Hypercholesterolemia Impairs Transduction of Vasodilator Signals Derived From Ischemic Myocardium
Myocardium–Microvessel Cross-Talk
Kouichi Sato, Tatsuya Komaru, Hiroki Shioiri, Satoru Takeda, Katsuaki Takahashi, Hiroshi Kanatsuka, Masaharu Nakayama, Kunio Shirato

Objective—Coronary microvessels are functionally coupled to the myocardial metabolic state. In hypercholesterolemia, the coronary vascular dysfunction extends to microvascular levels. We hypothesized that the vasodilator signal transduction from ischemic heart is impaired in the coronary microvascular wall of hypercholesterolemia.

Methods and Results—Rabbits were fed with normal chow (control group) or 2% high-cholesterol diet (hypercholesterolemia group) for 8 weeks. Coronary microvessels isolated from rabbit hearts were pressurized and gently placed on a beating canine heart. Myocardial ischemia was produced in the beating heart and the diameter of the isolated microvessel was observed using an intravital microscope with a floating objective. In control group, the isolated microvessels significantly dilated 2 minutes after the onset of ischemia, and a plateau was observed at 10 minutes. In contrast, the microvessels from hypercholesterolemia group did not dilate during ischemia. Dihydroethidium fluorescence microscopy revealed an elevated superoxide level in the microvessels of hypercholesterolemia group. The application of tiron (free radical scavenger) significantly dilated the isolated microvessels only from hypercholesterolemic animals.

Conclusions—We conclude that the transduction of vasodilator signals derived from ischemic myocardium is impaired in the coronary microvascular wall of hypercholesterolemia. Enhanced oxidative stress in hypercholesterolemia may alter the microvascular function. (Arterioscler Thromb Vasc Biol. 2004;24:2034-2039.)

Key Words: coronary circulation  
hyperlipoproteinemia  
ischemia  
reactive oxygen species  
vasodilation

The coronary microvascular network is closely linked to the myocardium functionally and anatomically.1 A tight correlation between coronary flow and the metabolic state of the heart has long been recognized.1,2 However, it has not been well-clarified how vasomotor signals from cardiac muscle cells modulate the tone of coronary microvessels because of the lack of adequate methods for evaluating the cross-talk between cardiac muscle and the coronary vascular bed. We have recently developed a new experimental method to analyze the cross-talk by combining an isolated microvessel with a beating heart preparation.3 With this method, we showed that pertussis toxin-sensitive G proteins (GPTX proteins) in the microvascular wall play a pivotal role in transmitting vasodilator signals from the ischemic heart to coronary microvessels.3 Previous in vivo studies have shown that GPTX proteins are critical in the microvascular control during autoregulation and metabolic stimulation.4,5

Hypercholesterolemia is one of the most important coronary risk factors. Although morphological changes like plaque formation take place only in conduit vessels, various functional changes are known to extend to the coronary microvessels.6,7 Early studies have shown that hypercholesterolemia impairs GPTX-mediated vascular responses such as serotonin-induced8,9 and acidosis-induced vasodilation.10 Accordingly, in the present study we tested the hypothesis that the transduction of vasodilator signals derived from ischemic myocardium is impaired in coronary microvessels of hypercholesterolemia by analyzing the cross-talk between ischemic myocardium and coronary microvessels.

Increasing evidence has shown that oxidative stress is an important deteriorating factor that produces structural and functional abnormalities in the blood vessels in atherosclerosis.11,12 Thus, we also investigated the involvement of reactive oxygen species in the impaired microvascular function.

Methods

The present studies conform with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (National Institutes of Health Publication 85-23, revised 1996), and the experimental protocols were approved by the institutional committee for animal experiments.
To clarify whether the coronary microvessels of hypercholesterolemia dilate in response to signals released from ischemic myocardium, we used a new bioassay method combining an isolated microvessel preparation with a beating-heart preparation.3

**Detector Microvessels**

Male Japanese white rabbits (5 weeks old) were fed with normal diet (control group, n=16) or 2% high-cholesterol diet (hypercholesterolemia group, n=12) for 8 weeks. Rabbits were prepared as described in our previous studies.3 Briefly, a coronary microvessel perfusing the left ventricle was dissected. One end of the microvessel was cannulated to a polyethylene micropipette filled with filtered physiological salt solution (PSS) (in mmol/L: NaCl 120.0, KCl 4.7, NaHCO3 25.0, CaCl2 2.0, MgSO4 1.17, Na2HPO4 1.2, calcium disodium EDTA 0.02, glucose 5.0, bubbled with 5% CO2, 95% O2) and the other end was ligated. Intraluminal pressure was monitored and microvessels with any leakage were discarded. The microvessel was incubated in oxygenized warm PSS (38°C) bath until use.

