Reduced coronary NO production in conscious dogs after the development of alloxan-induced diabetes

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Reduced coronary NO production in conscious dogs after the development of alloxan-induced diabetes. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H268–H278, 1999.—The role of nitric oxide (NO) in the control of coronary blood flow (CBF) during the development of diabetes is unknown. To study this, mongrel dogs were chronically instrumented using sterile techniques for measurements of systemic hemodynamics and CBF. With heart rate controlled (150 beats/min), veratrine (1–10 µg/kg) caused dose-dependent increases in CBF; e.g., 5 µg/kg of veratrine increased CBF by 57 ± 7% from 41 ± 1.3 ml/min (P < 0.05). The dogs developed diabetes 4–5 wk after injection of alloxan (40–60 mg/kg iv, blood glucose levels were 384 ± 18 mg/dl). After diabetes the same doses of veratrine caused smaller increases in CBF; i.e., 5 µg/kg of veratrine increased CBF by 32 ± 2% (P < 0.05 compared with control) from 28 ± 4 ml/min. ACh- and adenosine-induced coronary vasodilation were reduced after diabetes as well. In anesthetized dogs after diabetes, vagal stimulation caused smaller increases in CBF. ACh and bradykinin caused smaller increases in NO2- production in coronary microvessels from diabetic dogs. Furthermore, despite the fact that mRNA for endothelial cell NO synthase from the aorta was increased twofold with the use of Northern blotting, the protein for aortic endothelial constitutive NO synthase was reduced by 66% after diabetes, as determined by Western blotting. Our results indicate that the NO-dependent coronary vasodilation by the Bezold-Jarisch reflex is impaired in conscious dogs after diabetes. The mechanism responsible for the impaired endothelium-dependent coronary vasodilation is most likely the decreased release of NO from the endothelium.

Bezold-Jarisch reflex; adenosine; nitric oxide; coronary microvessels; nitrite production

DIABETES MELLITUS is one of the leading causes of death in the United States, largely because of associated coronary artery diseases, which manifest as myocardial infarction, angina, and silent ischemia (12). However, the mechanisms responsible for the cardiovascular mortality have not been defined completely. An early study (4) showed that adenosine-induced coronary vasodilation was reduced in diabetic lambs, suggesting that one mechanism responsible for regulation of the coronary circulation is depressed after diabetes.

Other studies have provided conflicting results concerning changes in endothelium-dependent vasorelaxation in diabetic animals, in which endothelium-dependent vasorelaxation was reduced, unchanged, and even enhanced (13, 27). However, subsequent studies have documented that endothelium-dependent vasodilation was depressed in the experimental diabetic animals (2, 10, 15, 20, 35, 37, 38). For instance, Kiff et al. (20) reported a selective impairment of hindquarter vasodilator responses to bradykinin in conscious Wistar rats with streptozotocin-induced diabetes. In vitro studies showed improvement of ACh-mediated endothelium-dependent relaxation in isolated mesenteric and hindlimb resistance arteries from diabetic rats (2, 10, 15, 37). Data from Matsunaga et al. (25) suggested that ACh-induced coronary vasodilation was selectively impaired in anesthetized dogs with alloxan-induced diabetes, whereas adenosine-induced coronary vasodilation was preserved. Whether endothelium-dependent coronary vasodilation is impaired in conscious dogs has not been addressed.

It is well known that activation of the carotid chemoreflex or the Bezold-Jarisch reflex causes cholinergic coronary vasodilation and bradycardia (16, 19). Our previous studies have indicated that the reflex cholinergic coronary vasodilation induced by activation of the carotid chemoreflex or the Bezold-Jarisch reflex was mediated by NO, since the administration of nitro-L-arginine (L-NNA), a nitric oxide (NO) synthase (NOS) inhibitor, blocked the coronary vasodilation induced by these two reflexes (34, 44). We have further indicated that the reflex cholinergic NO-dependent coronary vasodilation was selectively impaired after pacing-induced heart failure and enhanced after short-term exercise training in conscious dogs (44, 45). The reflex cholinergic bradycardia, which is mediated by an NO-independent mechanism, was still preserved after the development of pacing-induced heart failure or after short-term exercise training, indicating a specific defect in reflex cholinergic control of the coronary circulation. The changes in reflex cholinergic NO-dependent coronary vasodilation after heart failure or exercise training were proportional to the altered release of NO from the vascular endothelium due to changed gene expression of endothelial constitutive NOS (eNOS) (32, 36).

The goals of this study were 1) to determine the time course for changes in baseline hemodynamics and in the coronary circulation in conscious dogs during the development of diabetes, 2) to correlate changes in plasma glucose with alteration in endothelial function in vivo, 3) to determine the changes in reflex cholinergic NO-dependent and endothelium-dependent and endothelium-independent coronary vasodilation in conscious dogs after the development of diabetes, and 4) to determine the potential mechanism(s) responsible for
the altered reflex cholinergic NO-dependent coronary vasodilation after the development of alloxan-induced diabetes.

**MATERIALS AND METHODS**

The protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conform to the guiding principles for the use and care of laboratory animals of the National Institutes of Health and the American Physiological Society. The loss of body weight in the diabetic dogs was specifically discussed at the meeting of the Institutional Animal Care and Use Committee.

