Endothelium-mediated Coronary Blood Flow Modulation in Humans

Effects of Age, Atherosclerosis, Hypercholesterolemia, and Hypertension

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Abstract

The effects of age, atherosclerosis, hypertension, and hypercholesterolemia on vascular function of the coronary circulation were studied by subselective intracoronary infusions of acetylcholine, which releases endothelium-derived relaxing factor, and papaverine, which directly relaxes vascular smooth muscle, in normal patients (n = 18; no risk factors for coronary artery disease), in patients with evidence of early atherosclerosis but normal cholesterol levels and normal blood pressure (n = 12), in patients with hypertension without left ventricular hypertrophy (n = 12), and in patients with hypercholesterolemia (n = 20). Papaverine-induced maximal increases in coronary blood flow were significantly greater in normals, but no differences were noted between the groups of patients with early atherosclerosis, with hypertension, and with hypercholesterolemia. The capacity of the coronary system to increase blood flow in response to acetylcholine was similar in normal and normocholesterolemic patients with epicardial atherosclerosis and/or hypertension but was significantly impaired in patients with hypercholesterolemia, irrespective of evidence of epicardial atherosclerotic lesions. Age ($r = -0.62$, $P < 0.0001$) and total serum cholesterol levels ($r = -0.70$; $P < 0.0001$) were the only significant independent predictors of a blunted coronary blood flow response to acetylcholine. Thus, hypercholesterolemia and advanced age selectively impair endothelium-mediated relaxation of the coronary microvasculature in response to acetylcholine, whereas endothelial dysfunction is restricted to epicardial arteries in age-matched normocholesterolemic patients with evidence of coronary atherosclerosis and/or hypertension. (*J. Clin. Invest.* 1993. 92:652–662.) Key words: endothelium-derived relaxing factor • coronary artery disease • acetylcholine • coronary vasomotor tone • risk factors

Introduction

The endothelium covers the inner surface of all blood vessels and has been recognized as playing a major role in modulating vascular smooth muscle tone by synthesizing and metabolizing vasoactive substances (1), including an endothelium-derived relaxing factor (EDRF)[1] (2), which is released after the stimulation of muscarinic receptors on endothelial cells by acetylcholine as well as by other agonists or physical stimuli (3, 4). Produced by endothelial cells, this factor traverses the subendothelial space and activates smooth muscle cell guanylate cyclase to increase cyclic guanosine monophosphate levels, leading to smooth muscle relaxation (5, 6). Evidence has been provided that nitric oxide, derived from L-arginine, or a related compound, may account for the biological activity of EDRF (7, 8).

Recently, it has become apparent that atherosclerosis markedly impairs this important function of the endothelium in large conduit vessels from a variety of experimental animals (9–11) as well as in humans (12–14). The observation that endothelium-dependent vasodilation of conduit vessels is abnormal in the presence of atherosclerosis is not surprising, because the thickened intima associated with atherosclerosis may act as a barrier to vasoactive substances released from the endothelium as well as because progression of atherosclerosis leads to an injury of the endothelium itself (15). Indeed, we have recently demonstrated a hierarchical structure in the impairment of endothelium-dependent responses with progressive disease in human epicardial conductance vessels in vivo (16).

Although possibly important in the genesis of vascular spasm, this abnormality of conduit vessel function probably contributes little to the regulation of coronary blood flow in the absence of spasm, because myocardial perfusion is regulated predominantly by microvessels < 200 μm in diameter (17). Although a functional abnormality of the coronary microvasculature has been recognized as a cause of chest pain in patients with angiographically normal coronary arteries and angina (18), little is known about the endothelium-mediated regulation of the coronary microvasculature in humans. Unlike the walls of larger arteries, the walls of resistance vessels do not develop overt atheroma after exposure to high levels of cholesterol (19). Nevertheless, several recent experimental studies did show that endothelium-dependent relaxation is abnormal in the resistance vessels of cholesterol-fed atherosclerotic animals (20–22). These results suggest that, despite the heterogeneity in the vascular susceptibility to develop overt atheroma in response to the atherogenic substance, the exposure of blood vessels to increased levels of circulating cholesterol induces abnormalities in vascular function in both conductance and resistance vessels. Yet, early in the course of coronary atherosclerotic disease before the development of hemodynamically significant epicardial artery lesions, an altered endothelial regulation of blood vessels would be of substantial pathophysiological importance if it would occur at flow-regulating sites within the coronary circulation. In addition, the presence of

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1. *Abbreviations used in this paper:* CAD, coronary artery disease; EDRF, endothelium-derived relaxing factor.

conditions contributing to the development of coronary atherosclerosis, such as hypertension and advanced age, may independently affect endothelial function of the coronary microvasculature and thereby exacerbate the pathophysiological consequences of coronary artery disease.

Thus, the present study was designed to assess the responsiveness of the coronary microvasculature in patients with early stages of epicardial atherosclerosis without flow-limiting stenoses; to evaluate the effects of plasma cholesterol levels on endothelium-mediated coronary blood flow modulation; to determine the effects of atherosclerosis associated with arterial hypertension on endothelium-mediated vascular function of the coronary circulation; and to evaluate whether age independently affects vascular function at the level of the coronary microvasculature.

Methods

Patient population

The study population included 62 patients undergoing routine diagnostic cardiac catheterization. These patients were classified into four groups on the basis of their history and the presence or absence of atherosclerosis on the diagnostic coronary angiogram. Patients with unstable angina, recent myocardial infarction, a clinical history suggestive of variant angina, valvular heart disease, clinical evidence of heart failure, and diabetes mellitus were excluded. In addition, no patient had angiographic or echocardiographic evidence of left ventricular hypertrophy. The epicardial artery vasomotor responses of 22 of these patients have been previously reported (16). Written informed consent was obtained from all patients before the study. The study protocol was approved by the Ethical Committee of the University of Freiburg.

Normal control group. The normal control group comprised 18 patients (6 women, 12 men). Normalcy was defined as: absence of a history of arterial hypertension (defined as chronically elevated blood pressure ≥ 150/95 mmHg) or hypercholesterolemia (total serum cholesterol level within the 75th percentile adjusted for age and sex); angiographically normal, smooth coronary arteries without luminal irregularities and a vasodilator response of the left anterior descending coronary artery (the territory under study) to the intracoronary infusion of acetylcholine up to a dosage of 7.2 μg/min; no evidence of segmental wall motion abnormalities and a left ventricular ejection fraction > 55% assessed by biplane cineventriculography; and a normal coronary flow reserve exceeding 4.0 in response to 7 mg papaverine subselectively infused into the left anterior descending coronary artery.

