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Acetylcholine-Induced Response of Coronary Resistance Arterioles in Cholesterol-Fed Rabbits

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ABSTRACT—The extent to which reported abnormal responses of the human coronary circulation to acetylcholine in patients with hypercholesterolemia reflect endothelial injury is not clear. We used an in vitro rabbit model to determine whether these reactions involve endothelial or vascular smooth muscle dysfunction. Coronary resistance arterioles were isolated from hearts of rabbits fed 1% cholesterol to induce hypercholesterolemia or a control diet for 4 weeks. Arterioles were double cannulated with glass micropipettes and pressurized in a no-flow state. Acetylcholine contracted the arterioles in a concentration-dependent manner whether or not the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine was added. In control but not hypercholesterolemic preparations, contraction was significantly greater when endothelium was removed. In the hypercholesterolemic group, contraction significantly exceeded that in controls. In control but not high-cholesterol preparations, substance P dilated vessels with intact endothelium in a concentration-dependent manner. Addition of N^G-monomethyl-L-arginine inhibited this response. With or without endothelium, norepinephrine contracted arterioles to a greater extent in the hypercholesterolemic group than in controls. We concluded that severe hypercholesterolemia decreased endothelially dependent factors by injuring endothelium and independently increased contractility of vascular smooth muscle.

Keywords: Acetylcholine, Coronary resistance arteriole, Hypercholesterolemia, Vascular smooth muscle, Endothelium

Hypercholesterolemia is an important risk factor for ischemic heart disease (1–3). In patients with hypercholesterolemia, the vascular endothelium is damaged and platelet activity is increased, making thrombosis more likely (4). The coronary arteries of hypercholesterolemic patients often contract in response to acetylcholine, which frequently is interpreted as a sign of endothelial injury (5–7). Like acetylcholine, substance P induces endothelially dependent release of nitric oxide (EDNO), but substance P also dilates coronary arteries that have been contracted by acetylcholine (8). This evidence indicates that contraction of vessels induced by acetylcholine in hypercholesterolemia cannot be assumed to simply reflect endothelial injury. Since acetylcholine receptors are present on both endothelial and vascular smooth muscle cells, imbalances at either or both receptor locations may exaggerate the reactivity of vascular smooth muscle in response to acetylcholine. Even though many reports have examined the effect of hypercholesterolemia on vascular reactivity, most studies have involved the aorta or carotid arteries (9, 10). Furthermore, many studies on coronary arteries have used large, superficial conduit arteries. Very few in vitro studies have examined the effect of hypercholesterolemia on the coronary resistance vessels that essentially determine coronary blood flow (11).

In the present study, we sought to address the questions of whether hypercholesterolemia changes the reactivity of coronary resistance arteries to acetylcholine and whether such charges primarily involve endothelium or vascular smooth muscle. In vitro experiments were chosen to eliminate effects of neuroendocrine factors and shear stress and to limit the study focus to coronary resistance vessels.

MATERIALS AND METHODS

Animals

Male New Zealand white rabbits were divided into two groups: control rabbits fed a regular diet for 4 weeks (n=12; average body weight, 2.1±0.6 kg) and rabbits fed

a 1% cholesterol diet for 4 weeks (n=12; average body weight, 2.2±0.4 kg). Rabbits were housed and cared for at Hyogo College of Medicine according to the institutional rules regulating animal experiments.

Arteriolar preparation

After rabbits had been sufficiently anesthetized using 0.1 mg/g of pentobarbital sodium, tracheostomy was performed to permit use of a mechanical ventilator (Harvard Instruments, Buckinghamshire, UK). After opening the chest and confirming a continuing heartbeat, the heart was removed and immediately placed in dissection solution consisting of 145 mM sodium chloride, 4.7 mM potassium chloride, 2.0 mM calcium chloride, 1.2 mM magnesium sulfate, 5.0 mM glucose, 2.0 mM pyruvic acid, 2.0 mM MOPS (3-[*N*-morphorino] propanesulfonic acid), 0.02 mM EDTA, 1.2 mM dibasic sodium phosphate (pH 7.4) and 1% w/v bovine serum albumin. The left ventricular muscle was excised and promptly fixed on a silicone pad in a chamber containing dissection solution at 4℃. Next, coronary resistance vessels in the left ventricular muscle were separated from the surrounding connective tissue under a microscope using ultrafine scissors and forceps.

