

# Endothelium-dependent hyperpolarization-mediated vasodilatation compensates nitric oxide-mediated endothelial dysfunction during ischemia in diabetes-induced canine coronary collateral microcirculation in vivo

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## Abstract

**Objectives:** It has been previously demonstrated that endothelial caveolin-1 plays crucial roles to produce an endothelium-derived hyperpolarizing factor in mouse mesenteric arteries. We examined whether this mechanism is involved in the endothelium-dependent hyperpolarization-mediated responses to compensate reduced NO-mediated responses in diabetes mellitus during coronary occlusion in dogs in vivo.

**Methods:** Canine subepicardial collateral coronary small arteries ( $\geq 100 \mu\text{m}$ ) and arterioles ( $< 100 \mu\text{m}$ ) were observed by an intravital microscope. Experiments were performed during occlusion of the left anterior descending coronary artery (90 minutes) under the following conditions ( $n = 6$  each); (i) control, (ii) diabetes mellitus, and (iii) diabetes mellitus+L-NMMA+K<sub>Ca</sub> channel blockade. Vascular and myocardial levels of caveolin-1, eNOS, and caspase-3 were measured by ELISA.

**Results:** Caveolin-1 levels in the ischemic area were greater in coronary microvessels than in conduit arteries in the control group. NO-mediated coronary vasodilatations of small arteries to bradykinin did not increase in diabetes mellitus associated with decreased eNOS phosphorylation at Ser1177 compared with baseline of controls and were restored by compensation of endothelium-dependent hyperpolarization and were suppressed by K<sub>Ca</sub> channel blockade.

**Conclusions:** NO-mediated vasodilatations of small coronary arteries during coronary occlusion are impaired in diabetes mellitus and are compensated by endothelium-dependent hyperpolarization of arterioles in dogs in vivo.

## KEYWORDS

caveolin-1, coronary, diabetes mellitus, endothelium-dependent hyperpolarization, microcirculation

**Abbreviations:** AMC, 7-amino-4-methylcoumarin; Apm, Apamin; Cav-1<sup>-/-</sup>, Cav-1-knockout; Cav-1, caveolin-1; CBF, coronary blood flow; CCD, charge coupled device; CTx, charybdotoxin; DEVD, Asp-Glu-Val-Asp; DM, diabetes mellitus; EDH, endothelium-dependent hyperpolarization; eNOS, endothelial nitric oxide synthase; eNOS-Tg, eNOS transgenic; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; K<sub>Ca</sub> channels, Ca<sup>2+</sup>-activated K<sup>+</sup> channels; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; LVP, left ventricular pressure; LVP, left ventricular pressure; MBP, Mean blood pressure; MI, myocardial infarction; NO, nitric oxide; Occlusion<sub>15</sub> and Occlusion<sub>85</sub>, coronary occlusion at 15 min and 85 min; SOD, superoxide dismutase; Tg, transgenic.

## 1 | INTRODUCTION

The endothelium plays a crucial role in maintaining vascular homeostasis by synthesizing and releasing several vasodilating factors, including vasodilator prostaglandins, NO, and EDH factor.<sup>1,2</sup> Endothelium-derived H<sub>2</sub>O<sub>2</sub> is a primary EDH factor mesenteric arteries in mice and humans,<sup>3,4</sup> where endothelial Cu, Zn-SOD plays an important role as an EDH synthase.<sup>5,6</sup> EDH/H<sub>2</sub>O<sub>2</sub>-mediated responses are dependent on the eNOS system in mouse mesenteric arteries,<sup>7</sup> where H<sub>2</sub>O<sub>2</sub> acts as an EDH factor through activation of Ca<sup>2+</sup>-activated K<sup>+</sup> (K<sub>Ca</sub>) channels.<sup>8,9</sup>

eNOS is functionally inhibited in mesenteric arteries through Cav-1-dependent mechanism, switching its function from NO-generating enzyme to EDH/H<sub>2</sub>O<sub>2</sub>-generating enzyme in mice.<sup>10</sup> Excessive endothelium-derived NO with reduced EDH impairs cardiovascular homeostasis *in vivo*, causing cardiac hypertrophy in Cav-1<sup>-/-</sup> mice and hypotension in endothelium-specific eNOS-Tg mice.<sup>11</sup> Endothelial Cav-1 plays crucial roles of H<sub>2</sub>O<sub>2</sub> production as an EDH in mice.<sup>10,11</sup>