The mean age of these patients was 48.2±9.3 yr, ranging from 22 to 60 yr. Mean total serum cholesterol level was 200.9±21.3 mg%, ranging from 176 to 241 mg% at the time of the study. 16 patients underwent diagnostic coronary angiography for evaluation of atypical chest pain, and intermittent left bundle branch block was the cause for referral in two patients.

Coronary artery disease (CAD) group. 12 patients had angiographic evidence of coronary atherosclerosis, but < 30% luminal narrowing of the left anterior descending artery. Of these patients, four had > 50% luminal narrowing of either the right coronary artery or the left circumflex artery. The left anterior descending artery was angiographically normal in five patients, and demonstrated luminal irregularities with < 30% luminal narrowing in seven patients. All patients had normal total serum cholesterol levels ranging from 176 to 234 mg% (mean 209.8±17.6 mg%). None of these patients had a history of arterial hypertension requiring the initiation of antihypertensive therapy by the primary physician. Their mean age was 50.2±6.7 yr, ranging from 38 to 60 yr. Two patients were women and 10 were men.

Hypertension group. 12 patients had a history of arterial hypertension requiring the initiation of antihypertensive therapy by the primary physician. None of these patients had angiographic or echocardiographic evidence of left ventricular hypertrophy. In seven of these patients, the left anterior descending artery appeared angiographically normal, whereas luminal irregularities were visible in five patients. All patients had normal total serum cholesterol levels ranging from 179 to 232 mg% (mean 212.8±21.3 mg%). Their mean age was 55.9±6.5 yr, ranging from 47 to 68 yr, and three were women.

Hypercholesterolemia group. 20 patients had total serum cholesterol levels exceeding the 75th percentile adjusted for age and sex. Their cholesterol levels ranged from 246 to 346 mg% (mean 284.2±30.0 mg%). 13 patients had an angiographically normal appearing left anterior descending artery, whereas luminal irregularities were visible in seven patients. Their mean age was 52.7±11.7 yr, ranging from 22 to 63 yr. 10 of these patients had a history of arterial hypertension, but no patient had angiographic or echocardiographic evidence of left ventricular hypertrophy. 3 patients were women and 17 were men. None of the patients in this study had a history of myocardial infarction in the territory of the left anterior descending coronary artery. All patients demonstrated a normal left ventricular contraction pattern in the anterior and septal left ventricular wall and a normal global ejection fraction as assessed by biplane cineventriculography. Left ventricular enddiastolic pressure was ≤ 13 mmHg in all patients.

Study protocol

Vasoactive medications, including calcium channel blockers, angiotensin-converting enzyme inhibitors, and long-acting nitrates, were withheld ≥ 24 hours before cardiac catheterization. No patient received beta-adrenergic blockers within 48 h before the study. A total of eight patients were on aspirin therapy during the study (one in the normal, three in the CAD, two in the hypercholesterolemia, and two in the hypertension group). Diagnostic left heart catheterization and coronary angiography were performed by a standard percutaneous femoral approach. After completion of the diagnostic catheterization, an additional 5,000 U of heparin were given intravenously and an 8 F guiding catheter (Schneider, Zurich, Switzerland) was introduced into the left main coronary artery. A 3 F catheter (Monorail-Doppler; Schneider) with a 20 MHz pulsed Doppler crystal was advanced into the left anterior descending artery via a 0.014-in guide wire. The Doppler catheter was carefully positioned to obtain a stable flow velocity signal. Before introducing the Doppler catheter into the guiding catheter, the flow velocity recordings were referenced to zero and calibrated.

After stable baseline conditions were obtained, acetylcholine was selectively infused into the left anterior descending artery via the Doppler catheter to assess endothelium-dependent increases in coronary blood flow. Increasing dosages of acetylcholine (0.036, 0.36, and 3.6 μg/ml) were infused at an infusion rate of 2 ml/min, lasting 3 min for each concentration. The lowest dose of 0.036 μg acetylcholine/ml corresponds to an estimated blood concentration in the coronary bed of 10^{-8} M, assuming a blood flow of 80 ml/min. Stepwise acetylcholine infusions were terminated either when vessel occlusion occurred or when the largest dose (3.6 μg/ml) was reached.

10 min after acetylcholine infusion, 7 mg papaverine was subselectively injected into the left anterior descending artery via the Doppler catheter to assess endothelium-independent coronary flow reserve in the territory of the left anterior descending artery. Previous studies (23) have demonstrated that the dose of 7 mg papaverine, subselectively infused into the left anterior descending artery, elicits a maximal increase in coronary blood flow without affecting global hemodynamic parameters.

Throughout the study, phasic and mean intracoronary blood flow velocity, heart rate, and aortic pressure (via the guiding catheter) were continuously measured. Serial hand injections of nonionic contrast material (Ultravist; Schering AG, Berlin, FRG) were performed during control, at the end of each acetylcholine infusion period, at recontrol after acetylcholine infusion, and after subselective infusion of papaverine.

Quantitative coronary angiography

The method of quantitative coronary angiography has been previously described (16, 23, 24). In brief, using a simultaneous biplane multidi-

rectional isocentric x-ray system (Siemens Bicor, Erlangen, FRG), the coronary arteries under study were positioned near the isocenter, biplane cine-angiograms were recorded at a frame rate of 25 frames/s, and enddiastolic cine frames were videodigitized and stored in the image analysis system (Mipron I; Kontron Electronics, Eching, FRG) in a 512 × 512 matrix with an 8-bit gray scale. Using the 12-cm field of view, the resulting pixel density was 7.3 pixels/mm. Automatic contour detection was performed by a previously described and validated method using a geometric edge differentiation technique (23, 24), and the exact radiological magnification factor of the measured segment was calculated to scale the data from pixels to millimeters (25). The accuracy and precision of this technique, as well as the reproducibility of serial measurements under routine clinical conditions, have been established in previous studies (23, 24).

Quantitative angiography of the epicardial artery was performed for two purposes. First, to determine cross-sectional area of the artery immediately distal to the radiopaque tip of the Doppler catheter to convert the Doppler-derived flow velocity to an estimate of coronary arterial flow. Second, to exclude limitations of coronary artery flow due to epicardial coronary artery constriction in response to acetylcholine by measuring the most constricting epicardial artery segment distal to the tip of the Doppler catheter, as previously suggested by Treasure et al. (26). To determine cross-sectional area of the artery, a 5–7-mm segment was measured immediately distal to the tip of the Doppler catheter. A series of diameter measurements was obtained for each scanline for the length of the arterial segment, displayed in graph form, showing diameter versus segment length, and the mean diameter value was calculated. Whenever possible, measurements were performed in both views of the biplane images using the radiopaque tip of the Doppler catheter for identification of corresponding vessel segments, and the vessels' cross-sectional area was calculated from both views assuming an elliptical shape. Only single-plane analysis was performed for those coronary segments demonstrating overlapping with other parts of the coronary tree in one view. In those cases (19 of 62 patients = 31%), vessel cross-sectional area was calculated assuming a circular shape. Measurement of the most constricting artery segment was performed in a similar fashion. However, instead of calculating the mean diameter value, the minimal absolute diameter of the analyzed segment was identified in both views and minimal cross-sectional area was calculated. Flow-limiting constriction was defined as > 50% cross-sectional area reduction compared with preacetylcholine cross-sectional area of the identical segment.