Study protocol

Coronary resistance vessels were transferred to a tissue bath filled with Krebs solution (118.5 mM sodium chloride, 4.7 mM potassium chloride, 2.55 mM calcium chloride, 1.19 mM magnesium sulfate. $7H_2O$, 11.6 mM glucose, 1.19 M dibasic sodium phosphate (pH 7.4), and 19.9 mM sodium bicarbonate). One end of the isolated resistance vessel was connected via a micropipette to a chamber in which pressures could be adjusted. To eliminate the mechanical effect of fluid flow through the vessel, its other end was connected to a micropipette with a closed tip and ligated using 11-0 nylon. Additionally, in all experiments, 0.6 M propranolol was added to the Krebs solution. The tissue bath was installed upon the stage of an inverted microscope (Diaphot-TMD; Nikon, Tokyo). The internal pressure of the coronary resistance vessels was maintained at 40 mmHg, representing their physiologic internal pressure according to previous reports (11). In half of each experimental group, after connection of the micropipette to one end of the vessel prior to ligation, the endothelium was stripped by the passage of air bubbles according to procedures described in a previous report (12). The internal diameter of the resistance vessels was measured from images captured by a CCD camera designed for microscopy (Hamamatsu Photonics, Hamamatsu) at a magnification of ×800. The Argus 10 Image Processor (Hamamatsu Photonics) was used. The tissue bath was perfused with Krebs solution at a rate of 4 ml/min and was surrounded by warm water from a tank kept at a constant-temperature to maintain the solution at 37℃. Moreover, tissue bath conditions were regulated using oxygen and nitrogen to maintain pH, PaO_2, and $PaCO_2$ in the Krebs solution at 7.40, 98 to 100 mmHg, and 35 to 40 mmHg, respectively.

As a first observation, the intraluminal diameter (C1) and wall thickness (WT) of the vessels were measured at room temperature. Since morphologic differences or irregularities in vessel diameter (D) could influence the study results, relative wall thickness (D/WT) was calculated. After confirming that the temperature of the Krebs solution in the tissue bath remained at 37℃ and that coronary resistance vessels were able to contract in response to stimuli, we measured their intraluminal diameter during maximal contraction induced by 30 μM norepinephrine and maximal dilation induced by 30 μM nitroprusside. Next, after confirming intrinsic tone, the intraluminal diameter of the vessels was measured at baseline pressure (control diameter, C2). The internal pressure was increased above the baseline pressure by 15 mmHg and then by 30 mmHg to confirm the myogenic response. After obtaining data for the first concentration-response curve, a 60-min washout period was allowed. We plotted the concentration-response curve using cumulative additions of each drug to the bath (duration of exposure to each concentration, 15 min). Vessels without endothelium also were tested after confirming reduction of their dilation response to substance P in a preparation from control-diet rabbits.

Vessels and lipid analysis

Basic vascular properties and lipid content were compared between preparations from control and hypercholesterolemic rabbits.

Protocol 1. Endothelially dependent reactions in hypercholesterolemia: Although acetylcholine is an endothelially-dependent vasodilator, previous in vitro studies have found that acetylcholine contract the coronary arteries (13). Substance P, another endothelially dependent vasodilator, has a mechanism of action which differs from that of acetylcholine (14). Using preparations from normocholesterolemic and hypercholesterolemic animals, concentration-response curves for acetylcholine were plotted to investigate the effects of elevated cholesterol and determine which of these depended upon the presence of endothelium. In controls, concentration-response curves for acetylcholine were compared with and without N^G-monomethyl-L-arginine (10^{-6} M, L-NMMA), an inhibitor of nitric oxide synthesis. We also recorded concentration-response curves for substance P in vessels with intact endothelium with and without addition of L-NMMA.