**Surgical Preparation**

Mongrel dogs (24.5–29 kg body wt, n = 19) were premedicated with acepromazine (0.3 mg/kg im) and anesthetized with pentobarbital sodium (25 mg/kg iv) and then intubated and ventilated with room air. A thoracotomy was performed in the left fifth intercostal space by use of sterile surgical techniques. Tygon catheters (Cardiovascular Instruments, Wakefield, MA) were placed in the descending thoracic aorta and in the left atrial appendage for the measurement of pressures and injection of drugs. A solid-state pressure gauge (model P 6.5, Konigsberg Instruments, Pasadena, CA) was placed in the apex of the left ventricle (LV) for the measurement of LV systolic pressure (LVSP) and calculation of the first derivative of LV pressure over time (LV dp/dt). A Doppler flow transducer (Craig Hartley, Houston, TX) was placed on the left circumflex coronary artery for the measurement of coronary blood flow (CBF). A pair of pacing electrodes was sutured on the LV for controlling heart rate (HR). The chest was closed in layers. The wires and the catheters were run subcutaneously and exited from the back of the dog’s neck. The dogs were allowed 10–14 days to recover fully and were trained to lie quietly on the laboratory table. On the day of the experiment, an intravenous catheter was inserted into a peripheral vein and attached to an infusion line so that drugs could be administered without disturbing the dogs.

**Recording Techniques**

Arterial pressure was measured by connecting the previously implanted catheter to a strain gauge transducer (model P23 1D, Statham, Newark, NJ), and mean arterial pressure (MAP) was derived using a 2-Hz low-pass filter. LV pressure was measured from the solid-state pressure gauge, and LV dp/dt was calculated using a microprocessor that was set as a differentiator with a frequency response flat to 700 Hz (model LM 324, National Semiconductor, Edison, NJ). Left circumflex CBF was measured from the previously placed ultrasonic flow transducer with use of a pulsed Doppler flowmeter (System 6, Triton Technology, San Diego, CA), and mean CBF was derived using a 2-Hz low-pass filter. Late diastolic coronary resistance (LDCR) was chosen as the index of coronary vascular resistance, since it is least sensitive to the compressive effect of ventricular contraction on coronary microvessels, and was calculated as the quotient of late diastolic arterial blood pressure and CBF (17, 22). HR was monitored from the pressure pulse interval with the use of a carotidachometer (Beckman Instruments, Newark, NJ).

**Experimental Protocols**

Effects of activation of the Bezold-Jarisch reflex by veratrine. On the day of the experiment, with the dog lying on the laboratory table quietly, when the hemodynamic parameters and CBF were stable, the experiments were begun. Veratrine (5 μg/kg) was administered as a bolus injection (1 ml) into the left atrium through the implanted catheter with the heart in spontaneous cardiac rhythm. The implanted pacing electrodes were then attached to an external pacemaker (model EV 3434, Pace Medical, Waltham, MA), and the HR was increased to 150 beats/min. With HR controlled, bolus intravenous injections of veratrine at 1, 2.5, 5, and 10 μg/kg were given. Each measured variable was allowed to return to control for ≥10 min before the next injection was given.

Effects of ACh and adenosine. ACh (5 μg/kg) and adenosine (0.5 μmol/kg) were administered intravenously with HR controlled. ACh and adenosine were used as coronary vasodilators, one endothelium-derived relaxing factor (EDRF) dependent and the other EDRF independent.

**Induction of diabetes.** After the control experiment the dogs were injected with alloxan monohydrate (40–60 mg/kg iv) over 1 min. Alloxan was prepared as a 5% solution in citrate buffer (pH 4–4.5). The blood gases and plasma glucose were measured on days 3 and 7 after alloxan injection. In some dogs in which plasma glucose levels were <200 mg/dl at day 3 after injection of alloxan, the second injection of alloxan was given. Only dogs with blood glucose >200 mg/dl (after fasting for 12 h) at day 7 were studied. The above protocols were repeated in conscious dogs after the development of diabetes.

Two dogs in which blood glucose levels remained normal after two injections of alloxan were used as time controls.

**Before and after the development of diabetes, the dogs were given the same amount of food and free access to water. Effects of electrical stimulation of vagus nerve.** On the day of the experiment, some of the normal (n = 6) and diabetic dogs (n = 3) were anesthetized with pentobarbital sodium and ventilated (Harvard, Boston, MA). Through a midline cervical incision, the cervical vagal trunks were isolated bilaterally, doubly ligated, and sectioned to interrupt descending parasympathetic outflow to the heart. The cardiac end of the left vagus nerve was placed on bipolar electrodes and immersed in mineral oil for electrical stimulation. The vagus nerve was stimulated for 20 s with the use of an electrical biphasic pulse of 0.4-ms duration with constant voltage (10 V) and frequencies of 2–20 Hz. During the experiments, the HR was kept constant (150 beats/min) and the dogs were given propranolol (1 mg/kg) to avoid the effects of changes in HR and contractility on CBF.