Data analysis

For estimation of directional changes in coronary blood flow, a coronary flow index was calculated by multiplying the mean Doppler-derived blood flow velocity with the computed cross-sectional area of the vessel segment immediately distal to the tip of the Doppler catheter. Since the injection of contrast material into the coronary circulation resulted in the typical biphasic response of coronary blood flow velocity with an initial decrease followed by an increase in flow velocity due to the hyperemic effects of the contrast material, the mean blood flow velocity immediately before the contrast injection was used for estimation of coronary blood flow. Blood flow responses to intracoronary acetylcholine infusion were analyzed in two ways and yielded similar results. First, as previously described (26), the slope of the dose–response relation to acetylcholine was calculated to correct for the fact that severe vasoconstriction with > 50% cross-sectional area reduction of the most constricting segment precluded the assessment of acetylcholine-induced increases in coronary blood flow. Using linear regression, the slope of the acetylcholine dose–response relation (% change in coronary blood flow index/dosage of acetylcholine) was calculated from the available doses for each patient. In the dose range of acetylcholine used in this study, a linear relation occurred between acetylcholine dose and the percent change in coronary blood flow index in each individual patient. The mean correlation coefficient was 0.91±0.07, ranging from 0.68 to 0.99, indicating good fit for the calculated regression lines used for the slope calculation. Second, because all patients received the 0.36 μg/ml dose of acetylcholine and no patient demonstrated > 50% constriction of the most constricting epicardial artery segment at this dose, the analysis was performed with the response to the single 0.36 μg/ml dose of acetylcholine.

Statistical analysis

All data are expressed as mean±SD, unless otherwise stated. Statistical comparisons were made by analysis of variance followed by the Student–Newman–Keul test. Linear-regression analysis was used to compare blood flow responses with age and serum cholesterol levels. Multivariate analysis using multiple stepwise regression techniques was performed to examine potential interactions between age, gender, cholesterol level, the presence or absence of angiographically visible epicardial artery atherosclerosis, and hypertension upon acetylcholine-induced blood flow responses. Statistical significance was assumed if a null hypothesis could be rejected at the 0.05 probability level.

Results

Baseline hemodynamic characteristics. The baseline systemic and coronary hemodynamic characteristics are summarized in Table I for the four groups of patients. There was no significant difference in heart rate, baseline epicardial artery cross-sectional area, or coronary blood flow index between the four groups of patients. As expected as a result of the enrollment criteria, mean aortic pressure was significantly higher in the group of patients with a history of hypertension.

Responses to acetylcholine and papaverine. No significant changes in mean aortic pressure or heart rate occurred during subselective infusion of either acetylcholine or papaverine. Fig. 1 illustrates the epicardial artery vasomotor response to the various interventions in a patient with hypercholesterolemia. In the normal control group, epicardial artery cross-sectional area increased during the acetylcholine infusion from 10.6±7.3 mm^2 at baseline to 12.8±7.9 mm^2 at the 3.6 μg/ml dose of acetylcholine corresponding to a 23.2±15.0% increase in lu-

Table I. Basal Systemic and Coronary Hemodynamic Characteristics

	Normals $n = 18$	CAD $n = 12$	Hypertension $n = 12$	Hypercholesterolemia $n = 20$
Heart rate (min^{-1})	71.7±9.7	73.8±9.9	69.8±10.3	70.9±9.6
MAP (mmHg)	90.1±5.3	89.1±8.4	101.2±7.0*	93.2±7.9
Epicardial artery luminal area (mm^2)	10.6±7.3	9.0±4.9	6.3±2.8	7.0±4.0
Coronary blood flow index (kHz × mm^2)	70.3±55.3	43.7±27.2	68.3±66.0	49.3±27.9

Values are mean±SD. MAP, mean aortic pressure; coronary blood flow index = Doppler-derived mean flow velocity × epicardial artery luminal area. * $P < 0.05$ versus normals, CAD, and hypercholesterolemia groups.