Coronary microcirculation plays an important role in maintaining blood flow and vascular homeostasis under both physiological and pathological conditions. We have confirmed that EDH plays an important compensatory role during coronary autoregulation<sup>12</sup> and reperfusion injury in ischemic<sup>13</sup> and collateral<sup>14</sup> microcirculation in dogs *in vivo* through compensation of NO. Endothelial dysfunction has been implicated in the reduced dilatation of porcine coronary microcirculation.<sup>15</sup> It is reported that the risk factors, such as DM, cause endothelial dysfunction by NO- and EDH-mediated vasodilatation.<sup>16</sup> We examined whether this mechanism is involved in the endothelial Cav-1 and EDH-mediated responses as a compensatory mechanism of NO in DM during coronary occlusion in canine coronary collateral microcirculation *in vivo*.

## 2 | METHODS

This study conformed to the Guideline on Animal Experiments of Kawasaki Medical School and the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health.

### 2.1 | Animal preparation

Mongrel dogs (7-20 kg, n = 18) of either sex were anesthetized with ketamine (10 mg/kg, IM) and sodium pentobarbital (25 mg/kg, IV). After intubation, each animal was ventilated with a ventilator (model VS600, IDC, Pittsburgh, PA) with room air supplemented by 100% oxygen. Aortic pressure and LVP were continuously monitored with a catheter (SPR-350, Millar, TX). Blood flow of the LCX was continuously monitored by a transonic flow probe (T206, Transonic Systems, Ithaca, NY). The heart rate was kept constant at 100 beats/min during the experiment by right ventricular pacing after atrioventricular node blocking with 37% formaldehyde.<sup>17</sup>

### 2.2 | Measurements of coronary diameter with an intravital microscope

We continuously monitored coronary vascular responses with an intravital microscope (VMS 1210, Nihon Kohden, Tokyo, Japan) with a needle-probe (magnification of ×200) *in vivo*. Briefly, we gently placed the needle-probe on coronary subepicardial microvessels. Collateral coronary small arteries (≥100 μm) and arterioles (<100 μm) were visually traced between the LAD and LCX with an injection of indocyanine green. When a clear vascular image was obtained, end-diastolic vascular images were taken with 30 pictures/s.<sup>17</sup>

### 2.3 | Plasma levels of glucose

Measurement for plasma levels of glucose was performed in the blood obtained from the cephalic vein at AM 9:00 (SRL, Tokyo, Japan).

### 2.4 | Measurement of caveolin-1

Measurement of Cav-1 level for coronary artery (LAD and LCX) and myocardium (LAD and LCX area) was performed by ELISA method (Canine, ELISA Assay, Cloud-Clone Corp. Wuhan). We prepared 30 mg of myocardium and coronary conduit artery in 1 mL of cell lysis buffer, homogenized them for 2 minutes at 25 Hz, sonicated them for detection of nuclear localized proteins, and spinned them for 10 minutes at 14 000 rpm. This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for Cav-1 was precoated onto a microplate. Standards and samples were pipetted into the wells and any Cav-1 present was bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for Cav-1 was added to the wells. After washing, avidin-conjugated HRP was added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and color develops in proportion to the amount of Cav-1 bound in the initial step. The color development was stopped and the absorbance was measured at 450 nm using a microplate reader.

