Cholesterol feeding exacerbates myocardial injury in Zucker diabetic fatty rats

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1First Department of Medicine, Osaka University School of Medicine, Suita 565-0871; and 2Cardiovascular Division, Osaka Rosai Hospital, Sakai 591-8025, Osaka, Japan.

Hoshida, Shiro, Nobushige Yamashita, Kinya Otsu, Tsunehiko Kuzuya, and Masatsugu Hori. Cholesterol feeding exacerbates myocardial injury in Zucker diabetic fatty rats. Am. J. Physiol. Heart Circ. Physiol. 278: H256–H262, 2000.—We measured infarct size after coronary occlusion (30 min) and reperfusion (24 h) in genetic non-insulin-dependent Zucker diabetic fatty (ZDF) rats with and without 4-wk cholesterol feeding. Infarct size was similar in ZDF rats and lean control rats but was significantly larger in cholesterol-fed diabetic rats than in cholesterol-fed lean rats (P < 0.05). Plasma levels of glucose, insulin, and triglycerides were significantly higher in diabetic rats and were not influenced by cholesterol feeding. The increase in total plasma cholesterol induced by cholesterol feeding was significantly greater in diabetic rats than in lean rats (P < 0.05). A significant positive correlation was found between total plasma cholesterol and infarct size (P < 0.05). Myeloperoxidase activity, as an index of neutrophil accumulation, was significantly higher and expression of P-selectin was more marked in the ischemic myocardium of cholesterol-fed diabetic rats than of cholesterol-fed lean rats. Acetylcholine-induced endothelium-dependent relaxation (EDR) of aortic rings was markedly impaired in cholesterol-fed diabetic rats. Thus cholesterol feeding significantly exacerbated myocardial injury produced by coronary occlusion-reperfusion in non-insulin-dependent diabetic rats, possibly because of enhanced expression of P-selectin and impairment of EDR in the coronary bed.

adhesion molecules; hypercholesterolemia; infarct size; myeloperoxidase; non-insulin-dependent diabetes

ENHANCED LEUKOCYTE-ENDOTHELIUM interaction in the coronary microcirculation may result in increased leukocyte-induced myocardial cell damage (21, 22, 27), leading to exacerbation of ischemia-reperfusion myocardial injury. Augmented expression of adhesion molecules involved in the interaction between leukocytes and coronary microvascular endothelium leads to enhanced leukocyte accumulation in the ischemic myocardium (2, 39). One of the adhesion molecules, P-selectin, located in Weibel-Palade bodies of endothelial cells, is rapidly translocated to the endothelial cell surface on activation of the endothelium with agonists such as thrombin and histamine (26, 28). P-selectin was also shown to be induced by cytokines (12), oxygen radicals (34), oxidized low-density lipoprotein (23), and lysophosphatidylcholine (30) but to be suppressed by nitric oxide (9, 30). Hypercholesterolemia induces microvascular dysfunction characterized by loss of endothelium-derived nitric oxide, increased rolling and adherence of leukocytes, and increased expression of P-selectin (10). Hypercholesterolemia also impairs coronary and aortic endothelium-dependent relaxation (EDR) in humans (5, 24) and animal models (3, 20, 32). Previous studies showed that infarct size after coronary occlusion-reperfusion is significantly larger in animals with acute or chronic hypercholesterolemia than in normal animals (11, 15, 18). In our preliminary study, long-term cholesterol feeding did not increase total plasma cholesterol markedly in normal rats. However, cholesterol feeding induces hypercholesterolemia more markedly in diabetic than nondiabetic animals (6, 29).

In the present study, we evaluated the severity of myocardial injury after occlusion-reperfusion in relation to total plasma cholesterol, P-selectin expression and neutrophil accumulation in the ischemic myocardium, and aortic EDR in genetic non-insulin-dependent diabetic rats with and without cholesterol feeding.