**NO production from coronary microvessels.** Normal and diabetic dogs were killed with an overdose of pentobarbital sodium (50 mg/kg iv), and the hearts were obtained immediately. The myocardium was cut into small pieces, chopped using a Macilwain tissue chopper, and suspended in ice-cold PBS. The resulting suspension was homogenized for 50 s at maximum speed. The homogenate was poured over a 100-μm nylon mesh sieve, and microvessels (including arterial, venous, capillary, and lymphatic vessels <100 μm in diameter) were isolated using the method of Gerdesen and Printz (14), Seyedi et al. (33), Sessa et al. (32), and Zhang and Hintze (43). NO production stimulated by ACh and bradykinin was measured in coronary microvessels (20 mg wet wt) from normal and diabetic dogs. N-nitro-L-arginine methyl ester (L-NAME) was used to block NO production in vitro, and HOE-140 was used to block kinin B1 receptor. The formation of NO was measured as nitrite with the use of the Griess reaction and a spectrophotometer at 540-nm absorbance. Chronically instrumented dogs were used as the normal group.

**Measurements of mRNA and protein for NO synthase in aortic endothelium.** After the dogs were killed with an overdose of pentobarbital sodium, the thoracic aorta was
isolated and the endothelium was scraped for measurements of mRNA and protein for eNOS with the use of Northern and Western analysis, respectively, as previously described (32, 36).

Measurement of plasma nitrate/nitrite, blood gases, plasma insulin, and plasma glucose. After hemodynamics and CBF were stable, arterial and venous blood samples were taken from the catheters with a syringe containing 0.2 ml of heparin (1,000 USP U/ml). The samples were centrifuged (3,000 rpm at 4°C) for 15 min, and the plasma was used for measurements of nitrate/nitrite, insulin, and glucose in plasma. Another 3 ml of arterial and venous samples were taken into a syringe with 0.1 ml of heparin; the syringes were capped and put into ice immediately for the measurement of blood gases.

The concentration of nitrate/nitrite in plasma was measured with the method established by our laboratory (42). PO₂, PCO₂, and pH of arterial and venous blood were measured with a pH/blood gas analyzer (Instrumentation Laboratories, Lexington, MA). The concentration of glucose in plasma was measured with a glucose analyzer (Beckman Instruments, Newark, NJ). Concentration of insulin in plasma was measured with a 125I RIA kit (Diagnostic Products, Los Angeles, CA).

Chemicals

Veratrine, ACh, adenosine, alloxan, propranolol, and bradykinin were purchased from Sigma Chemical (St. Louis, MO). L-NAME was purchased from Aldrich (Milwaukee, WI). HOE-140 was provided by Hoechst-Marion-Roussel.

Data Analysis

Values are means ± SE. In in vivo studies the responses are the peak values after administration of each agent or electrical stimulation. The statistical significance (null hypothesis) of differences was determined with a paired t-test for each peak response, and differences between groups were evaluated with repeated-measures ANOVA. When the ratio of F values indicated a significant difference, significance was determined using a Tukey's test (GBStat). The changes in NO production from canine coronary microvessels or for vagal stimulation were determined with Student's group t-test. Significant changes were considered at P < 0.05. The data of diabetes collected on the last day were used as the end-point data.

RESULTS

Time Course of Changes in Baseline Hemodynamics and Coronary Circulation, Plasma Levels of Glucose and Insulin, and Blood Gases in Conscious Dogs During Development of Diabetes

The time course of baseline hemodynamics and baseline CBF in conscious dogs during the development of diabetes is shown in Table 1. After injection of alloxan, MAP gradually decreased and reached statistical significance at 5 wk. The other hemodynamic indexes were not different from those of the control. CBF also gradually decreased after injection of alloxan, but the changes in CBF did not reach statistical significance. However, if the end point (the last time point for each dog) was considered, the baseline CBF values were significantly decreased after the development of diabetes (36 ± 5 vs. 24 ± 3 ml/min, n = 7, P < 0.05). Baseline LDCR was increased, but the change in baseline LDCR (2.54 ± 0.25 vs. 3.62 ± 0.64 mmHg·ml⁻¹·min⁻¹) did not reach statistical significance.

The time course for glucose and insulin levels in plasma and body weight are shown in Fig. 1. Insulin levels in plasma and body weight were significantly decreased, whereas glucose levels in plasma were significantly increased during the development of diabetes (all P < 0.05). All the diabetic dogs had polyuria and glucosuria.

The time course for blood gases is shown in Table 2. There were no statistical differences in blood gases during the development of diabetes. There was a slight decrease in hematocrit; however, this did not reach statistical significance.

Effects of Veratrine on Hemodynamics in Spontaneous Cardiac Rhythm

Veratrine stimulates cardiac receptors and causes reflex bradycardia (vagal mechanism), peripheral vasodilation (withdrawal of sympathetic mechanism), and coronary vasodilation (vagal mechanism). The afferent and efferent pathways of the Bezold-Jarisch reflex are vagal.