Protocol 2. Endothelially independent reactions in hypercholesterolemia: Since norepinephrine contracts vascular smooth muscle by a mechanism unrelated to that of acetylcholine (15, 16), concentration-response curves for norepinephrine were recorded to investigate the effects of hypercholesterolemia and presence of endothelium.

Drugs

Acetylcholine hydrochloride, propranolol, L-NMMA and substance P were purchased from Sigma (St. Louis, MO, USA). Norepinephrine, sodium nitroprusside and pentobarbital sodium were obtained from Wako Pure Chemical Industries (Osaka). Acetylcholine hydrochloride, norepinephrine, sodium nitroprusside and substance P were dissolved in double-distilled H_2O (each at a concentration of 10^{-2} M).

Statistical analyses

Lipid content, control diameter and % of maximum response are expressed as the mean±S.D. An unpaired Student's *t*-test was used for analysis. Intrinsic tone was expressed as $[(C1 - C2) / C2] \times 100$. Bonferroni procedures were used to make multiple comparisons between concentration-response data. A level of $P<0.05$ was accepted as statistically significant.

RESULTS

Serum lipid concentrations and vessel reactivity

While serum concentrations of total cholesterol, triglyceride and low-density lipoprotein (LDL) cholesterol were significantly higher in the cholesterol-fed group, no significant difference was seen between groups in concentrations of high-density lipoprotein (HDL) cholesterol (Table 1).

The mean diameter of excised vessels was 93±8 μm, with no significant difference between high-cholesterol and control groups. No significant differences in maximum contraction induced by norepinephrine or maximum dilation induced by nitroprusside were seen between the excised vessels (Table 2).

Protocol 1

As reported in previous studies (13), acetylcholine caused coronary resistance vessels with intact endothelium to contract in a concentration-dependent manner. The degree of contraction induced was significantly greater in preparations from rabbits fed the high-cholesterol diet than in controls. Even without endothelium, the degree of contraction was significantly greater in the cholesterol-fed group. The degree of contraction and percentage of maximum constriction induced by acetylcholine were significantly greater in preparations from control

Table 1. Serum lipids in rabbits

Group		n	Body weight (kg)	T-cho (mg/dl)	TG (mg/dl)	LDL-cho (mg/dl)	HDL-cho (mg/dl)
Controls	EC(+)	6	2.1±0.6	98.1±22.3	24.7±10.5	80.6±5.5	12.2±3.1
	EC(−)	6	2.1±0.9	97.3±30.1	26.0±11.7	78.4±7.4	9.9±8.6
Cholesterol-fed	EC(+)	6	2.2±0.5	788.2±46.5*	71.8±39.9*	752.4±50.1*	21.6±3.2
	EC(−)	6	2.2±0.7	741.7±30.3*	75.4±50.6*	733.7±41.6*	22.5±4.0

n, Number of animals in each group; EC(+)/EC(−), with/without endothelial cells; T-cho, total cholesterol; TG, triglyceride; LDL-cho, low-density lipoproteine cholesterol; HDL-cho, high-density lipoproteine cholesterol; *$P<0.05$ vs the respective control.

Table 2. Baseline control arteriolar diameters

Group		n	Control diameter		Intrinsic (%)	NP (μm)	NE (μm)
			Cl (μm)	C2 (μm)			
Controls	EC(+)	6	124±4	92±9	26.1±3.1	125±6	60±5
	EC(−)	6	124±5	94±4	24.2±1.9	126±5	63±7
Cholesterol-fed	EC(+)	6	135±7	96±8	26.7±2.2	138±4	56±6
	EC(−)	6	126±5	93±6	25.0±2.0	129±5	57±5

n, Number of vessels in each group; EC(+)/(−), with/without endothelial cells; Cl, control diameter before experiment; C2, control diameter after warm in bath; Intrinsic, the tone after warm in bath; NP, nitroprusside (30 μM); NE, norepinephrine (30 μM).