### 2.5 | Measurement of eNOS levels

The PathScan<sup>®</sup> Phospho-eNOS (Ser1177, stimulate site of eNOS, Cell Signaling Technology, Japan) sandwich ELISA Kit is a solid phase sandwich ELISA that detects endogenous levels of phosphorylated eNOS at Ser1177. We prepared 30 mg of myocardium (LAD and LCX area) in 1 mL of cell lysis buffer, homogenized it for 2 minutes at 25 Hz, sonicated it for detection of nuclear localized proteins, and spinned it for 10 minutes 14 000 rpm. This assay employs the quantitative sandwich enzyme immunoassay technique. A rabbit monoclonal antibody for phospho-eNOS (Ser1177) was coated onto the microwells. After incubation with cell lysates, phospho-eNOS protein was captured by the coated antibody. Following extensive washing, a mouse monoclonal antibody for eNOS was added to

detect captured eNOS protein phosphorylated at Ser1177. HRP-linked streptavidin was then used to recognize the bound detection antibody. HRP substrate, TMB, was added to develop color. The magnitude of the absorbance for this developed color is proportional to the quantity of eNOS phosphorylated at Ser1177. Absorbance was then immediately measured at 450 nm.

## 2.6 | Measurements of caspase-3

The PathScan® Caspase-3 Activity Assay Kit is a fluorescent assay that detects the activity of caspase-3 in cell lysates (Cell Signaling Technology, Japan). During the assay, activated caspase-3 cleaves this substrate between DEVD and AMC, generating highly fluorescent AMC that can be detected using a fluorescence reader with excitation at 380 nm and emission between 420-460 nm.

## 2.7 | Measurement of plasma BH<sub>4</sub>

Measurement of BH<sub>4</sub> samples was performed with HPLC as previously reported.<sup>18</sup> The mobile phase was 0.1 M sodium phosphate buffer (pH 3.0) containing 5% (v/v) methanol, 3 mmol/L sodium octyl sulphate, 0.1 mmol/L disodium EDTA, and 0.1 mmol/L ascorbic acid (to prevent oxidation). The flow-rate was maintained at 1.0 mL/min. The mobile phase was filtered through a 0.45- $\mu$ m membrane filter (Millipore) and then degassed under vacuum before use. The measurement of plasma samples was also performed.

## 2.8 | Experimental protocols

After the surgical procedure and instrumentation, at least 30 minutes was allowed for stabilization while monitoring hemodynamic variables (Figure 1). To evaluate the role of EDH, we examined vasodilating responses of coronary microvessels in response to bradykinin (100 ng/kg/min, 0.5 mL/min, LCX, IC) before and after myocardial occlusion (15 minutes, Occlusion<sub>15</sub> and 85 minutes,

Occlusion<sub>85</sub>) by proximal LAD occlusion (90 minutes) under the following three conditions (n = 6 each) with cyclooxygenase blockade (ibuprofen, 12.5 mg/kg iv, an inhibitor of the synthesis of vasodilator prostaglandins to evaluate the role of EDH and NO without PGI<sub>2</sub>) before the onset of the ischemia; (i) control condition, (ii) DM (alloxan 40 mg/kg iv, 1 week prior to the experiment), (iii) DM+ K<sub>Ca</sub> channel blockade (apamin 1  $\mu$ mol/L, an inhibitor of small-conductance K<sub>Ca</sub> channels + charybdotoxin 100 nmol/min, an inhibitor of large and intermediate-conductance K<sub>Ca</sub> channels) + L-NMMA (NOS inhibitor, 2  $\mu$ mol/min, IC) before the onset of the ischemia. In the DM group, the dogs were injected with alloxan monohydrate. Only dogs with blood glucose >200 mg/dL (fasted for at least 16 hours) on day 7 were included in the DM group as previously described.<sup>19</sup>

## 2.9 | Drugs

All drugs were obtained from Sigma-Aldrich Chemical Co. (Japan) and were diluted in a physiological saline immediately before use.