**Beating-Heart Preparation**

Mongrel dogs of either sex (n=28; body weight, 2.5 to 12.3 kg; mean, 6.5 ± 0.4 kg) were used and prepared as described in our previous in vivo studies.4,5,13,14 Briefly, the dogs were anesthetized and ventilated. A thoracotomy was performed, and the pericardium and ventricles were cut to expose the heart surface. The heart was paced at 100 to 110 bpm by left atrial pacing after the suppression of sinoatrial node activity by injecting formaldehyde (10%, 0.5 mL) to the myocardium of the sinoatrial nodal area. The main trunk of the left anterior descending coronary artery (LAD) was dissected, and a snare was placed around it for coronary occlusion. In several experiments, a small branch of the LAD distal to the occlusion point was cannulated with a small catheter (outer diameter 0.67 mm) to measure the distal coronary pressure.

**Bioassay Method**

The incubated detector microvessel was gently placed on the LAD perfusion area of the beating left ventricle of the dog and pressurized to 60 cm H2O to produce intrinsic tone. Only one detector microvessel was used for each experiment. The detector microvessel and the heart surface were kept moist throughout the experiment by continuous superfusion of PSS at a rate of 60 mL/h. The entire superfusion line was continuously warmed by a warm circuit using a thermostat to keep the superfusate temperature at 37°C on the heart surface. For visualization of the detector microvessels, placed on the beating left ventricles, an intravital microscope system equipped with a floating objective was used as described in our previous study.3,13 With this system, the surface of the beating heart can be continuously observed throughout the cardiac cycle. An objective lifter was used to carefully adjust the distance between the objective and microvessels, so as not to compress the detector microvessel. Epi-illumination with a stroboscopic light source was applied to obtain the images of the detector microvessel. Obtained images were monitored and recorded using a highly sensitive TV camera (model C 1000–12; Hamamatsu Photonics) or charge-coupled device camera (KV–26B, Hitachi). The spatial resolution of this system was 2 μm.

Vascular diameters were measured at least 3 times during the end-diastolic phase. To evaluate the diameter changes for each intervention, the diameters were measured at the same point using the polyethylene micropipette or the tying thread as a reference point.

**Experimental Protocols**

Coronary vascular responses caused by signals released from the ischemic myocardium were compared between control and hypercholesterolemia. After detector microvessels of the control group (n=11) or hypercholesterolemia group (n=6) were placed on the beating hearts, ~1 hour was allowed for all hemodynamic variables to stabilize and for the intrinsic tone of the detector microvessels to develop. When the intrinsic tone of the detector microvessel was not observed (baseline diameter ≥90% of the maximal diameter produced by nitroprusside), the vessel was discarded.

After the measurement of the monitored baseline variables, the LAD of the beating heart was completely occluded to produce myocardial ischemia. Images of the detector microvessel were collected at 2, 3, 5, and 10 minutes after the onset of ischemia. Hemodynamic variables were collected 5 and 10 minutes after the onset of ischemia. In each experiment, ischemia of the myocardium on which the detector microvessel was placed was confirmed by the dyskinetic wall motion and cyanotic color change of the heart. When ventricular fibrillation occurred during the protocol, the experiment was excluded from the data analysis. At the end of the experiment, sodium nitroprusside (100 μmol/L; Wako Chemicals) was superfused for 5 minutes to produce maximal dilation.

The involvement of oxidative stress in the microvascular tone was investigated in the control (n=5) and hypercholesterolemia (n=6) groups. After the development of the spontaneous tone of the detector vessels, tiron, a cell-permeable free radical scavenger (10 mmol/L; Wako Chemicals)15 was superfused for 30 minutes and images of the detector microvessel were recorded. At the end of the experiment, nitroprusside (100 μmol/L) was superfused for 5 minutes to produce maximal dilation.

