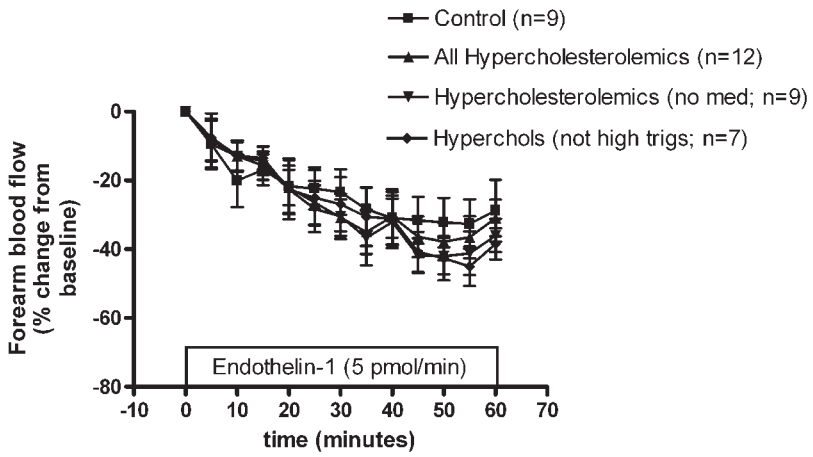

Fig. 1. Forearm blood flow responses to intra-arterial infusion of endothelin-1 (5 pmol/min) in healthy control subjects (Control), patients with hypercholesterolemia (All Hypercholesterolemics; $P = 0.784$), patients with hypercholesterolemia who have never been on medication [Hypercholesterolemics (no med); $P = 0.109$], and patients with hypercholesterolemia who have triglyceride levels of <3.5 mmol/l [Hyperchols (not high trigs); $P = 0.691$]. Values represent means ± SEM. Analysis was by two-way repeated-measures ANOVA comparison against controls.

lesterol levels on the role of endothelin in humans have been confounded with the use of these selective antagonists, particularly BQ-123, at high concentrations, which have been demonstrated to have modest systemic effects (9, 14, 15). Because systemic effects may involve changes in sympathetic output, central effects, hormonal responses, and alterations in blood pressure that can make changes in forearm blood flow difficult to interpret (19), the use of these receptors at such concentrations is clearly suboptimal. In the current study, at the lower concentrations used, neither BQ-123 nor BQ-788 affected blood pressure or heart rate. Furthermore, BQ-123 infused at the higher concentration of 100 nmol/min (as was done in both previous studies) has been shown (17) to achieve local concentrations greater than that deemed selective for endothelin A receptors, and unwanted, nonspecific inhibition at the endothelin B receptor is also achieved. Thus, a careful reexamination of the responses to infusions of BQ-123 used in similar studies is warranted, particularly in normal, healthy control groups used to remove confounding interpretation of data resulting from differences in disease severity or etiology. In the current study, the lower concentration of BQ-123 (10 nmol/l) induced an increase in blood flow of 35%; in the Cardillo et al. study (4), BQ-123 at 100 nmol/min achieved no change in basal blood flow; in the study by Nohria et al. (9), an increase of 20% was observed with BQ-123 at 100 nmol/min; in the Ferro et al. study (19) (a different research team from the previous two), an increase of 35% was observed with the higher concentration. Thus, the use of BQ-123 at 10 nmol/min

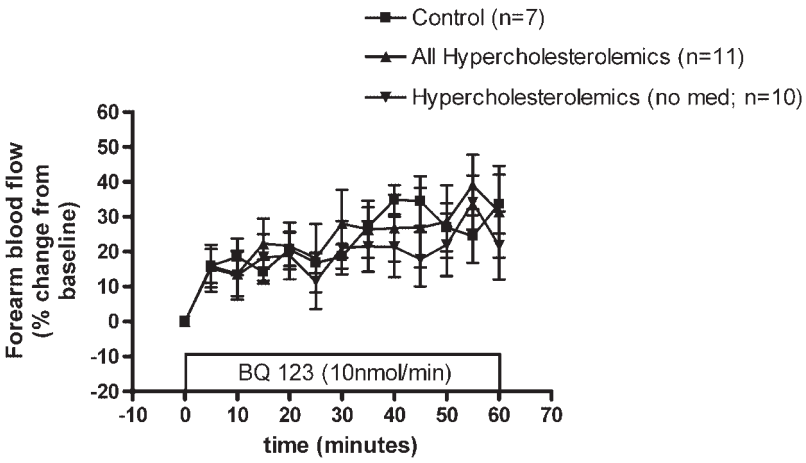

Fig. 2. Forearm blood flow responses to intra-arterial infusion of BQ-123 (10 nmol/min) in healthy control subjects (Control), patients with hypercholesterolemia (All Hypercholesterolemics; $P = 0.899$), and patients with hypercholesterolemia who have never been on medication [Hypercholesterolemics (no med); $P = 0.632$]. Values represent means ± SEM. Analysis was by two-way repeated-measures ANOVA comparison against controls.