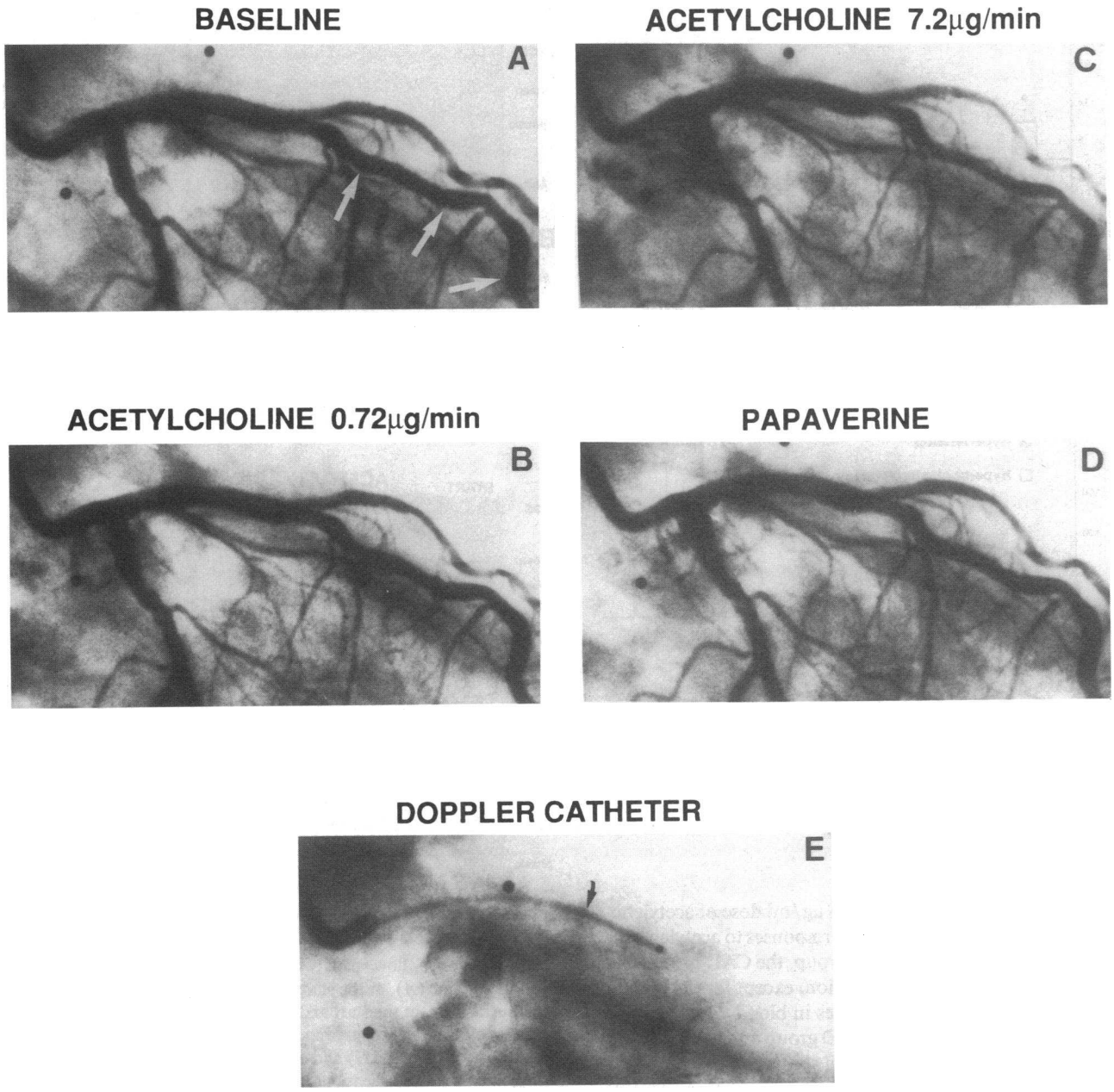

Figure 1. Coronary angiogram of a patient with hypercholesterolemia at baseline (*A; white arrows* indicate left anterior descending coronary artery distal to the Doppler infusion catheter), during 0.72 μg/min acetylcholine infusion (*B*), during 7.2 μg/min acetylcholine infusion (*C*), immediately after papaverine infusion (*D*), and position of the contrast-filled Doppler catheter (*small arrow, E*).

minal area (Fig. 2 *A*). In contrast, acetylcholine elicited a dose-dependent vasoconstrictor response of epicardial conductance vessels in patients with atherosclerosis, hypertension, and hypercholesterolemia (Fig. 2 *A*). Mean epicardial artery cross-sectional area decreased from 9.0±4.9 mm^2 at baseline to 6.7±3.6 mm^2 at the 3.6 μg/ml acetylcholine dose in patients with atherosclerosis, from 6.3±2.8 to 4.6±2.1 mm^2 in patients with hypertension, and from 7.0±4.0 to 4.9±3.9 mm^2 in patients with hypercholesterolemia. The extent of epicardial artery vasoconstriction was similar in all three groups of patients at each individual concentration of acetylcholine (Fig. 2 *A*). Moreover, the vasoconstrictor response did not significantly differ in angiographically normal arteries and arteries with angiographically visible wall irregularities.

Fig. 3 illustrates the intracoronary flow velocity tracings corresponding to the angiograms shown in Fig. 1 at the various interventions. As shown in Fig. 2 *B*, the increases in coronary blood flow indexes in response to acetylcholine were significantly different in the four groups of patients. Compared with the patients with normal total serum cholesterol levels (normal control group, CAD group, and hypertension group), the vasodilator response of the coronary microcirculation to acetylcholine was blunted in patients with hypercholesterolemia. This blunted coronary blood flow response achieved statistical signif-

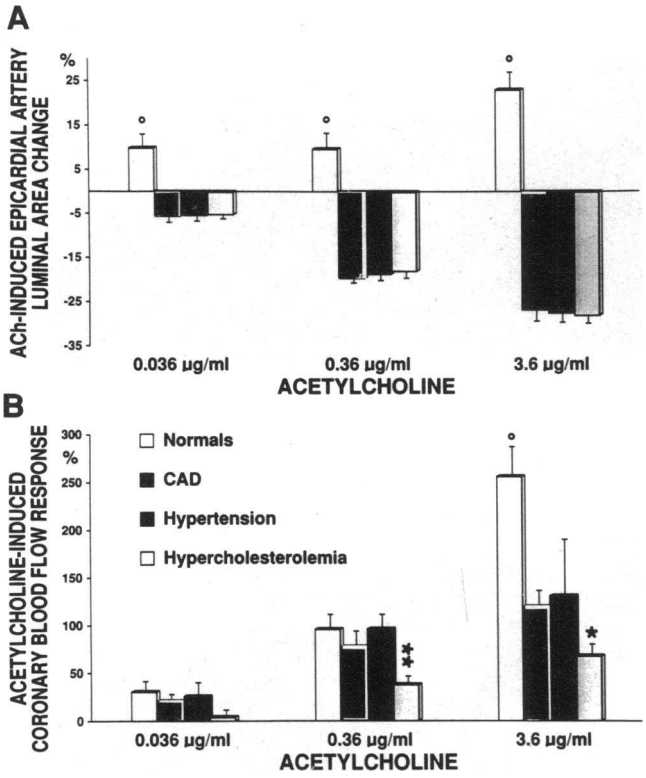

Figure 2. Dose-dependent effects of acetylcholine on epicardial artery luminal area (percentage change from baseline, *A*) and coronary blood flow (percentage change from baseline, *B*). Mean±SEM. (*Open circle*) $P < 0.01$ vs. CAD, hypertension, and hypercholesterolemia groups; (*) $P < 0.05$ vs. CAD and hypertension, and $P < 0.01$ vs. normals group; (**) $P < 0.05$ vs. normals, CAD, and hypertension group.