MATERIALS AND METHODS

Animals. Male Zucker diabetic fatty rats (ZDF/Gmi: fa/fa) and lean control rats (ZDF/Gmi-lean: fa/+ or +/+ ) were obtained from Genetic Models (Indianapolis, IN). Rats were divided into four groups and fed for 20 wk beginning after the age of 7 wk, when diabetes begins to develop in ZDF rats, as follows: untreated lean control rats (n = 12); lean control rats fed a diet containing 4% cholesterol plus 0.5% cholic acid for the last 4 wk of the 20-wk feeding period (n = 10); untreated ZDF rats (n = 13); and ZDF rats fed a diet containing 4% cholesterol plus 0.5% cholic acid for the last 4 wk (n = 16). Rats were fed a standard laboratory chow (MF, Oriental Yeast, Tokyo, Japan) supplemented with 10% confectioner’s sugar (20 g daily). They were maintained in individual cages at 24°C with a 12-h light-dark cycle (Nihon Bioservice Center, Hashima, Japan). Plasma levels of glucose (hexokinase-glucose 6-phosphate dehydrogenase), insulin (Inostar, Stillwater, MN), total cholesterol, and triglycerides were measured between 8:00 AM and 9:00 AM at designated intervals until the animals were subjected to myocardial ischemia and reperfusion at 26 wk of age.

Infarction protocol. Rats were anesthetized with pentobarbital sodium (25 mg/kg ip), intubated orally, and ventilated with a small-animal respirator (model 683, Harvard Apparatus, Cambridge, MA). The right femoral artery was cannul-
lated with a polyethylene tube for measurement of blood pressure. Myocardial infarctions were induced as described previously (15, 17). After the heart was exposed, silk thread was passed around the left coronary artery (LCA). The ends of the thread were passed through a small vinyl tube. The LCA was occluded by pulling the snare and clamping it into position. After a 10-min stabilization period, the LCA was occluded for 30 min followed by 24-h reperfusion. The surgical wounds were repaired 30 min after reperfusion, and the rats were returned to their cages to recover. An aseptic surgical technique was used throughout, and benzylpenicillin (30,000 U/kg) was injected intramuscularly as infection prophylaxis. The size of the incision was measured as follows. After the heart was exposed and the LCA was reoccluded, Evans blue dye (2%) was injected via the right femoral vein to estimate heart was exposed and the LCA was reoccluded, Evans blue dye (2%) was injected via the right femoral vein to estimate the size of the ischemic area. The left ventricle was cut and incubated with triphenyltetrazolium chloride (1%, 37°C) to stain the noninfarcted region. Infarct size and ischemic area are expressed as percentage of the weight of the ischemic region at risk and of the left ventricle, respectively. This experiment was performed in accordance with guidelines set forth by the Animal Care and Use Committee of Osaka University School of Medicine.

Assessment of neutrophil accumulation. Myeloperoxidase (MPO) activity was assayed as described previously (14). In brief, myocardial tissue obtained from nonischemic tissue and the border region of ischemic tissue of each heart was frozen rapidly in liquid nitrogen. After centrifugation of the tissue at 40,000 g for 15 min in a potassium phosphate buffer (pH 6.0), MPO activity of the supernatant was assayed spectrophotometrically. A unit of MPO activity was defined as the amount capable of degrading 1 mol peroxidase/min at 30°C.

Immunohistochemistry of adhesion molecules. Myocardial samples for immunohistochemical assessment were obtained from the border region of ischemic tissue of each heart. Immunohistochemistry was performed as previously described (16). Myocardial samples were placed on cryostat chucks with OCT embedding medium (Lab-Tek Products, Naperville, IL) on dry-iced acetone and stored at −80°C. Several cryostat sections (5 μm) of each heart were fixed in 100% acetone at 4°C for 10 min. To inhibit endogenous peroxidase, we incubated the sections for 10 min with 0.3% hydrogen peroxide and 0.1% sodium azide in PBS and exposed them to 10% (vol/vol) normal goat serum for 10 min at room temperature to prevent nonspecific staining. The sections were then incubated with a monoclonal mouse anti-P-selectin (WAPS 12.2, Endogen, Cambridge, MA) for 30 min at room temperature to prevent nonspecific staining. The sections were washed and allowed to equilibrate to baseline. Vasoconstriction of smooth muscle cells was assessed using sodium nitroprusside (SNP, 1 nM to 10 μM).

Statistical analysis. Values are expressed as means ± SE. Differences in infarct size, hemodynamic changes, MPO activity, and relaxation of aortic rings were determined by ANOVA with Scheffé’s test. A level of P < 0.05 was accepted as statistically significant.