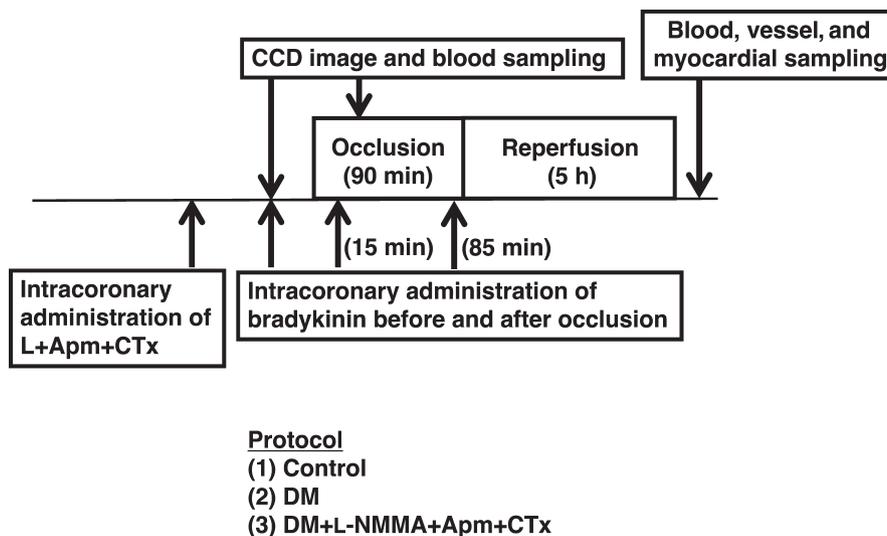
## 2.10 | Statistical analysis

Results are expressed as means  $\pm$  SEM. Vascular responses of coronary small artery and arterioles to bradykinin were analyzed by one-way analysis of variance followed by Scheffe's post hoc test for multiple comparisons. The power of this analysis is greater than that of using the animal as the unit of analysis, giving smaller P values. The criterion for statistical significance was set at  $P < .05$ .

## 3 | RESULTS

### 3.1 | Plasma glucose, hemodynamics, and blood gases during coronary occlusion

Diabetic dogs had elevated plasma glucose levels compared with controls (Table 1).



**FIGURE 1** Experimental protocols. Apm, CCD, CTx

**TABLE 1** Glucose and hemodynamics before and after coronary occlusion during administration of bradykinin

	Control (n = 6)		DM (n = 6)	DM+L-NMMA+Apm+CTx (n = 6)	
Glucose (mg/dL)	96 ± 3		308 ± 32 <sup>##</sup>	262 ± 30 <sup>##</sup>	
MBP(mm Hg)	Baseline	Bradykinin2 min	CBF(mL/min)	Baseline	Bradykinin2 min
Bradykinin before occlusion					
Control(n = 6)	85 ± 6	78 ± 2	Control(n = 6)	21 ± 3	56 ± 9 <sup>*††</sup>
DM(n = 6)	91 ± 6	88 ± 6	DM(n = 6)	22 ± 2	56 ± 2 <sup>**††</sup>
DM+L-NMMA+Apm+CTx(n = 6)	76 ± 9	67 ± 8	DM+L-NMMA+Apm+CTx(n = 6)	17 ± 3	20 ± 3
Bradykinin after occlusion 15 min					
Control(n = 6)	80 ± 4	72 ± 4	Control(n = 6)	22 ± 3	51 ± 7 <sup>*</sup>
DM(n = 6)	70 ± 9	63 ± 9	DM(n = 6)	18 ± 3	40 ± 8 <sup>*</sup>
DM+L-NMMA+Apm+CTx(n = 6)	74 ± 10	70 ± 8	DM+L-NMMA+Apm+CTx(n = 6)	23 ± 2	30 ± 4
Bradykinin after occlusion 85 min					
Control(n = 6)	79 ± 7	76 ± 6	Control(n = 6)	19 ± 3	47 ± 8 <sup>*</sup>
DM(n = 6)	70 ± 7	67 ± 7	DM(n = 6)	25 ± 6	40 ± 4 <sup>*</sup>
DM+L-NMMA+Apm+CTx(n = 6)	79 ± 9	76 ± 10	DM+L-NMMA+Apm+CTx(n = 6)	21 ± 3	25 ± 4

Plasma glucose levels were elevated in DM compared with control. CBF in control and DM before and after LAD occlusion during administration of bradykinin significantly increased compared with baseline, and those in DM+L-NMMA+Apm+CTx did not increase compared with baseline. Results are shown as mean±SEM.

MBP, mean blood pressure; apamin, Apm; charybdotoxin, CTx; CBF, coronary blood flow; Bradykinin 2 min, 2 min after bradykinin administration.

\* $P < .05$ , \*\* $P < .01$  vs baseline; <sup>##</sup> $P < .01$  vs control; <sup>††</sup> $P < .01$  vs DM+L-NMMA+Apm+CTx (Bradykinin 2 min).