**Superoxide Detection**

Dihydroethidium was used to detect superoxide production in rabbit coronary microvessels (control group, n=3; hypercholesterolemia group, n=3) as previously described.16 Briefly, fresh and unfixed heart tissue from control and hypercholesterolemic rabbits was cut into several blocks and frozen in optimal cutting temperature compound (Tissue-Tek, Sakura Fine Chemical) until use. Transverse sections (20-μm-thick) were cut with a cryostat and placed on poly-L-lysine–coated glass slides. Then, the glass slides from control and hypercholesterolemic rabbits were incubated at room temperature for 30 minutes with dihydroethidium (10 μmol/L) in one container to expose the samples from both groups to dihydroethidium in exactly the same condition in terms of the incubation time and dye concentration. Coronary microvessels, 100 to 200 μm in diameter, were selected for observation and images were obtained using a laser scanning confocal microscope (MRC–1024; BioRad). The excitation wavelength was 488 nm, and emission fluorescence was detected with a 585-nm long-pass filter.

**Responses of Isolated Microvessels to Acetylcholine and Nitroprusside**

The responses of isolated rabbit coronary microvessels to acetylcholine and nitroprusside in vitro was normal-

**Data Analysis**

All variables were expressed as the mean value ± SEM. The aortic pressure (phasic and mean) and distal coronary pressure (mean) were recorded on a Rectigraph (model 8K; San-ei Sokki). The responses

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of the detector microvessel diameters to myocardial ischemia and tiron were expressed as the percent change in diameter.

Changes in body weight and plasma cholesterol were analyzed by Student t test for paired samples. Aortic pressure and vascular diameters were statistically analyzed by using 1-way analysis of variance for repeated measures and the Student t test for paired samples, with the Bonferroni correction applied to detect the time point when significant changes occurred. Concentration–response curves of isolated microvessels for acetylcholine and nitroprusside were analyzed by comparing ED50 and maximal responses with Prism v3.0 (GraphPad Software Inc). At P<0.05, the differences were accepted as significant.

Results

In the control rabbits, the cholesterol level did not change between before (59±14 mg/dL) and after feeding (42±6 mg/dL). However, in the hypercholesterolemia rabbits, the cholesterol level significantly increased after feeding (2574±404 mg/dL versus 46±1 mg/dL, P<0.05). There were no differences in body weight between the control and hypercholesterolemia group before (1.4±0.3 kg versus 1.3±0.2 kg) and after feeding (2.5±0.2 kg versus 2.7±0.1 kg).

The blood gases and pH of dogs were kept within physiological ranges during the experiments (Table). The aortic pressure slightly decreased at 5 minutes but not at 10 minutes after the induction of myocardial ischemia in the hypercholesterolemia group (Table). In 6 cases (3 in the control group, 3 in the hypercholesterolemia group), the coronary occlusion produced a significant reduction in the perfusion pressure in the control (before ischemia, 91±12 mm Hg; 5-minute ischemia, 23±3 mm Hg; 10-minute ischemia, 24±4 mm Hg) and the hypercholesterolemia groups (before ischemia, 121±15 mm Hg; 5-minute ischemia, 26±7 mm Hg; 10-minute ischemia, 31±9 mm Hg).

Figure 1A shows the detector microvessel responses in the control group (11 microvessels; baseline diameter, 235±24 μm) and hypercholesterolemia group (6 microvessels; baseline diameter, 210±19 μm) during myocardial ischemia. In the control group, significant dilation was observed at 2, 3, 5, and 10 minutes after the induction of ischemia. In striking contrast, myocardial ischemia did not produce any significant dilation of the detector microvessels in the hypercholesterolemia group. The difference between the 2 groups was statistically significant.

Figure 1B shows the detector microvessel responses to nitroprusside (100 μmol/L) superfusion. In both groups, the microvessels dilated significantly. The magnitude of the dilation was not different between the 2 groups.

Figure 2 shows the detector microvessel responses to tiron. Tiron did not change the diameter of the detector microvessels in the control group (n=5; baseline diameter, 155±19 μm) but caused dilation in the hypercholesterolemia group (n=6; baseline diameter, 145±16 μm). The difference between the 2 groups was statistically significant. Nitroprusside superfusion after tiron produced dilation of the detector microvessels in both groups. The magnitude of the dilation was not different between the 2 groups. Those observations indicated that oxidative stress is critically involved in determining basal microvascular tone in hypercholesterolemia.