Bolus intra-atrial injection of veratrine (5 μg/kg) resulted in a typical cardiac inhibitory response, includ-

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Table 1. Baseline Hemodynamics and CBF in Conscious Dogs after Alloxan

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 7)</th>
<th>1 wk (n = 7)</th>
<th>2 wk (n = 7)</th>
<th>3 wk (n = 7)</th>
<th>4 wk (n = 7)*</th>
<th>5 wk (n = 5)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDP, mmHg</td>
<td>8 ± 0.7</td>
<td>9 ± 0.6</td>
<td>10 ± 1.1</td>
<td>8 ± 0.9</td>
<td>8 ± 0.8</td>
<td>8 ± 0.7</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>126 ± 4</td>
<td>135 ± 4</td>
<td>128 ± 4</td>
<td>121 ± 4</td>
<td>120 ± 5</td>
<td>128 ± 3</td>
</tr>
<tr>
<td>LDp/dt, mmHg/s</td>
<td>2,793 ± 123</td>
<td>3,106 ± 217</td>
<td>2,900 ± 100</td>
<td>2,785 ± 95</td>
<td>2,804 ± 125</td>
<td>2,812 ± 139</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>101 ± 2</td>
<td>99 ± 3</td>
<td>96 ± 4</td>
<td>97 ± 2</td>
<td>94 ± 3</td>
<td>91 ± 3‡</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>85 ± 2</td>
<td>81 ± 4</td>
<td>82 ± 9</td>
<td>86 ± 10</td>
<td>80 ± 8</td>
<td>79 ± 12</td>
</tr>
<tr>
<td>CBF, ml/min</td>
<td>36 ± 5</td>
<td>29 ± 4</td>
<td>30 ± 5</td>
<td>28 ± 5</td>
<td>25 ± 5</td>
<td>22 ± 8</td>
</tr>
</tbody>
</table>

Values are means ± SE. LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; LDp/dt, 1st derivative of left ventricular pressure over time; MAP, mean arterial pressure; HR, heart rate; CBF, coronary blood flow. For CBF: *n = 6; †n = 3; ‡P < 0.05 compared with control.
Table 2. Arterial pH, Po2, PCO2, CaO2, and hematocrit in conscious dogs after alloxan

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 7)</th>
<th>1 wk (n = 7)</th>
<th>2 wk (n = 7)</th>
<th>3 wk (n = 7)</th>
<th>4 wk (n = 7)</th>
<th>5 wk (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.42 ± 0.01</td>
<td>7.43 ± 0.02</td>
<td>7.40 ± 0.02</td>
<td>7.41 ± 0.01</td>
<td>7.43 ± 0.01</td>
<td>7.37 ± 0.03</td>
</tr>
<tr>
<td>Po2, mmHg</td>
<td>90 ± 3</td>
<td>87 ± 3</td>
<td>87 ± 2</td>
<td>83 ± 3</td>
<td>83 ± 3</td>
<td>84 ± 4</td>
</tr>
<tr>
<td>PCO2, mmHg</td>
<td>35 ± 0.4</td>
<td>35 ± 1</td>
<td>36 ± 0.8</td>
<td>35 ± 1.0</td>
<td>36 ± 1.6</td>
<td>34 ± 2.8</td>
</tr>
<tr>
<td>CaO2, %</td>
<td>16 ± 0.8</td>
<td>16 ± 0.9</td>
<td>17 ± 0.4</td>
<td>15 ± 0.7</td>
<td>15 ± 0.6</td>
<td>14 ± 0.5</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>35 ± 1</td>
<td>35 ± 1</td>
<td>34 ± 1</td>
<td>31 ± 2</td>
<td>31 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. CaO2, arterial O2 content. *n = 4.

Table 3. Effects of veratrine on hemodynamics and CBF in conscious dogs before and after diabetes

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 7)</th>
<th>Diabetes (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Response</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>131 ± 6</td>
<td>91 ± 8†</td>
</tr>
<tr>
<td>LV dP/dt, mmHg/s</td>
<td>2,892 ± 145</td>
<td>2,023 ± 197†</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>104 ± 3</td>
<td>43 ± 6†</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>84 ± 1</td>
<td>36 ± 5†</td>
</tr>
<tr>
<td>CBF, ml/min</td>
<td>34 ± 4</td>
<td>42 ± 10</td>
</tr>
</tbody>
</table>

Values are means ± SE. Veratrine was administered at 5 µg/kg.
*End points of diabetic dogs are as follows: 1 at week 3, 3 at week 4, 3 at week 5. †P < 0.05 compared with baseline; ‡P < 0.05 compared with control.

summarized in Table 3. There were no significant increases in CBF. There was still a marked bradycardia after veratrine injection in conscious dogs after the development of alloxan-induced diabetes (Table 3).

Effects of Veratrine on Coronary Circulation and Hemodynamics in Conscious Dogs With HR Controlled

Because veratrine resulted in a significant decrease in HR, which caused dramatic hypotension and prevented the increases in CBF, the heart was paced at 150 beats/min. With HR controlled, 1–10 µg/kg of veratrine caused dose-dependent increases in CBF and decreases in LDCR. Figure 2 shows changes in CBF and LDCR in response to intra-atrial injection of veratrine at 1–10 µg/kg in the same conscious dog before and after the development of diabetes. Veratrine also decreased MAP and LVSP. The changes in systemic hemodynamics induced by veratrine at 5 µg/kg are shown in Table 4, and the changes were not significantly different before and after the development of diabetes. Figure 3 shows the time course of increases in CBF induced by veratrine at 5 µg/kg. CBF responses to veratrine were not significantly decreased until 4 wk after injection of alloxan.

Two dogs did not develop diabetes after injection of alloxan (twice), and the dogs were used as time controls; i.e., 4–5 wk after the control experiment the second experiment was repeated. In the two dogs the coronary vasodilatory response to veratrine at 5 µg/kg was not altered during two experiments 4–5 wk apart (increase in CBF was 15 vs. 18 ml/min), suggesting that the impaired coronary vasodilation after diabetes is not...
due to direct action of alloxan. This also demonstrates that the coronary vasodilation in response to activation of the Bezold-Jarisch reflex is not altered over a 4- to 5-wk period.