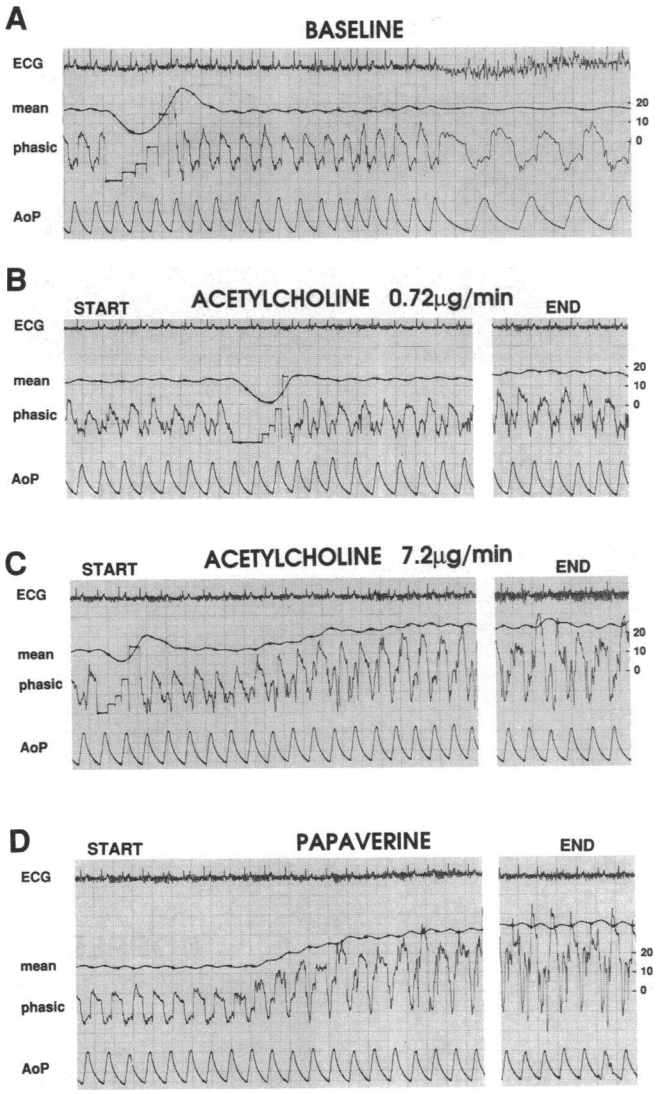

Figure 3. Coronary flow velocity tracings of the patient illustrated in Fig. 1 at baseline (*A*), at the start and end of 0.72-µg/min acetylcholine infusion (*B*), at the start and end of 7.2-µg/min acetylcholine infusion (*C*), and at the start and end of papaverine infusion (*D*). Note that heart rate (*ECG*) and aortic pressure (*AoP*) are unchanged during the various interventions, whereas mean blood flow velocity increases by 40% at the 0.72-µg/min acetylcholine concentration (*B*), by 90% at the 7.2-µg/min acetylcholine concentration (*C*), and by 280% after 7 mg papaverine (*D*).

icance ($P < 0.05$) at the 0.36 and 3.6 µg/ml dose of acetylcholine. The dose-dependent blood flow responses to acetylcholine were similar in the normal control group, the CAD group, and the group of patients with hypertension, except for the highest dose of acetylcholine, where increases in blood flow were also significantly blunted in both the CAD group and the hypertension group compared with the normal control group (Fig. 2 *B*). The slope of the acetylcholine dose–response relation (percent change in coronary blood flow index/dosage of acetylcholine) was 81.9±39.3 in the normal control group, 43.4±13.2 in the CAD group ($P < 0.05$ vs. normal control group), 50.5±27.6 in the hypertension group ($P < 0.05$ vs. normal control group), and 24.6±14.9 in the hypercholesterolemia group ($P < 0.01$ vs. normal control group and $P < 0.05$ vs. both coronary artery disease and hypertension groups). These data demonstrate that, despite similar vasoconstrictor responses of the epicardial conductance vessels, acetylcholine-induced dilation of the coronary microvasculature is impaired in patients with hypercholesterolemia compared with patients with atherosclerosis and hypertension but normal total serum cholesterol levels. Fig. 4 illustrates that there was no relation between basal epicardial artery luminal area and acetylcholine-induced blood flow responses, indicating that intrinsic coronary artery size did not determine blood flow responses to acetylcholine.

To determine whether hypercholesterolemia affects smooth muscle function directly, the coronary blood flow responses to the smooth muscle relaxant papaverine were examined. Coronary blood flow in response to papaverine increased by 476.1±127.7% in the normal control group, by 335.2±104.1% in the CAD group ($P < 0.05$ vs. normal control group), by 336.7±105.7% in the hypertension group ($P < 0.05$ vs. normal control group), and by 356.9±150.6% in the hypercholesterolemia group ($P < 0.05$ vs. normal control group). Thus, although the normal control group demonstrated a significantly higher endothelium-independent coronary blood flow reserve, there were essentially no differences between the groups of patients with atherosclerosis, hypertension, or hypercholesterolemia.

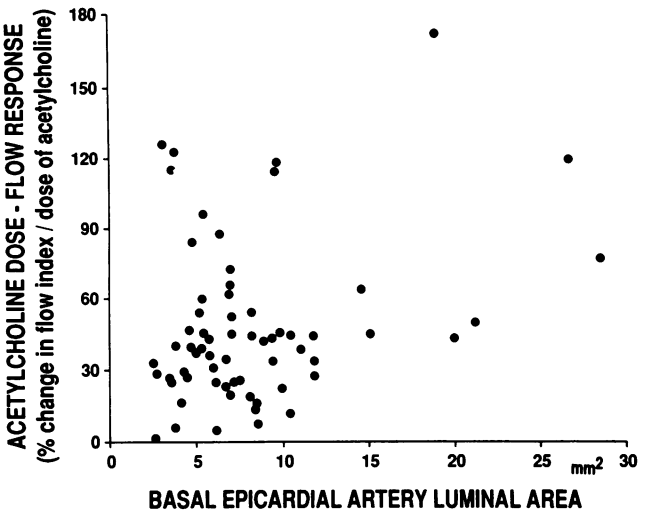

Figure 4. Relation between basal epicardial artery luminal area and acetylcholine-induced coronary blood flow changes.

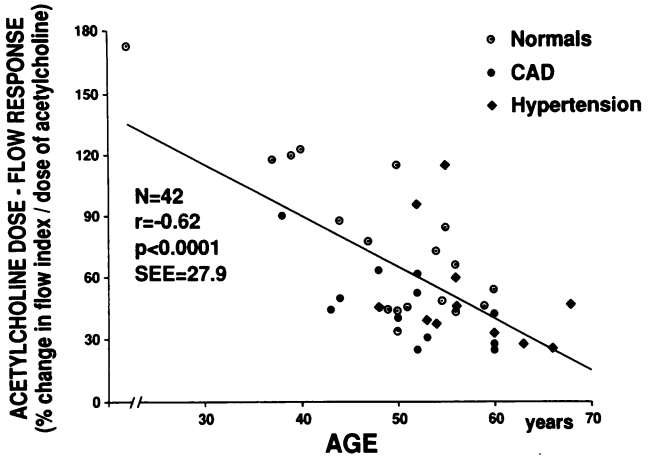

Figure 6. Correlation between age and acetylcholine dose–coronary blood flow response for all normocholesterolemic patients.