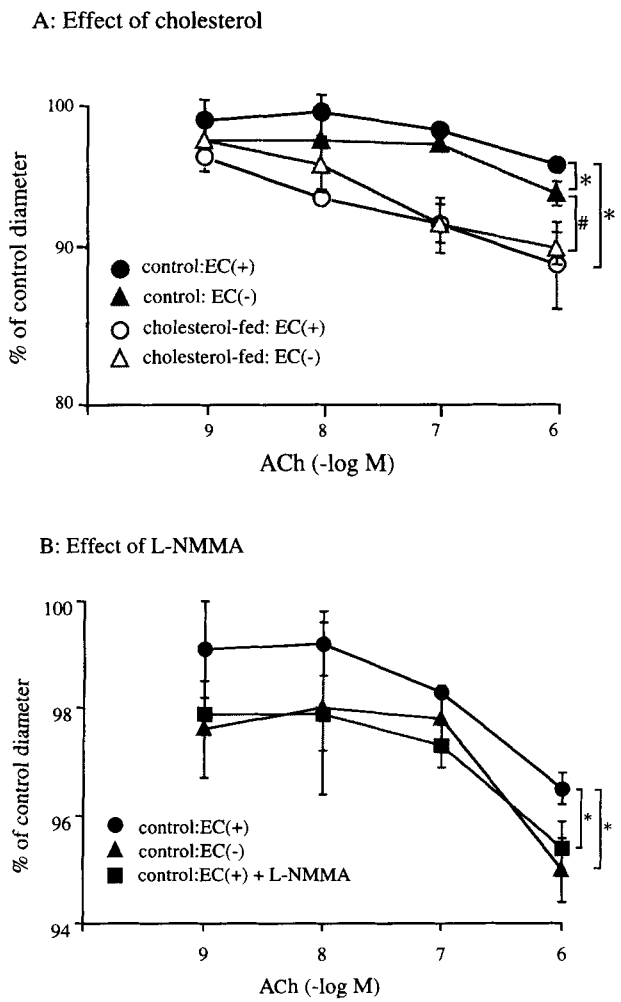

Fig. 1. Responses to acetylcholine (ACh). Panel A shows concentration-response curves for acetylcholine with a significant difference between controls (n=6, both with and without endothelial cells) and the cholesterol-fed group (n=6, both with and without endothelium). The degree of contraction was significantly greater without endothelium in controls. However, no difference related to the presence of endothelium was seen in the cholesterol-fed group. N^G-Monomethyl-L-arginine (L-NMMA) enhanced vasoconstriction in controls (panel B). Bars represent S.D. *P<0.05 vs control EC(+); #P<0.05 vs control EC(−), at each ACh concentration tested.

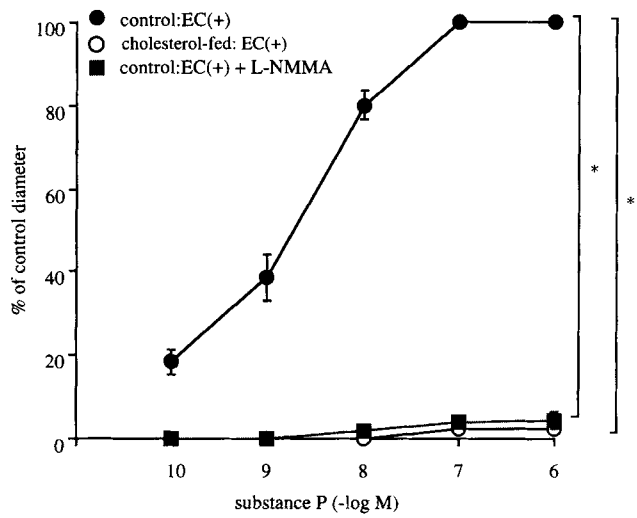

Fig. 2. Responses to substance P. Concentration-response curves for substance P showed a dose-response relationship in controls (n=6), but not in the cholesterol-fed group (n=6) or with N^G-monomethyl-L-arginine (L-NMMA). In both groups, vessels had intact endothelium. Bars represent S.D. *P<0.05 vs control EC(+) at each substance P concentration tested.

animals that lacked endothelium than in those with intact endothelium. In contrast, no significant differences were attributable to the presence of endothelium in preparations from cholesterol-fed animals. In the presence of L-NMMA, the degree of contraction in controls did not differ significantly between preparations with and without endothelium (Fig. 1, Table 3). In the control-diet group, substance P dilated vessels with intact endothelium in a concentration-dependent manner, but no such effect occurred in the high-cholesterol group, even with the endothelium in place (Fig. 2, Table 3). L-NMMA inhibited the vascular response obtained when the endothelium was intact (Fig. 2).