Effects of ACh

Our previous results have shown that controlling HR (150 beats/min) did not affect the coronary vasodilation response to ACh (38); therefore, we only studied the coronary vasodilation in response to ACh with HR controlled. Bolus intravenous injection of ACh (5 µg/kg) resulted in significant increases in CBF by 44 ± 6 ml/min and decreases in LDCR by 69 ± 2.1%. ACh also caused decreases in LVSP and MAP and reflex increases in LV dP/dt (Table 4). After the development of diabetes, ACh at 5 µg/kg only increased CBF by 32 ± 3 ml/min.

Table 4. Effects of veratrine, ACh, and adenosine on hemodynamics and coronary circulation in conscious dogs with HR controlled before and after diabetes

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 7)</th>
<th>Diabetes (n = 7)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Response</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veratrine (5 µg/kg)</td>
<td>132 ± 6</td>
<td>111 ± 7†</td>
</tr>
<tr>
<td>ACh (5 µg/kg)</td>
<td>127 ± 6</td>
<td>98 ± 6†</td>
</tr>
<tr>
<td>Adenosine (0.5 µmol/kg)</td>
<td>125 ± 8</td>
<td>108 ± 8†</td>
</tr>
<tr>
<td>LV dP/dt, mmHg/s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veratrine (5 µg/kg)</td>
<td>2,460 ± 189</td>
<td>2,217 ± 47†</td>
</tr>
<tr>
<td>ACh (5 µg/kg)</td>
<td>2,356 ± 141</td>
<td>3,280 ± 295†</td>
</tr>
<tr>
<td>Adenosine (0.5 µmol/kg)</td>
<td>2,339 ± 182</td>
<td>2,969 ± 157†</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veratrine (5 µg/kg)</td>
<td>115 ± 6</td>
<td>87 ± 5†</td>
</tr>
<tr>
<td>ACh (5 µg/kg)</td>
<td>111 ± 4</td>
<td>60 ± 6†</td>
</tr>
<tr>
<td>Adenosine (0.5 µmol/kg)</td>
<td>108 ± 6</td>
<td>84 ± 7†</td>
</tr>
<tr>
<td>Mean CBF, ml/min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veratrine (5 µg/kg)</td>
<td>41 ± 4</td>
<td>64 ± 6†</td>
</tr>
<tr>
<td>ACh (5 µg/kg)</td>
<td>38 ± 3</td>
<td>83 ± 6†</td>
</tr>
<tr>
<td>Adenosine (0.5 µmol/kg)</td>
<td>39 ± 4</td>
<td>112 ± 6†</td>
</tr>
<tr>
<td>LDCR, mmHg·ml⁻¹·min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veratrine (5 µg/kg)</td>
<td>2.4 ± 0.14</td>
<td>-48 ± 3%‡</td>
</tr>
<tr>
<td>ACh (5 µg/kg)</td>
<td>2.3 ± 0.19</td>
<td>-69 ± 2%‡</td>
</tr>
<tr>
<td>Adenosine (0.5 µmol/kg)</td>
<td>2.1 ± 0.15</td>
<td>-72 ± 4%‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. LDCR, late diastolic coronary resistance. *End points of diabetic dogs are as follows: 1 at week 3, 2 at week 4, 3 at week 5. †P < 0.05 compared with baseline. ‡P < 0.05 compared with control.
ml/min and decreased LDCR by $61 \pm 2.0\%$ (both $P < 0.05$ compared with values before diabetes). After the development of diabetes, ACh still caused significant decreases in LVSP and MAP and an increase in LV $dP/dt$ in conscious dogs (Table 4).

### Effects of Adenosine

With HR controlled, adenosine (0.5 µmol/kg) increased CBF by $73 \pm 5$ ml/min and decreased LDCR by $72 \pm 3.8\%$. Adenosine also reduced MAP and LVSP and increased LV $dP/dt$. The changes in hemodynamic and coronary circulation in response to adenosine with the HR controlled are shown in Table 4. After the development of diabetes, the coronary vasodilation produced by adenosine was attenuated. Adenosine increased CBF by $44 \pm 6$ ml/min and decreased LDCR by $61 \pm 1.5\%$ (both $P < 0.05$ compared with those before diabetes).

### CBF Response to Electrical Stimulation of the Left Cervical Vagus Nerve in Anesthetized Dogs

In normal anesthetized dogs, electrical stimulation of the left vagus nerve resulted in frequency-dependent increases in CBF (Fig. 4). For instance, 10 V of 10-Hz electrical stimulation increased CBF by $56 \pm 7\%$ from $24 \pm 3$ ml/min. In dogs after the development of diabetes, the same electrical stimulus caused smaller increases in CBF (Fig. 4). Stimulation of 10 Hz with 10 V increased CBF by only $34 \pm 2\%$ from $27 \pm 5$ ml/min ($n = 3, P < 0.05$ compared with normal group).