To assess the capacity of the coronary system to increase blood flow in response to acetylcholine, the acetylcholine dose–response relation was expressed as relative proportion of the maximally obtainable coronary blood flow response to papaverine. Fig. 5 illustrates that this proportion was significantly lower in patients with hypercholesterolemia compared with the three groups of patients with normal total serum cholesterol levels. When the group of patients with hypercholesterolemia was divided into those with and without an additional history of hypertension, the capacity to increase blood flow in response to acetylcholine was exactly comparable with 21.3±9.5% for the patients with hypercholesterolemia alone (n = 10) and 22.9±15.9% for the patients with both hypercholesterolemia and a history of hypertension (n = 10). Thus, the presence of a history of hypertension did not further impair acetylcholine-induced blood flow responses in patients with elevated serum cholesterol levels. Moreover, there was essentially no difference in the endothelium-dependent dilator capacity of the coronary microvasculature between the age-matched normal control group and the patients with atherosclerosis or hypertension, despite opposite vasomotor responses of the epicardial conductance vessels in these patients. These data indicate that endothelium-dependent vasodilation of the coronary microvasculature is selectively impaired in patients with hypercholesterolemia.

Relationship of acetylcholine-induced blood flow responses with age and serum cholesterol levels. Multivariate analysis using stepwise multiple-regression techniques revealed a significant negative correlation of acetylcholine-induced coronary blood flow responses with total serum cholesterol level ($P < 0.0001$) and with age ($P < 0.0001$), whereas gender, arterial blood pressure at the time of the study, a history of hypertension, and the presence or absence of angiographically visible luminal epicardial artery irregularities were not independently related to the acetylcholine-induced coronary blood flow response.

Fig. 6 illustrates the significant negative relationship between the acetylcholine dose–response relation and age of the individual patients with normal serum cholesterol levels. A similar relationship was found when the effects of acetylcholine were expressed as proportion of the papaverine effects ($r = -0.58$, $P < 0.001$). Both, the epicardial artery vasomotor

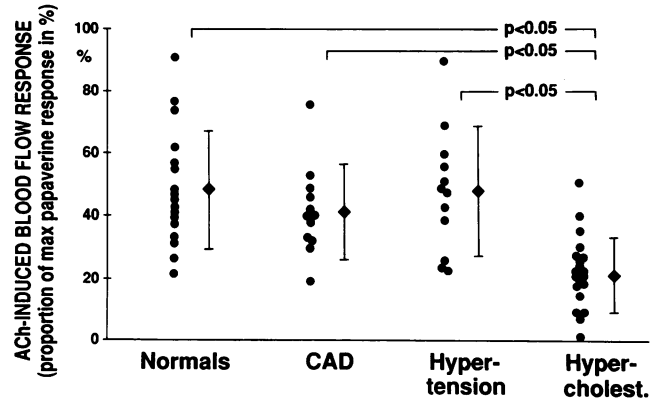

Figure 5. Capacity of the coronary system to increase blood flow in response to acetylcholine (expressed as percentage of the maximal coronary blood flow response to papaverine) of each individual patient in the four groups. Mean±SD.

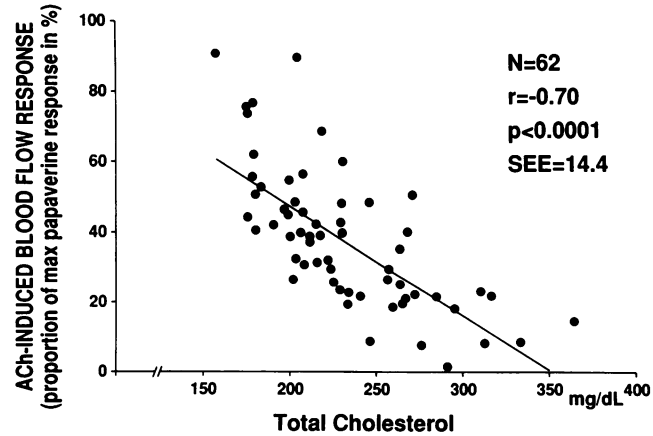

Figure 7. Correlation between total serum cholesterol level and acetylcholine-induced dilator capacity of the coronary system (expressed as percentage of the maximal papaverine response) for all patients.

response to acetylcholine as well as the papaverine-induced increases in blood flow were not significantly correlated with age ($r = 0.13$ and $r = 0.29$, $P > 0.05$, respectively). When the regression line correlating acetylcholine-induced blood flow changes and age was calculated only for the patients demonstrating a dilator response of their epicardial arteries (normal control group), the correlation coefficient improved to $r = -0.79$. Thus, the significant negative relationship between age and acetylcholine-induced blood flow responses is not solely explained by an epicardial artery vasocontrictor response in the older patients.

The strongest single predictor of a blunted coronary blood flow response to acetylcholine was the total serum cholesterol level. Moreover, Fig. 7 demonstrates the significant negative correlation between the capacity of the coronary system to increase blood flow in response to acetylcholine and the total serum cholesterol level at the time of the study.

Discussion

The present study is the first to assess coronary blood flow responses to the endothelium-dependent agonist acetylcholine in a large series of patients with coronary atherosclerosis and different risk factors implicated in the pathogenesis of CAD. There are three important new findings. First, despite similar vasoconstrictor effects upon epicardial conductance vessels, the acetylcholine-induced increase in coronary blood flow was markedly blunted in patients with hypercholesterolemia compared with patients with atherosclerosis but normal total serum cholesterol levels. Moreover, there was a close direct relationship between total serum cholesterol and the impairment in coronary blood flow responses to acetylcholine. Second, the acetylcholine-induced dilator capacity of the coronary microvasculature decreased with advanced age irrespective of the presence or absence of epicardial artery dysfunction. Third, arterial hypertension without established left ventricular hypertrophy had no apparent effect upon acetylcholine-induced increases in coronary blood flow in the intact human coronary circulation.