RESULTS

Mortality and characteristics of experimental groups. Eleven rats died of ventricular fibrillation during coronary occlusion, and four rats died on the first day after the operation, probably because of arrhythmia or heart failure. Although the mortality rates for the two groups of cholesterol-fed rats appeared higher than those for the other two groups, the difference was not statistically significant (Table 1). The 36 surviving rats were subjected to various analyses. There was a significant difference in body weight between lean and diabetic rats before and after the 20-wk feeding period (Table 2). However, cholesterol feeding did not affect body weight.

Table 1. Mortality rate in experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Rats</th>
<th>CF (n=10)</th>
<th>CF+ (n=6)</th>
<th>CF (n=10)</th>
<th>CF+ (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF (-)</td>
<td>12</td>
<td>2</td>
<td>0</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>CF (+)</td>
<td>10</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td>40</td>
</tr>
<tr>
<td>Diabetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF (-)</td>
<td>13</td>
<td>3</td>
<td>0</td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>CF (+)</td>
<td>16</td>
<td>4</td>
<td>2</td>
<td>10</td>
<td>38</td>
</tr>
</tbody>
</table>

Late death indicates death on 1st postoperative day. VF, ventricular fibrillation; CF, cholesterol feeding. No significant difference was seen in mortality among all 4 groups.

After a stable plateau contraction was achieved, EDR was assayed by the addition of cumulative concentrations of ACh (1 nM to 10 μM). After the response stabilized, the rings were washed and allowed to equilibrate to baseline. Vasoconstriction of smooth muscle cells was assessed using sodium nitroprusside (SNP, 1 nM to 10 μM).

Table 2. Characteristics of experimental groups

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>192±7</td>
<td>192±6</td>
</tr>
<tr>
<td>Post</td>
<td>439±9</td>
<td>432±14</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>124±4</td>
<td>123±3</td>
</tr>
<tr>
<td>Post</td>
<td>113±6</td>
<td>109±6</td>
</tr>
<tr>
<td>Insulin, mg/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>4.0±0.6</td>
<td>4.1±0.6</td>
</tr>
<tr>
<td>Post</td>
<td>5.6±0.7</td>
<td>5.4±0.7</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>81±1</td>
<td>81±1</td>
</tr>
<tr>
<td>Post</td>
<td>97±2</td>
<td>123±4</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
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<td></td>
</tr>
<tr>
<td>Pre</td>
<td>42±3</td>
<td>50±5</td>
</tr>
<tr>
<td>Post</td>
<td>115±19</td>
<td>111±26</td>
</tr>
</tbody>
</table>

Data are means ± SE. n is no. of rats. Pre, before 20-wk feeding period; Post, after 20-wk feeding period. *P < 0.05 vs. lean; †P < 0.05 vs. Pre value; ‡P < 0.05 vs. CF –.
Total plasma cholesterol and triglycerides increased significantly after the 20-wk feeding period in lean and diabetic rats with and without cholesterol feeding. The increase in total plasma cholesterol was significantly greater in diabetic rats with and without cholesterol feeding compared with lean rats. The plasma level of glucose increased significantly after the feeding period only in diabetic rats.

Hemodynamics. There was no significant difference in mean arterial pressure among groups during coronary occlusion and reperfusion. The rate-pressure product was also similar in all groups (Fig. 1).

Infarct size. There was no significant difference in infarct size between diabetic rats and lean rats without cholesterol feeding (Fig. 2). Cholesterol feeding did not affect infarct size in lean rats but significantly increased infarct size in diabetic rats ($P < 0.05$ vs. other 3 groups) in association with a marked increase in total plasma cholesterol. A significant positive correlation was found between total plasma cholesterol and infarct size ($r = 0.616$, $P < 0.05$; Fig. 3). The ischemic region did not differ significantly among groups.

Accumulation of neutrophils. MPO activity in the nonischemic myocardium did not differ among groups but was significantly higher in the ischemic myocardium compared with the nonischemic myocardium in all groups (Table 3). MPO activity in the ischemic myocardium did not differ significantly between lean and diabetic rats. Cholesterol feeding significantly increased MPO activity in the ischemic myocardium of diabetic, but not lean, rats.