### NO Production From Coronary Microvessels

There was less basal nitrite production in coronary microvessels from diabetic dogs ($n = 5$) than from normal dogs ($n = 9$) used in the present study ($52 \pm 4$ vs. $71 \pm 4$ pmol/mg tissue, $P < 0.05$). In coronary microvessels from normal dogs, ACh and bradykinin caused concentration-dependent increases in nitrite production. The actual changes in nitrite production in coronary microvessels from normal and diabetic dogs are shown in Fig. 5. Nitrite production in coronary microvessels from diabetic dogs in response to ACh and bradykinin was significantly less than that in microvessels from normal dogs. The increase in nitrite production in response to ACh and bradykinin was blocked by L-NAME. The increase in nitrite production by bradykinin was blocked by HOE-140 in coronary microvessels from normal dogs as well as diabetic dogs (data not shown).

### Concentration of Nitrate/Nitrite in Plasma

After injection of alloxan, concentration of nitrate/nitrite in plasma was gradually decreased and reached statistical significance at week 3. Nitrate/nitrite in plasma is shown in Fig. 6. The time course for blood creatinine is also shown in Fig. 6. There was no statistically significant change in blood creatinine during the development of diabetes.

### mRNA and Protein for ecNOS in Aortic Endothelium

Figure 7 shows the change in mRNA for ecNOS from aortic endothelium after the development of alloxan-induced diabetes. There was a doubling of mRNA for ecNOS in aortic endothelium from diabetic dogs compared with that from normal dogs. In contrast, the protein for ecNOS decreased by $66\%$ in aortic endothelium compared with that from normal dogs (Fig. 8).

### DISCUSSION

The most striking finding of the present study was the reduction in NO-dependent coronary vasodilation...
in vivo and NO production in vitro in coronary microvessels due to the decreased protein for ecNOS, despite the enhanced mRNA for ecNOS in aortic endothelium from diabetic dogs. Thus, during diabetes, unlike heart failure (44) or exercise training (45), there are differential changes in mRNA and protein for ecNOS. Furthermore, this is most likely not due to acute hyperglycemia, since plasma glucose levels were elevated at day 3 but endothelial dysfunction was not observed for 4–5 wk.

In this study we observed the time course for changes in baseline hemodynamics and coronary circulation in conscious dogs during the development of diabetes. Our results have indicated that MAP and CBF were significantly decreased in conscious dogs after the development of diabetes, whereas the other hemodynamic parameters were not altered.

Our results also showed that reflex cholinergic NO-dependent coronary vasodilation after activation of the Bezold-Jarisch reflex was selectively depressed in conscious dogs after the development of diabetes, whereas reflex cholinergic bradycardia was still preserved. Endothelium-dependent (ACh induced) and endothelium-independent (adenosine induced) coronary vasodilation were impaired as well. The depressed reflex cholinergic NO-dependent coronary vasodilation is related to the decreased release of NO from the vascular endothelium rather than to the altered outflow of vagus nerves to the heart. This is evidenced by the fact that electrical stimulation of the vagus nerve at the same frequency caused smaller increases in CBF in anesthetized dogs after diabetes and that similar doses of ACh and bradykinin resulted in smaller increases in NO production in coronary microvessels from diabetic dogs than in microvessels from normal dogs.

Alloxan-induced diabetes was occasionally found in 1943 when Dunn et al. (5) and Rerup (31) investigated the effect of a series of uric acid derivatives, including alloxan, on the kidney. Histological studies revealed a partial or even total necrosis of pancreatic islets without damage to other tissues after injection of alloxan in rats (5, 31). Our results have shown significant increases in blood glucose levels and a decrease in body weight in conscious dogs after injection of alloxan. We have further demonstrated that insulin levels in plasma were significantly decreased in conscious dogs after injection of alloxan, providing direct evidence for the development of diabetes in conscious dogs after injection of alloxan. In our study the time course for changes in baseline hemodynamics and coronary circulation was observed in conscious dogs during the development of diabetes as well. The results obtained by Matsunaga et al. (25) showed no significant differences in baseline
hemodynamics and CBF in anesthetized dogs after the development of alloxan-induced diabetes over 4 wk. However, our results have indicated that MAP and CBF were significantly decreased in conscious dogs after the development of diabetes. In the present study, other hemodynamic indexes and LDCR were not significantly altered in conscious dogs after the development of alloxan-induced diabetes; these findings are consistent with the results of Matsunaga et al. In the present study and that of Matsunaga et al., the dose of alloxan (40–60 and 40 mg/kg) and the time after injection of alloxan (4–5 wk and 4 wk) were similar. The only difference is the state of the dogs (conscious vs. anesthetized). It should be pointed out that the pentobarbital used in their study has significant effects on the cardiovascular system in the dog (23). In addition, our results also showed no statistical differences in PO$_2$, PCO$_2$, pH, arterial O$_2$ content, hematocrit, and plasma creatinine in conscious dogs during the development of diabetes.

In the early 1980s, Downing et al. found that adenosine-induced coronary vasodilation was depressed in anesthetized lambs after the development of alloxan-induced diabetes (4) and that the reduced adenosine-induced coronary vasodilation was reversed by insulin (3). Using a perfused heart preparation from spontaneous diabetic rat, Durante et al. (6) observed the reduced coronary vasodilation to adenosine compared with normal rats. However, Matsunaga et al. (25) showed that adenosine-induced coronary vasodilation was not altered in anesthetized dogs after the development of diabetes. Our results from conscious dogs have demonstrated that adenosine-induced coronary vasodilation was impaired after the development of diabetes, consistent with the studies of Downing et al. The mechanism(s) responsible for the depressed adenosine-induced coronary vasodilation after the development of diabetes is unknown.