Effects of acetylcholine on the human coronary vasculature

Isolated normal epicardial human coronary arteries have been shown to relax in response to acetylcholine via an endothelium-dependent mechanism, and this muscarinic endothelium-mediated relaxation is impaired in atherosclerotic arteries, leaving the direct vasoconstrictor effects of acetylcholine on the muscarinic receptors of the vascular smooth muscle unopposed (12). Clinical studies have shown that intracoronary infusion of acetylcholine dilates normal epicardial arteries in patients without risk factors for CAD (14, 16, 24), but vasoconstricts epicardial vessels in patients with risk factors for coronary atherosclerosis irrespective of the presence or absence of angiographically detectable atherosclerotic lesions (16, 27–29). The vasoconstrictor response to acetylcholine in angiographically normal epicardial arteries of patients with risk factors for CAD has been interpreted to reflect early atherosclerosis at a stage not detectable by angiography or a disturbance of endothelial function that precedes the development of atherosclerosis (16, 28). However, there is still some debate whether acetylcholine-stimulated release of EDRF also mediates vasorelaxation of the coronary resistance vasculature. This controversy stems from experimental in vitro studies, which failed to demonstrate endothelium-dependent relaxations to acetylcholine in ring preparations of microvessels (30, 31). However, when the microvascular effects of acetylcholine were investigated in pressurized arteries exposed to flow, which much more closely resembles the in vivo situation, it could be demonstrated that acetylcholine produces vascular relaxation by the release of EDRF also in the resistance vasculature of all species examined this far (21, 22, 32–34). Importantly, when methylene blue, an inhibitor of soluble guanylate cyclase, is infused into the human coronary circulation in vivo, the dilator effects of acetylcholine upon coronary microvessels are reversed to constrictor effects with dramatical increases in coronary vascular resistance (35). These results do provide indirect evidence that acetylcholine, at least in part, mediates relaxation of the human coronary resistance vasculature by the release of EDRF. In addition, when acetylcholine was infused into the brachial artery of humans, previous α-adrenoceptor blockade, as well as the administration of acetylsalicylic acid did not alter the significant reduction in forearm vascular resistance, indicating that the vascular effects of acetylcholine in the human circulation are independent of prostaglandins and adrenergic neurotransmission (36–38). However, these studies do not rule out that the endothelium-dependent dilator effect of acetylcholine is in part mediated by the release of a hyperpolarizing factor (39) nor that the dilator effect is counteracted by the concomitant release of an endothelium-derived constricting factor (33, 40). In addition, experimental studies demonstrated that flow activates an endothelial calcium–activated potassium channel to release nitric oxide (41). Moreover, although aspirin or indomethacin do not inhibit the effects of flow-stimulated endothelial cells on vascular reactivity (42), flow-induced prostacyclin release has been previously demonstrated (43). Thus, acetylcholine-induced increases in blood flow might be modulated by flow-dependent mechanisms unrelated to its effect upon endothelial muscarinic receptor stimulation. In the present study, acetylcholine increased coronary arterial blood flow by ~ 250% in those patients, who demonstrated a dilator response of their epicardial arteries to acetylcholine. This endothelium-mediated increase in coronary blood flow corresponds to roughly one half of the maximally achievable increase in blood flow by the smooth muscle relaxant papaverine. Very similar results have been recently published by Treasure et al. (26) in a comparable group of patients using a similar methodology to assess vascular reactivity of the coronary circulation in humans. Thus, the stimulated release of EDRF activity by acetylcholine can substantially modify coronary vascular resistance in the intact human coronary circulation in vivo.

Effects of atherosclerosis, hypercholesterolemia, hypertension, and age on coronary vascular reactivity

Atherosclerosis. The demonstration of opposite effects of acetylcholine upon epicardial conductance and microvessels in patients with evidence of atherosclerosis in the present study confirms previous reports from our laboratory (16) as well as by others (35, 44). Compared with the normal control patients, the blunted increase in coronary blood flow in these patients, especially at the highest dose of acetylcholine, might be explained by the constriction of the epicardial arteries, leading to a reduced vascular conductivity and thereby limiting

increases in blood flow especially during high-flow states. However, in the same subjects, the response to the endothelium-independent smooth muscle relaxant papaverine was also significantly reduced compared with the normal control group. When the coronary blood flow effects of acetylcholine were expressed as a proportion of the maximum papaverine-inducible effects, there was essentially no difference between these two age-matched groups of patients. Thus, the blunted increase in coronary blood flow in patients with atherosclerosis implicates functional abnormalities intrinsic to the vascular smooth muscle rather than the consequence of an abnormal response of either large or small coronary vessels to acetylcholine.

Hypercholesterolemia. In contrast, in patients with hypercholesterolemia, the coronary blood flow response to acetylcholine was considerably impaired in comparison to the patients with normal cholesterol levels despite similar reductions in epicardial artery cross-sectional areas and similar papaverine-induced increases in coronary blood flow. Thus, the patients with hypercholesterolemia exhibited a selective impairment of their coronary microvasculature to relax in response to acetylcholine consistent with our previous observation (16). In contrast to hypercholesterolemic patients, impaired endothelial function appears to be restricted to epicardial arteries in age-matched patients with evidence of coronary atherosclerosis but normal cholesterol levels.

Importantly, the blunted blood flow response to acetylcholine was directly related to the total serum cholesterol level. The mechanisms responsible for the blunted acetylcholine-induced relaxation of human coronary microvessels in hypercholesterolemia remain to be determined. It has been debated whether hypercholesterolemia per se or the atherosclerotic process is responsible for abnormal endothelium-dependent responses observed in experimental animals made atherosclerotic by high-cholesterol diets (22, 45). Histological specimens examined by light microscopy did not reveal any appreciable structural alterations within the vessel wall of resistance vessels obtained from cholesterol-fed animals (20, 46), although electron microscopy demonstrated the presence of vacuoles likely representing lipid droplets within the endothelium (22). It is conceivable that the accumulation of lipids with associated oxidative processes may be in large part responsible for decreased production and/or increased intracellular destruction of EDRF. Interestingly, in this context, a very recent study indicated that oxidized LDL interferes with receptor-operated signal transduction mechanisms linked to the formation of EDRF, specifically with the receptor-mediated intracellular availability of L-arginine (47). Indeed, the supplementation of L-arginine either by intravenous infusion in vivo or by in vitro exposure normalizes endothelium-dependent responses in conduit and resistance vessels of hypercholesterolemic animals without affecting endothelium-independent vascular function but has no effects in normal animals (48, 49). We have previously demonstrated that very early in the process of atherosclerosis during hypercholesterolemia in humans, endothelium-dependent responses are impaired first by a depressed receptor-mediated initiation of the production and/or release of EDRF, whereas flow-dependent dilation, which is strictly endothelium-dependent but bypasses receptor-mediated mechanisms, is well preserved (16). More importantly, we have recently demonstrated that the administration of L-arginine normalizes coronary blood flow responses to acetylcholine in vivo in hypercholesterolemic humans but has no effect on acetylcholine-induced blood flow responses in patients with atherosclerosis and normal levels of cholesterol (50). Thus, an interference of lipoproteins with the receptor-mediated intracellular availability of L-arginine, the precursor of EDRF, might indeed play an important role for the blunted acetylcholine-induced increase in coronary blood flow observed in patients with elevated serum cholesterol levels.

Hypertension. A history of hypertension had no apparent effect upon coronary blood flow responses to acetylcholine in our patients, who had no evidence of left ventricular hypertrophy as well as similar endothelium-independent blood flow responses to papaverine as the patients with atherosclerosis and the patients with hypercholesterolemia. In addition, the combination of hypertension and hypercholesterolemia did not further impair acetylcholine-induced blood flow responses compared with hypercholesterolemia alone. These results suggest that as long as hypertension has not already induced left ventricular hypertrophy and thereby affected endothelium-independent coronary flow reserve to papaverine, endothelial dysfunction of the coronary circulation in hypertensive patients is confined to the large epicardial vessels, which are continuously exposed to high pulsatile pressure and shear stress (51, 52). These findings contrast with the results of studies in the human forearm circulation, where arterial hypertension has been shown to be associated with a blunted blood flow response to acetylcholine despite normal responses to nitroprusside (36, 37). These discrepancies might be simply related to the different vascular beds. Whereas the epicardial arteries, such as the large arteries of the cerebral and limb circulation, are known targets of hypertension (53), hypertensive vascular disease rarely develops in the large vessels of the human forearm circulation.