<table>
<thead>
<tr>
<th>Mean Aortic Pressure (mm Hg)</th>
<th>Blood pH</th>
<th>Blood Gases</th>
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</thead>
<tbody>
<tr>
<td>Control group (n=11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before ischemia</td>
<td>105±8</td>
<td>7.40±0.01</td>
</tr>
<tr>
<td>5-min ischemia</td>
<td>98±7</td>
<td></td>
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<tr>
<td>10-min ischemia</td>
<td>98±7</td>
<td></td>
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<tr>
<td>Hypercholesterolemia group (n=6)</td>
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<td></td>
</tr>
<tr>
<td>Before ischemia</td>
<td>107±9</td>
<td>7.39±0.01</td>
</tr>
<tr>
<td>5-min ischemia</td>
<td>91±12*</td>
<td></td>
</tr>
<tr>
<td>10-min ischemia</td>
<td>111±15</td>
<td></td>
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</tbody>
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*P<0.05 compared with before ischemia.

Data are expressed as the mean±SEM.

Figure 1. The responses of the detector microvessels to myocardial ischemia (A) and nitroprusside (100 μmol/L; B) in the control and hypercholesterolemia groups. C indicates control group; H, hypercholesterolemia group. *P<0.05 versus control group.
Fluorescence microscopy with dihydroethidium showed that the superoxide production was enhanced in microvessels in the hypercholesterolemia group compared with those in the control group (Figure 3). We performed superoxide detection in three vessels for each group and the results were consistent. These results demonstrated that the coronary microvessels of hypercholesterolemia are exposed to enhanced oxidative stress and supported the results that tiron dilated only the detector vessels of hypercholesterolemia.

The responsiveness of isolated coronary microvessels to acetylcholine and nitroprusside in an in vitro setting is shown in Figure 4. There was no significant difference in vascular responses to acetylcholine between the control (6 microvessels; baseline diameter, 140 \( \pm \) 15 \( \mu \)m) and the hypercholesterolemia group (4 microvessels; baseline diameter, 124 \( \pm \) 15 \( \mu \)m) (Figure 4A). Again, there was no significant difference in the vascular responses to nitroprusside between the control (8 microvessels; baseline diameter, 120 \( \pm \) 11 \( \mu \)m) and the hypercholesterolemia group (5 microvessels; baseline diameter, 137 \( \pm \) 35 \( \mu \)m) (Figure 4B).

**Discussion**

The major findings of the present studies were as follows: (1) ischemic myocardium releases transferable vasodilator signals that are transmitted to coronary arterial microvessels; (2) the transduction of the ischemic myocardium-derived dilator signals is severely impaired in the microvascular walls that have been exposed to hypercholesterolemia; (3) superoxide production is increased in the microvascular walls of hypercholesterolemic animals; and (4) the enhanced oxidative stress participates in the formation of the basal microvascular tone.

**Methodological Considerations**

It is widely accepted that the myocardial metabolism is critically linked to the coronary flow regulation. However, because there are several other factors that are involved in the flow regulation such as myogenic responses, shear stress-induced flow regulation, and many neurohumoral factors, it has been difficult to determine the cross-talk between the myocardium and coronary microvasculature. In the condition of ischemia, for example, a decrease in the distending pressure, which reduces the myogenic tone, and a decrease in shear stress, which decreases nitric oxide release, could produce opposing coronary vasomotion. Further, in addition to the myocardium-derived factors, many neurohumoral factors that may produce vasodilation and constriction could participate in the determination of the coronary microvascular tone. Furthermore, extreme low perfusion could cause
vascular collapse, which masks all vasodilator signals. Thus, research on vessel–myocardium cross-talk has long been hampered.

The bioassay system we used in the present study is unique in that we can control the isolated microvessels and beating heart independently to analyze the interaction between them. In the present study, the intraluminal pressure (60 cm H2O) and shear stress (zero) of the detector microvessels were constant throughout the experiment, and changes in the neurohumoral environment around the detector microvessels could be considered minimal.

Using this system, we have recently elucidated that ischemic myocardium releases transferable dilator signals that specifically activate GPTX proteins.3 Because early studies showed that the vascular responses via G protein, which is pertussis toxin-sensitive, are specifically impaired in hypercholesterolemia,8-10 we tested whether the cross-talk between ischemic myocardium and coronary microvessels is impaired in the microvascular wall of hypercholesterolemia.