Early studies provided conflicting results concerning endothelium-dependent vasorelaxation in diabetic animals, in which endothelium-dependent vasorelaxation was reduced, unchanged, and even enhanced (13, 27). However, subsequent studies have documented that endothelium-dependent vasodilation was depressed in diabetic animals (2, 10, 15, 20, 35, 37, 38). For instance, Kiff et al. (20) reported a selective impairment of hindquarter vasodilator responses to bradykinin in conscious Wistar rats with streptozotocin-induced diabetes mellitus. In vitro studies showed that ACh-mediated endothelium-dependent relaxation in isolated mesenteric and hindlimb resistance arteries from diabetic rats was impaired (2, 10, 15, 37). The data from Matsunaga et al. (25) suggested that ACh-induced coronary vasodilation is selectively impaired in anesthetized dogs with diabetes, whereas adenosine-induced coronary vasodilation is preserved. Koltai et al. (21) also demonstrated that ACh-induced coronary vasodilation was depressed in anesthetized dogs after the development of alloxan-induced diabetes. Other studies further suggested that the reduced endothelium-dependent vasodilation was predominantly due to decreased NO release, since nitroprusside-induced relaxation (endothelium independent) was similar in arteries from normal and diabetic rats (2, 37). Our results also indicated that ACh-induced coronary vasodilation was impaired in conscious dogs after the development of alloxan-induced diabetes. It has been recognized that two components are involved in the vasorelaxation induced by ACh: NO and endothelium-derived hyperpolarizing factor (EDHF). There are conflicting results regarding the involvement of hyperpolarizing factor in the depressed ACh-induced vasorelaxation in diabetic animals. Using tetraethylammonium to block EDHF, Endo et al. (7) observed similar effects of tetraethylammonium on ACh-induced relaxation of aorta from streptozotocin-induced diabetic and normal rats, suggesting that EDHF did not contribute to the decreased endothelium-dependent relaxation in the aorta from streptozotocin-induced diabetic rats. In contrast, Fukao et al. (11) measured the membrane potentials directly, and their results showed that ACh-induced hyperpolarization in mesenteric arteries from streptozotocin-induced diabetic rats was depressed, although there were no significant differences in the resting membrane potentials of the arteries from control and diabetic rats. ACh-induced hyperpolarization in control and diabetic arteries was not altered by treatment with L-NNA, suggesting that an NO-independent mechanism is involved.

Activation of the Bezold-Jarisch reflex results in coronary vasodilation and bradycardia (8, 9, 46). Our previous results demonstrated that reflex cholinergic coronary vasodilation was mediated by NO, since L-NNA blocked the coronary vasodilation induced by activation of the Bezold-Jarisch reflex (44). Most important, we further indicated that reflex cholinergic NO-dependent coronary vasodilation was selectively depressed in conscious dogs after the development of pacing-induced heart failure (44), whereas the reflex cholinergic NO-dependent coronary vasodilation was enhanced in conscious dogs after short-term exercise training (45). Reflex bradycardia induced by activation of the Bezold-Jarisch reflex was still preserved after heart failure (44) or exercise training (45). The altered reflex cholinergic NO-dependent coronary vasodilation after heart failure or exercise training is related to the changed release of NO from the vascular endothelium and the alteration in the gene expression of NOS (32, 36). The present results further demonstrate that reflex cholinergic NO-dependent coronary vasodilation was also impaired in conscious dogs after the development of diabetes, whereas reflex bradycardia was still preserved. Cohen (1) found that after exposure to high glucose for 6 h the relaxation of rabbit aorta to ACh was reduced. Using intracoronary infusion of 10% glucose (resulting in coronary glucose levels of 350–400 mg/dl) for 4 h, Hollenbaugh et al. (18) observed depressed ACh-induced coronary vasodilation in anesthetized dogs. Williams et al. (40) also found that acute hyperglycemia (6 h) attenuated endothelium-dependent vasodilation of the forearm in humans. However, our results indicated that the depressed reflex cholinergic NO-
dependent coronary vasodilation in conscious dogs did not occur until 4–5 wk (Fig. 3) after injection of alloxan, suggesting that the hyperglycemia (at least in the short term) is not likely responsible for the depressed reflex cholinergic NO-dependent coronary vasodilation in conscious dogs after the development of diabetes, since blood glucose levels were increased to >200 mg/dl 3 days after injection of alloxan and remained at >350 mg/dl for 1–5 wk after injection of alloxan.

Five components are involved in a reflex: receptors, afferent nerves, reflex center (central nervous system), efferent nerves, and effectors. If the depressed reflex cholinergic NO-dependent coronary vasodilation after diabetes is due to the changes in receptors, in the central nervous system, or in the efferent activities of the vagus nerve to the heart, the outflow of the vagus nerve to the heart would be decreased. This is most likely not the case, since there was no resting tachycardia that could be attributed to vagal withdrawal (Table 1). To eliminate this possibility, electrical stimulation was used to control the efferent activity of the vagus nerve. In a previous study we indicated that vagal stimulation caused greater increases in CBF in anesthetized dogs after exercise training than in normal dogs, and the enhanced coronary vasodilation in response to vagal stimulation in dogs after exercise training was blocked by L-NAME, suggesting an NO mechanism (37).