Expression of adhesion molecules. Myocardial expression of P-selectin in the border region of ischemic tissue was not observed in lean rats or diabetic rats without cholesterol feeding. However, expression of P-selectin in the coronary bed was increased in cholesterol-fed diabetic rats compared with lean rats or diabetic rats without cholesterol feeding (Fig. 4), although all the coronary vasculature did not express P-selectin in cholesterol-fed diabetic rats. The expression of P-selectin on intravascular platelets could not be ruled out in cholesterol-fed diabetic rats. The expression of P-selectin was not observed in cholesterol-fed lean rats (data not shown).

Impairment of EDR. ACh induced vascular relaxation in a concentration-dependent manner in aortic rings from lean rats (Fig. 5). ACh-induced relaxation was moderately but significantly reduced in the aortic
rings of diabetic rats. Cholesterol feeding reduced relaxation in lean rats to a level similar to that seen in diabetic rats without cholesterol feeding. ACh-induced EDR was markedly impaired in cholesterol-fed diabetic rats. The vasodilatory response to SNP was similar in all groups (Fig. 5).

### DISCUSSION

Hyperglycemia and infarct size. The effect of diabetes on myocardial injury after ischemia-reperfusion is controversial. Some studies have shown that left ventricular function after myocardial ischemia deteriorates in diabetic rats and rabbits (7, 13, 38), but Tani and Neely (37) reported that the functional recovery of the left ventricle after temporal myocardial ischemia was enhanced in isolated diabetic rat hearts. The acquisition of tolerance to ischemia, assessed in terms of infarct size, has been observed in diabetic rats (25), although Vogel and Apstein (38) reported that there was no difference in the size of the infarct produced by myocardial ischemia followed by 48-h reperfusion between rabbits with and without diabetes. These previous studies involved animals with chemically induced insulin-dependent diabetes. In the present study, there was no difference in infarct size between the lean control and ZDF rats, which are a non-insulin-dependent model of diabetes. The plasma level of glucose in these rats at

### Table 3. Myocardial accumulation of leukocytes as assessed by myeloperoxidase activity

<table>
<thead>
<tr>
<th></th>
<th>Nonischemic</th>
<th>Ischemic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lean</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF−</td>
<td>8</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>CF+</td>
<td>6</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td><strong>Diabetic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF−</td>
<td>8</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>CF+</td>
<td>8</td>
<td>3.2 ± 0.3</td>
</tr>
</tbody>
</table>

Data are means ± SE. n is no. of rats. MPO, myeloperoxidase. *P < 0.05 vs. nonischemic; †P < 0.05 vs. lean and diabetic rats without CF.

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Fig. 3. Positive correlation between total plasma cholesterol and infarct size in Zucker diabetic (closed symbols) and lean control (open symbols) rats (r = 0.616). Circles, rats without cholesterol feeding; squares, rats with cholesterol feeding.

![Graph showing correlation between total plasma cholesterol and infarct size](image)

Fig. 4. Immunohistochemical assessment of expression of P-selectin in border region of ischemic myocardium. All panels show counterstaining with hematoxylin and have the same magnification. A: lean rats. B: diabetic rats. C: diabetic rats with cholesterol feeding. Calibration line represents 50 µm.

![Immunohistochemical assessment of expression of P-selectin](image)
the age of 26 wk is usually 300–500 mg/dl (1, 8) but may be as low as 200–300 mg/dl (31, 36). The plasma level of glucose in ZDF rats in the present study was relatively low, possibly because the amount of food was restricted to 20 g/day, and was similar to that seen in most patients with non-insulin-dependent diabetes mellitus. The relatively low plasma level of glucose may explain why the infarct size was similar in lean and ZDF rats.