Protocol 2

Like acetylcholine, norepinephrine contracted coronary resistance vessels in a concentration-dependent

Table 3. % of maximum response

Group		n	Acetylcholine (%)	Substance P (%)	Norepinephrine (%)
Controls	EC(+)	6	97.1±0.6	100	82.3±2.1
	EC(−)	6	94.4±1.2*	3.8±1.4*	84.6±1.8
Cholesterol-fed	EC(+)	6	88.6±1.6*		77.6±3.1*
	EC(−)	6	90.3±2.3#		77.3±1.9#

n, Number of vessels in each group; EC(+)/(−), with/without endothelial cells; *P<0.05 vs control EC(+); #P<0.05 vs control EC(−).

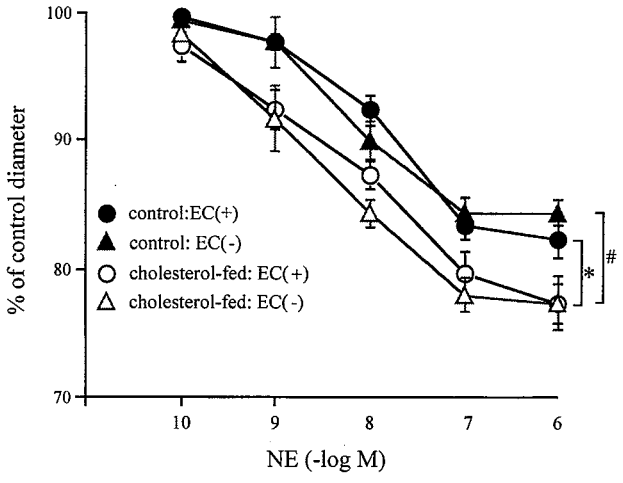

Fig. 3. Responses to norepinephrine (NE). Concentration-response curves for norepinephrine showed significant differences between controls and the cholesterol-fed group, both with and without intact endothelium. Bars represent S.D. $^{*}P<0.05$ vs control EC(+); $^{\#}P<0.05$ vs control EC(−), at each NE concentration tested.

manner. The degree of contraction and percentage of maximum contraction resulting from norepinephrine were significantly greater for the cholesterol-fed group than for the control group whether or not endothelium was present (Fig. 3, Table 3).

DISCUSSION

In the present study, preparations from rabbits with experimentally induced hypercholesterolemia were studied to determine the effect of hypercholesterolemia on the reactivity of the resistance vessels that regulate coronary blood flow. Coronary resistance vessels from hypercholesterolemic animals contracted in response to either acetylcholine or norepinephrine. With acetylcholine, the degree of contraction seen in control preparations was significantly greater when endothelium was absent. The presence or absence of endothelium did not affect contraction in cholesterol-fed groups.

Previous reports have demonstrated abnormalities of nitric oxide synthesis in the endothelium of coronary resistance vessels of rabbits with experimentally induced hypercholesterolemia (17, 18). This synthetic abnormality is believed to result in reduced secretion of EDNO, resulting in accelerated contraction of coronary resistance vessels in response to acetylcholine (19, 20). In the present study, dilation of vessels in response to substance P almost disappeared in preparations affected by hypercholesterelemia. Notably, however, hypercholesterolemia enhanced contraction of vessels in response to acetylcholine even when the endothelium was removed. Results were the same with norepinephrine, which contracts vascular smooth muscle by a different mechanism than acetylcholine.