Throughout the experiments, MBP at baseline was constant and comparable, and  $pO_2$ ,  $pCO_2$ , and pH were maintained within the physiological ranges (pH 7.35-7.45,  $pO_2 > 70$  mm Hg, and  $pCO_2$  25-40 mm Hg). MBP at baseline did not differ significantly before and after myocardial ischemia (Table 1). CBF (LCX) in control and DM before and after LAD occlusion during administration of bradykinin significantly increased compared with baseline, and those in DM with L-NMMA+K<sub>Ca</sub> channel blockade (apamin+charybdotoxin) did not increase compared with baseline (Table 1).

### 3.2 | Changes in caveolin-1 after coronary occlusion

Caveolin-1 in the LAD area was greater in coronary microvessels than in conduit arteries in the control group ( $P < .01$ ) and was unaltered in the DM group (Figure 2).

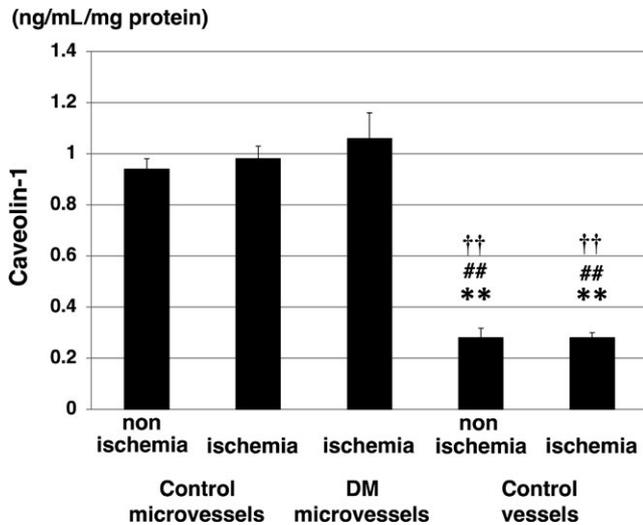
### 3.3 | Effects of EDH on vasodilatation and eNOS activity during coronary occlusion

NO-mediated coronary collateral vasodilatation of small arteries ( $4.8 \pm 1.0\%$ ) to bradykinin significantly decreased after coronary occlusion at 15 minutes (Occlusion<sub>15</sub>) and 85 min (Occlusion<sub>85</sub>) (Occlusion<sub>15</sub>,  $-5.8 \pm 1.1\%$  and Occlusion<sub>85</sub>,  $-5.6 \pm 3.0\%$ , both  $P < .05$ ) and were restored by compensation of EDH in arterioles (Occlusion<sub>15</sub>,  $6.5 \pm 0.8\%$ ,  $P < .05$  and Occlusion<sub>85</sub>,  $5.4 \pm 1.1\%$ ,  $P < .01$  vs before ischemia  $20.5 \pm 3.8\%$ ) (Figure 3A,B). NO-mediated coronary vasodilatation of small coronary arteries to bradykinin did not

increase in DM (baseline  $-0.4 \pm 2.0\%$ , Oc<sub>15</sub>,  $-4.1 \pm 1.0\%$  and Oc<sub>85</sub>,  $-4.4 \pm 1.0\%$ ) and DM+L-NMMA+K<sub>Ca</sub> channel blockade (baseline  $-0.3 \pm 2.3\%$ , Occlusion<sub>15</sub>,  $-4.4 \pm 1.4\%$  and Occlusion<sub>85</sub>,  $-4.2 \pm 1.5\%$ ) before and after ischemia (Figure 3A) with the decrease in eNOS phosphorylation at Ser1177 (Figure 4) compared with baseline of control and were restored by compensation of EDH after ischemia in arterioles of DM (Occlusion<sub>15</sub>,  $3.8 \pm 0.9\%$ ,  $P < .01$  and Occlusion<sub>85</sub>,  $3.1 \pm 1.2\%$ ,  $P < .01$  vs before ischemia) and were decreased after DM+L-NMMA+Apm+CTx (Occlusion<sub>15</sub>,  $-4.6 \pm 2.2\%$ , and Occlusion<sub>85</sub>,  $-3.4 \pm 2.8\%$ , vs before ischemia of control,  $P < .01$  and DM,  $P < .05$ ) (Figure 3B). The ratio of vasodilatation of arterioles to that of small coronary arteries in control and DM was significantly greater in arterioles than in small arteries (Figure 5A). The ratio of EDH-mediated arteriolar vasodilatations before coronary occlusion in the control group was significantly greater than that after occlusion in the Occlusion<sub>85</sub> of control ( $P < .05$ ), the Occlusion<sub>15</sub> ( $P < .05$ ), and Occlusion<sub>85</sub> ( $P < .01$ ) of DM group, and the before and after coronary occlusion in the DM+L-NMMA+Apm+CTx group (each  $P < .01$ ). The Occlusion<sub>15</sub> of control was not significantly decreased from baseline of control and was significantly decreased in Occlusion<sub>15</sub> in the DM+L-NMMA+Apm+CTx group. (Figure 5B).