Hypercholesterolemia and infarct size. We previously reported (18) that the severity of myocardial injury induced by coronary occlusion-reperfusion is increased in association with increased accumulation of neutrophils in the ischemic myocardium in rabbits with acute hypercholesterolemia (∼300 mg/dl) compared with normocholesterolemic rabbits. Cholesterol feeding has markedly different effects on total cholesterol levels in rats compared with rabbits. Therefore, we focused on the absolute value of total plasma cholesterol. In our preliminary study, total plasma cholesterol did not exceed 200 mg/dl in nondiabetic normal (Wistar-Kyoto) rats fed with 4% cholesterol plus 0.5% cholic acid for at least 20 wk. In the present study, total plasma cholesterol level did not exceed the level of 200 mg/dl with cholesterol feeding and infarct size was unaffected in lean rats. In diabetic rats, however, cholesterol feeding induced a higher total plasma cholesterol level (∼300 mg/dl) than that in lean rats and significantly exacerbated myocardial ischemia-reperfusion injury. Modest increases in cholesterol did not affect myocardial injury, and dramatic differences in plasma triglyceride levels (Table 2) had no effects on response to myocardial ischemia-reperfusion. Impairment of EDR and neutrophil accumulation in the ischemic myocardium were greater in the cholesterol-fed diabetic rats than in the other rats. These findings indicate that a high plasma level of cholesterol per se (∼300 mg/dl) may significantly exacerbate myocardial injury in rats. The infarct size in the cholesterol-fed lean rats was similar to that in lean and diabetic rats without cholesterol feeding, supporting this theory. A significant positive correlation was found between total plasma cholesterol and infarct size in these rats (Fig. 3), which may be similar in rabbits at ∼300 mg/dl of total plasma cholesterol (18). However, even at a much higher level of total plasma cholesterol infarct size would not be increased any more in rats, because there was no difference in infarct size between 300 and 2,000 mg/dl of total plasma cholesterol in rabbits (15, 18). Although diabetes mellitus is associated with increased leukocyte-endothelial cell adhesion in response to ischemia-reperfusion (33), the significance of the underlying diabetic metabolic state in the worsening of myocardial injury induced by hypercholesterolemia in rats remains unknown.

Mechanism for exacerbating myocardial injury. Hypercholesterolemia is a major risk factor for ischemic vascular disease. Hypercholesterolemia was more common in cholesterol-fed, non-insulin-dependent diabetic rats than in nondiabetic rats. The leukocyte-endothelium interaction may be enhanced during ischemia and reperfusion in the presence of hypercholesterolemia because of an increased expression of adhesion molecules (4, 35). In the present study, the expression of P-selectin was enhanced in cholesterol-fed diabetic rats in association with increased neutrophil accumulation in the ischemic myocardium compared with lean rats, leading to increased severity of myocardial injury, although neutrophil accumulation may have been increased because infarct size was increased by other mechanisms. Functional nitric oxide production in the coronary vasculature would be a key factor in the present results of our study, because hypercholesterolemia impairs EDR in humans and animal models and the expression of P-selectin can be suppressed by nitric oxide (9, 30). The effects of altered nitric oxide production caused by hypercholesterolemia on the P-selectin expression of the coronary bed (Fig. 4) and on the EDR of aortic tissue (Fig. 5) may differ. There may be a graded effect of nitric oxide on P-selectin expression, and this may manifest in no perceived change in
expression. We previously reported that the expression of P-selectin is enhanced in the coronary bed of hypercholesterolemic rabbits and that the enhancement is effectively inhibited by long-term treatment with an antioxidant, probucol (16), or an angiotensin-converting enzyme inhibitor, quinapril (19), associated with the restoration of the impaired EDR. The expression of other adhesion molecules in endothelium and neutrophils must be examined.

Study limitations. We examined infarct size by using a non-insulin-dependent diabetic model to obtain a high level of total plasma cholesterol. Further studies are needed to elucidate the differences in the severity of myocardial injury between diabetic and normal rats, both of which show a similar high level of total plasma cholesterol. Although we showed that P-selectin was upregulated in coronary bed of the cholesterol-fed, non-insulin-dependent diabetic rats, we did not have any quantitative data that support this contention. We could not rule out the expression of P-selectin on intravascular platelets. Additional studies using a P-selectin antibody would clarify the role of increased P-selectin expression more convincingly.

In conclusion, there was no difference in infarct size between lean control and non-insulin-dependent diabetic rats. Cholesterol feeding, however, significantly enhanced total plasma cholesterol in non-insulin-dependent diabetic rats, exacerbated myocardial injury resulting from coronary occlusion-reperfusion associated with enhanced P-selectin expression, increased neutrophil accumulation in the ischemic myocardium, and markedly impaired EDR in aortic rings. A positive correlation was found between infarct size and total plasma cholesterol, the level of which is similar to that seen in most patients with hypercholesterolemia. Further studies are needed to examine the correlation between total plasma cholesterol level and infarct size in other species.

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Received 12 April 1999; accepted in final form 27 August 1999.

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