Muscarinic receptors mediating responses to acetylcholine are distributed in both endothelium and vascular smooth muscle (13). Acetylcholine produced dose-dependent constriction in vessels from preparations with and without endothelium in our study. These results are in agreement with findings in previous reports (13, 21) that coronary arteriolar regulatory functions are mediated by alterations in endothelial cells, vascular smooth muscle cells, or both, and coronary arteriolar reactions are likely to reflect coronary smooth muscle cell sensitivity to muscarinic stimulation. Although acetylcholine can produce vasoconstriction on coronary arteries on the no-flow state in vitro (13), this neurotransmitter can produce vasodilation in coronary arteries when flow is present (14, 22). In the present study, with nitric oxide synthase inhibitor also added, acetylcholine produced vasoconstriction (Fig. 1). This finding suggests that the overall reaction of coronary resistance arteries in vitro to acetylcholine predominantly involved vascular smooth muscle cells, not endothelial cells. A study of responses of mesenteric resistance vessels to acetylcholine in hypertensive rats suggested that endothelially dependent hyperporlizing factor (EDHF) rather than NO has a central role (23). Dual involvement of NO and EDHF in tonus regulation has been reported in small coronary arteries (24). In contrast, our results showed a constrictive response to acetylcholine, and EDHF was not implicated in this study. On the other hand, substance P is a nonmuscarinic, endothelially dependent vasodilator that acts at tachykinin receptors on the endothelial cell surface (14). Substance P has been reported to release EDNO without exerting a direct effect on smooth muscle cells, either constriction or dilation (25, 26). Substance P produced vasodilation in our controls but had little effect in the high-cholesterol group (Fig. 2). This finding suggests that hypercholesterolemia injures the endothelium and vascular smooth muscle.

Responses to norepinephrine in the presence of propranolol reflect α-adrenoceptor sensitivity. Different α-adrenoceptor subtypes mediate constriction of different vessel types (27): while the α_2-adrenoceptor is distributed in the endothelium and mediates release of EDNO, this is not true for the α_1-adrenoceptor (28). Sensitivity of α_1-adrenoceptors may have been enhanced by hypercholesterolemia in the present study. Vascular injury can induce extensive proliferation of smooth muscle cells (29), and susceptibility to oxidative stress may be increased in atherosclerotic arteries in cholesterol-fed rabbits (30). Taken together, these findings suggest that hypercholesterolemia increases contractility of vascular smooth muscle in rabbit coronary resistance vessels and that this

might be the mechanism of increased coronary resistance vessel contraction in response to acetylcholine.

Investigating blood flow in the forearm in patients with hypercholesterolemia, Gilligan and colleagues have found that reactions to bradykinin and acetylcholine differed even though both agents are endothelially dependent. They concluded that hypercholesterolemia injures acetylcholine receptors or a related signal transduction pathway (31). The difference reported by Gilligan may reflect differing receptor locations: bradykinin receptors are found only on endothelial cells, while acetylcholine receptors are found on both endothelial and vascular smooth muscle cells (11). Therefore, as suggested by the results of the present study, hypercholesterolemia appears to exert effects at acetylcholine receptors and at α-adrenoceptors on vascular smooth muscle cells.

Many reports have described differences in drug responses attributable to differences in the diameters of coronary arteries (32, 33). The degree of vascular injury caused by cholesterol also has been reported to differ according to vessel diameter (34). When Cappelli-Bigazzi and colleagues fed rats genetically predisposed to hypercholesterolemia a high-cholesterol diet, both endothelium and vascular smooth muscle were damaged, while in similar rats fed a low-cholesterol diet, only the endothelium was damaged (35). Since our rabbits with experimentally induced hypercholesterolemia had markedly elevated serum cholesterol concentrations (mean, 760 mg/dl), the reactivity of vascular smooth muscle is likely to have been changed. Furthermore, since the degree of vascular injury may differ according to serum concentration of cholesterol, vascular reactions also may differ according to cholesterol concentration (36). Damage caused by cholesterol may be clarified by further investigation of the relationship between degree of damage and concentration of cholesterol.

In the present study, we induced hypercholesterolemia in rabbits to investigate its effect on the response of coronary resistance vessels to acetylcholine. The vascular endothelial injuries induced by hypercholesterolemia have received a great deal of attention, but the present study shows that severe hypercholesterolemia not only affects endothelially dependent vasoactive factors, but independently enhances the contractility of vascular smooth muscle. Details of the mechanisms of this effect still need to be clarified, but changes in receptors or intracellular information transfer systems may be involved.

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