Aging. Autopsy studies have shown that epicardial coronary atherosclerosis begins with fatty streaks in childhood and progresses with increasing age (54). In addition, the proximal segments of the left anterior descending artery have been shown to be particularly vulnerable to fatty streaks, as well as to more advanced lesions (55). Consequently, the loss of acetylcholine-induced epicardial artery dilation and its reversal to a vasoconstrictor response with increasing age observed in patients with angiographically normal appearing coronary arteries have been attributed to the presence of early atherosclerotic lesions in the older patients rather than to age per se (28, 29). In contrast, in the present study, the strongest relation between age and acetylcholine-mediated dilator capacity of the coronary microvasculature was observed in those patients who exhibited a dilator response of their epicardial conductance vessels to acetylcholine.

Since acetylcholine is invariably a constrictor of atherosclerotic large-size arteries (12) and atherosclerosis occurs more frequently in the proximal than in the distal segments of coronary arteries (55), and does not develop at all in the coronary resistance vasculature, a dilator response of epicardial conductance vessels excludes the presence of atherosclerotic lesions at least to the extent of impairing acetylcholine-mediated endothelial function. Thus, the reduction in the acetylcholine-induced dilator capacity of the coronary microvasculature with increasing age cannot solely be explained by the presence of atherosclerotic lesions in the older individuals of these patient groups. These results strongly suggest that aging per se contrib-

utes to the impaired acetylcholine-induced dilator capacity of the human coronary microvasculature.

The mechanisms responsible for the age-associated reduction in the ability of the coronary microvasculature to dilate in response to the pharmacological stimulation with acetylcholine remain to be determined. Experimental studies demonstrating thinning and loss of endothelial cells in aged animals (56) suggested an impaired production of EDRF (57). In addition, advanced glycosylation end products, which accumulate in the vascular subendothelium in aging (58), have been experimentally shown to quench nitric oxide and mediate defective endothelium-dependent vasodilation (59). Other possible mechanisms include a decline in endothelial muscarinic receptor density, the release of hyperpolarizing or constricting factors, or an increased sensitivity of vascular smooth muscle to the constrictor effect of acetylcholine with advancing age.

Limitations of this study

The methodology used to assess coronary vascular resistance provides no direct data to identify the specific site of the impaired dilator response of the coronary microvessels in hypercholesterolemia or advanced age. Although flow-limiting vasoconstriction of epicardial conductance vessels was excluded, the precise site of coronary vascular abnormalities downstream from the epicardial vessels cannot be assessed in the intact human coronary circulation. This might be important in light of recent experimental studies demonstrating that the control of coronary vascular resistance is extremely complex and different size classes of coronary microvessels exhibit heterogeneous responses to vasoactive stimuli (60).

It has been repeatedly demonstrated that in humans, the most important vasodilator action of acetylcholine is mediated through the release of EDRF (35–38, 61). Moreover, the demonstration that the supplementation of L-arginine normalizes the blunted coronary blood flow responses to acetylcholine in hypercholesterolemic subjects (50) does provide very strong support for the existence of an acetylcholine-stimulated L-arginine/nitric oxide pathway as an important mediator of acetylcholine-induced vasodilation in the intact human coronary circulation. However, we cannot exclude the possibility that the concomitant release of a hyperpolarizing factor (39) or even a constricting factor (33, 40) or functional alterations in the flow-induced activation of endothelial potassium channels (41) might modify the response to acetylcholine during aging or hypercholesterolemia. Finally, studies in the intact human coronary circulation do not allow us to differentiate whether the impaired acetylcholine-mediated vascular relaxation is due to an abnormal production or destruction of EDRF or to abnormalities of endothelial cell membrane receptor–second messenger interactions. Although papaverine is a potent smooth muscle relaxant to assess maximal vasodilator capacity of the coronary circulation, it does not act via the same mechanism as nitrovasodilators, including EDRF. However, nitroglycerine has minimal effects on small coronary resistance vessels (62) and nitroprusside in intracoronary dosages necessary to maximize coronary blood flow in humans profoundly affects systemic hemodynamics, thereby preventing interpretation of its effects on coronary vascular resistance. Thus, we cannot exclude that aging and hypercholesterolemia might reduce the effects of acetylcholine by inactivating guanylate cyclase of vascular smooth muscle. Nevertheless, even if vascular smooth muscle guanylate cyclase is altered in hypercholesterolemia and by aging, the net effect of EDRF activity released from the endothelium upon stimulation would be a diminished relaxation of vascular smooth muscle. Thus, our conclusion of an impaired acetylcholine-induced vasodilator capacity of the coronary microvasculature with increasing age and in hypercholesterolemia with all its implications would still be valid.

Clinical significance

We have recently demonstrated that patients with endothelial dysfunction of the coronary microvasculature exhibit an impaired coronary blood flow regulation during sympathetic stimulation associated with increased myocardial work (63). Thus, the blunted endothelium-mediated vasodilator capacity of the coronary microvasculature in patients with hypercholesterolemia and advanced age might contribute to the pathogenesis of myocardial ischemia. In addition, the functional integrity of the endothelium plays a pivotal role in guarding against the initiation of vascular events that may lead to the development of vasospasm and thrombosis (1). We have recently shown that intracoronary platelet aggregation causes profound constriction of atherosclerotic epicardial arteries in humans in vivo (64). Two recent studies (65, 66) demonstrated that the intracoronary infusion of serotonin induces a dilator response in normal coronary vessels but causes profound vasoconstriction of both epicardial conductance and coronary resistance vessels in patients with arteriosclerosis, thereby suggesting an important protective effect of the endothelium. Moreover, even short-term, diet-induced hypercholesterolemia considerably increases infarct size as well as ischemia-reperfusion injury in experimental animals (21, 67, 68). In light of recent experimental evidence that the endothelium of the coronary microcirculation is extraordinarily sensitive to ischemia, responding with loss of natural anticoagulant and vasorelaxing properties (69), it is very intriguing to hypothesize that the blunted endothelium-mediated dilator capacity of the coronary microvasculature may play an important pathophysiological role for the aggravated sequelae of ischemic events in elderly and hypercholesterolemic patients with ischemic heart disease.

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