**Hypercholesterolemia-Induced Impairment of the Cross-Talk**

Our present results clearly demonstrated that hypercholesterolemia severely impairs the transduction of ischemic myocardium-derived dilator signals in the microvascular wall. Because the dilator responses of the detector microvessels from hypercholesterolemic animals to acetylcholine and nitrorglyceryl were well preserved, the impairment of the dilation in hypercholesterolemia is not nonspecific phenomenon such as artificial vascular damage.

Because the myocardial ischemia was produced in dogs, which could be assumed not to be hypercholesterolemic, in both control and hypercholesterolemia groups, the signals from the ischemic heart can be considered to have been similar in the 2 groups. Actually, the distal coronary pressure during coronary occlusion was comparable between the 2 groups. Accordingly, we conclude that the impairment of signal transduction during ischemia caused by hypercholesterolemia takes place within the coronary microvascular wall.

Hypercholesterolemia is known to impair agonist-induced8,9,18,19 and flow-induced20 dilation in the coronary vasculature. The impairment of ischemia-induced microvascular dilation, which is a pathophysiologically important defense mechanism, was demonstrated for the first time in the present study.

The isolated rabbit coronary microvessels from the hypercholesterolemia group dilated in response to acetylcholine and the magnitude of the dilation was comparable to the control group. In general, hypercholesterolemia decreases bioavailability of nitric oxide in vascular tissue.18 It is possible that decreased nitric oxide bioavailability in hypercholesterolemia was compensated with endothelium-derived hyperpolarizing factors because the contribution of those factors is greater in the microvascular level.21

The vasodilator signals from ischemic myocardium have not been determined yet, and the identification of the signals is outside the scope of the present study. However, acidosis is one of the possible vasomotor signals from ischemic myocardium. We previously demonstrated that ischemic myocardium releases dilator signals that activate GPTX proteins.3 Furthermore, we and others have shown that acidosis-induced microvascular dilation is mediated by GPTX proteins.10,22,23 Additionally, we have recently shown that acidosis-induced dilation is severely impaired in hypercholesterolemic rabbits.30

Another possible mechanism of the abolished dilation of detector vessels from hypercholesterolemic animals could be the augmented responsiveness to constrictors masking the detector vessel dilation. Previous studies showed that the ischemic myocardium releases vasoconstrictors like endothelin24 and dilators. There are some articles reporting that several agonists (acetylcholine, serotonin, a thromboxane analogue, endothelin, etc) produce enhanced constriction in coronary microvessels from hypercholesterolemic animals.6,19,25,26 Because such augmented constriction has been reported in monkeys and pigs so far, it is not clear whether the enhanced constriction takes place in rabbit coronary microvessels. However, we cannot exclude the possibility that the enhanced constriction of coronary microvessels from hypercholesterolemia may have masked the ischemia-induced coronary dilation.

In the present study, we demonstrated that superoxide production is increased in the microvascular walls of hypercholesterolemic animals as reported in the conduit vessels.27 and that tiron, a free radical scavenger, produced significant dilation of the detector vessels from the hypercholesterolemic animals but not those from the control animals. These observations suggest that increased superoxide anions participate in the formation of the microvascular tone in hypercholesterolemia.

Reactive oxygen species are known to cause various vasomotor responses.28 Laurindo et al29 reported the superoxide-induced vasoconstriction of dog coronary arteries, and Suzuki et al30 reported a close positive correlation between oxidative stress and arteriolar tone in mouse mesenteric microvessels. It is interesting to speculate that the basal microvascular tone produced by superoxide causes the microvessels to become less responsive to the signals derived from ischemic myocardium. Armstead31 has recently demonstrated that superoxide inhibits the dilation of cerebral vessels mediated by GPTX protein. Furthermore, Pomerantz et al32 have shown that the Gα level of vascular smooth muscle cells is decreased under the condition of cholesterol enrichment by post-transcriptional mechanisms such as diminished isoprenylation of Gα, subunits, which could lead to low anchoring of G protein.

**Clinical Implications**

Based on our observations, the reduced cross-talk between ischemic myocardium and coronary microvessels in hypercholesterolemia could produce greater ischemic myocardial damage. Anderson et al33 reported that increases in coronary blood flow in response to atrial pacing are inversely related to the serum total cholesterol in human. These observations support our finding that the cross-talk between the myocardium and coronary microcirculation is impaired in hypercholesterolemia. This uncoupling could contribute to the propen-
sity for myocardial ischemia in the absence of critical stenosis in this diseased condition.34

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