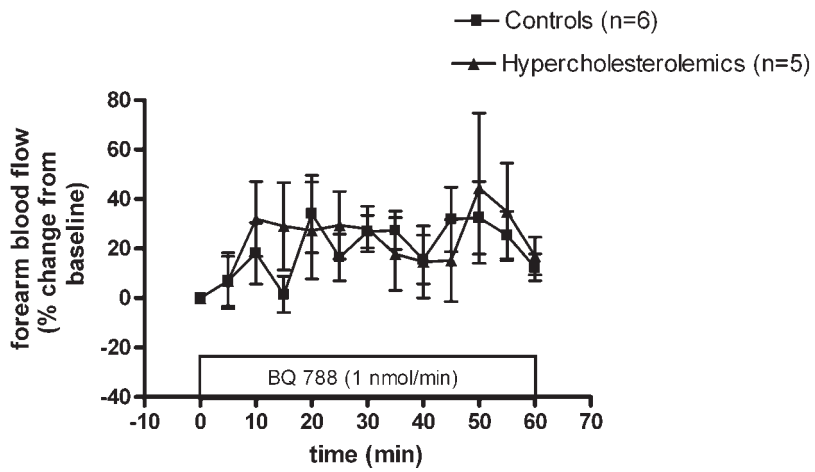

Fig. 3. Forearm blood flow responses to intra-arterial infusion of BQ-788 (1 nmol/min) in healthy control subjects (Control) and patients with hypercholesterolemia (Hypercholesterolemics; $P = 0.744$). Values represent means ± SEM. Analysis was by two-way repeated-measures ANOVA comparison against controls.

in the current study supports the contention (16) that the degree of vasodilatation achieved at this concentration is, if anything, more, certainly not less, than that obtained at 100 nmol/min in previous studies, suggesting that endothelin B receptors (causing a counterconstrictive effect when blocked) are less likely to be inhibited at the lower concentration and less likely to confound the interpretation of the data obtained. Similarly, in hypercholesterolemic subjects, BQ-123 (10 nmol/min in the current study) induced an increase in blood flow of 40% compared with 25% obtained with BQ-123 (100 nmol/min) in the studies of Cardillo et al. (4) and Nohria et al. (4), again suggesting that at the lower concentration a more selective effect on endothelin A receptors was observed. What is currently lacking is a selective endothelial-specific endothelin B receptor antagonist, which may help unravel some of the discrepancies in the data outcomes stated above (e.g., the difference in the scale of increase with BQ-123 at 10 nmol/min compared with 100 nmol/min in the control versus patient groups when such comparisons are made between some of the studies). Regardless of these anomalies, however, we would argue that the correct concentration for the selective blockade of endothelin A receptors has been used in the current study, thus removing at least the unwanted effects of blocking endothelin B receptors.

Similar to the findings of Cardillo et al. (4), it was observed in this study that vasoconstrictive responses to infusions of exogenous endothelin-1 were not influenced by hypercholesterolemia. This finding is of interest despite the fact that subjects in the current study displayed significantly higher, albeit clinically insignificant, increases in body mass index, blood pressure, and plasma triglyceride levels as well as higher plasma cholesterol levels compared with the matched controls; these additional factors did deter from the finding that the responses to endothelin were comparable to those observed in controls. Unlike the Cardillo et al. study (4), however, responses to BQ-123 at the lower concentration of 10 nmol/min in the current study were also not influenced by hypercholesterolemia. Although the cohort in the current study had a lower average plasma cholesterol level (total plasma cholesterol of 7 mmol/l compared with 7.5 mmol/l in the previous study), it is unlikely that such a marginal difference would account for the difference in response to BQ-123 observed. Indeed, when responses to all three of the drugs used (endothelin-1, BQ-123, and BQ-788) at 60 min of infusion were plotted against total plasma cholesterol (which ranged from 4 to 11 mmol/l), the correlation coefficients were 0.012 ($P = 0.96$), 0.109 ($P = 0.677$), and 0.217 ($P = 0.521$), respectively, demonstrating no evidence of an association between cholesterol levels versus vascular responsiveness to either exogenous or endogenous endothelin.

Responses to infusion of the selective endothelin B receptor antagonist BQ-788 at 1 nmol/min were, on the other hand, less informative. At this concentration, Verhaar et al. (16) demonstrated a consistent constrictive response in healthy controls. In the current study, the responses obtained with BQ-788 were highly inconsistent (neither consistently constrictive nor dilatory) in healthy control subjects as well as in patients with high plasma cholesterol levels. Although the discrepancy in findings in the control group may be age-dependent [healthy controls in the previous study were younger (ranging from 20 to 48 years)], this is unlikely in that other studies examining older age controls have demonstrated a constrictive (albeit marginal) response (2). On the other hand, the effects of BQ-788 have been reported to be contradictory and dependent on gender (20), which may explain some of the contradictory results in the current study.

In a previous study with patients with overt vascular disease (i.e., atherosclerosis) (2), the response to BQ-788 was significantly amplified. Responses to BQ-788 in our hypercholesterolemic cohort were not affected by plasma cholesterol levels, consistent with our hypothesis that responses to endogenous endothelin-1 were not affected by

this early-stage disease. Further support comes from the finding that plasma endothelin-1 levels were not different in the two groups in the current study.

We thus conclude that responses to neither exogenous nor endogenous endothelin are influenced by high plasma cholesterol levels in humans and that the endothelin system is unlikely to contribute to early vascular disease pathology in these subjects.

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