The present results indicate that, at a given frequency and voltage, electrical stimulation of the vagus resulted in smaller increases in CBF in anesthetized dogs after the development of diabetes. Vagal stimulation also causes bradycardia and β-adrenergic coronary vasodilation (8). In the present study, HR was kept constant and propranolol was used to block β1- and β2-receptors in the heart to avoid the changes in HR and β-adrenergic coronary vasodilation during vagal stimulation. Therefore, the bradycardia and β-adrenergic coronary vasodilation were not involved in the increases in CBF in response to vagal stimulation. The depressed coronary vasodilation in response to vagal stimulation obtained in the present study could not exclude the possibility of the involvement of the afferent activity of the vagi. The present results, however, demonstrated that the reflex control of HR by the Bezold-Jarisch reflex was not altered in conscious dogs after the development of diabetes. These results imply that the depressed reflex cholinergic NO-dependent coronary vasodilation after diabetes is not simply due to the altered vagal efferent activity to the heart.

Our previous results indicated that reflex cholinergic NO-dependent coronary vasodilation was impaired in conscious dogs after heart failure and enhanced after short-term exercise training. Direct evidence for the involvement of NO in the reflex cholinergic NO-dependent coronary vasodilation after heart failure or exercise training was from our observations of NO production from coronary microvessels (32, 36, 45). Our results showed that similar doses of ACh caused smaller increases in NO production in coronary microvessels from the failing heart (36), whereas the same doses of agonists (ACh, bradykinin, ANG II, and norepinephrine) resulted in greater increases in NO production in coronary microvessels from dogs after exercise training (45). The present results further demonstrate that ACh and bradykinin caused smaller increases in NO production in coronary microvessels from diabetic dogs than in coronary microvessels from normal dogs. In the present study the increases in NO production in coronary microvessels from normal and diabetic dogs in response to the agonists were blocked by L-NAME, indicating that the nitrite production reflects the activity of NOS in coronary microvessels.

The data from our laboratory suggest that the mechanism responsible for the altered release of NO from coronary microvessels after heart failure or exercise training is related to the down- or upregulation of ecNOS gene expression in the vascular endothelium (32, 36). The histochemical data obtained by Yamamoto et al. (41) showed that the expression of the neuronal NOS gene in the paraventricular and supraoptic nuclei from diabetic rats was significantly increased compared with that from normal rats. Our study indicates that the mRNA for ecNOS from the aortic endothelium of diabetic dogs was increased as well, suggesting that the mechanism responsible for the decreased release of NO in coronary microvessels from diabetic dogs is not related to downregulation of ecNOS. More recently, a decreased activity of constitutive NOS has been shown in platelets from patients with insulin-dependent and insulin-independent diabetes mellitus (24, 30). Furthermore, Prieper (28) indicated that tetrahydrobiopterin, a cofactor of NOS, improved the relaxation induced by ACh in aortic rings from diabetic rats, suggesting that tetrahydrobiopterin availability could play a role in the regulation of NO production in endothelium from diabetic rats. Taken together, it is probable that the decreased release of NO from the vascular endothelium is related to the decreased activity for ecNOS or a defect of cofactors of ecNOS rather than the downregulation of gene expression of ecNOS. This is in marked contrast to heart failure (44) or exercise training (45). Our results further demonstrate that the protein for ecNOS in aortic endothelium from diabetic dogs is decreased by 66% compared with that from normal dogs.

The present results also demonstrate that arterial nitrate/nitrite levels were significantly decreased during the development of diabetes. However, the present study could not provide the exact mechanism(s) for the decreased blood nitrate/nitrite. The possible mechanisms include 1) the decreased release of NO from the vascular endothelium, evidenced by reduced release of NO in coronary microvessels from diabetic dogs, or 2) the polyuria during the development of diabetes, since the kidney is the primary organ responsible for the excretion of nitrate (39). The latter probably only plays a minor role in the decreased arterial blood nitrate/nitrite, since polyuria occurred quickly (2–3 days after injection of alloxan), whereas the decreases in blood nitrate/nitrite did not reach statistical significance until 3 wk after injection of alloxan.

In addition to the decreased NO, inactivation of NO by superoxide may be involved in the depressed endothe-
lum-dependent vasorelaxation after diabetes, since inhibition of superoxide by superoxide dismutase could restore the impaired endothelium-dependent vasorelaxation (1, 26, 29). Our data cannot rule out this possibility, although we have found a 66% reduction in ecNOS protein.

In summary, our results indicate in conscious dogs after diabetes that 1) baseline MAP and baseline CBF were significantly decreased, 2) endothelium-dependent (ACh induced) and endothelium-independent (adenosine induced) coronary vasodilation were impaired, and 3) reflex cholinergic NO-dependent coronary vasodilation was selectively depressed. The most likely mechanism responsible for the depressed reflex cholinergic NO-dependent coronary vasodilation was the decreased release of NO from the vascular endothelium rather than the altered efferent activity of the vagus nerve to the heart. Finally, this is not due to reduced transcription of ecNOS (reduced mRNA) but most likely to some posttranscriptional event, since ecNOS protein was reduced.

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