### 3.4 | Changes in caspase 3 and BH<sub>4</sub> after coronary occlusion

Caspase-3 as one of the indices of an apoptosis in ischemic area (LAD) of the control and DM groups significantly increased compared with



**FIGURE 2** Distribution of caveolin-1 level in coronary arteries and microvessels. Caveolin-1 was comparable among control microvessels (nonischemia), control microvessels (ischemia), and DM microvessels (ischemia) and were greater than in control vessels (nonischemia) and control vessels (ischemia). Number of coronary arteries per animal used was 6 of 6 for each group and number of myocardium as microvessel of animals used was 6 of 6 for each group.  $**P < .01$  vs control microvessels (nonischemia),  $###P < .01$  vs control microvessels (ischemia), and  $\dagger\dagger P < .01$  vs DM microvessels (ischemia)

nonischemic area (LCX) of the control group (Figure 6). Plasma levels of  $BH_4$  in the DM group ( $1.5 \pm 0.3$  ng/mL) did not differ significantly from the control group ( $2.1 \pm 0.5$  ng/mL).

## 4 | DISCUSSION

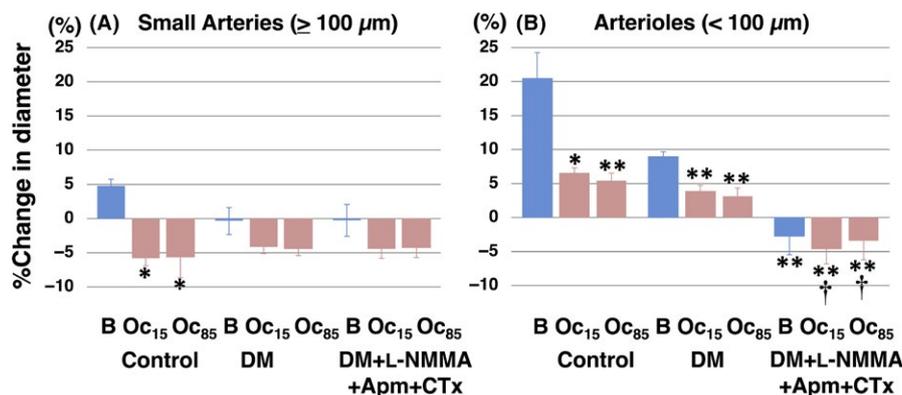
The major findings of the present study are as follows; (i) Cav-1 was significantly greater in EDH-mediated coronary microvessels than

in NO-mediated conduit arteries in the control group, while Cav-1 was maintained in coronary arterioles after ischemia in DM; (ii) NO-mediated vasodilations of small coronary arteries were impaired during coronary occlusion and were compensated by EDH of arterioles in the control and DM groups in vivo, because EDH plays a dominant role in endothelium-dependent vasodilatation to bradykinin.<sup>20</sup> These results indicate that EDH is involved in ischemia-induced coronary collateral vasodilatation in DM in vivo and that there are substantial compensatory interactions between NO and EDH. To the best of our knowledge, this is the first study that demonstrates the important protective roles of EDH and NO against myocardial ischemia and DM in vivo.

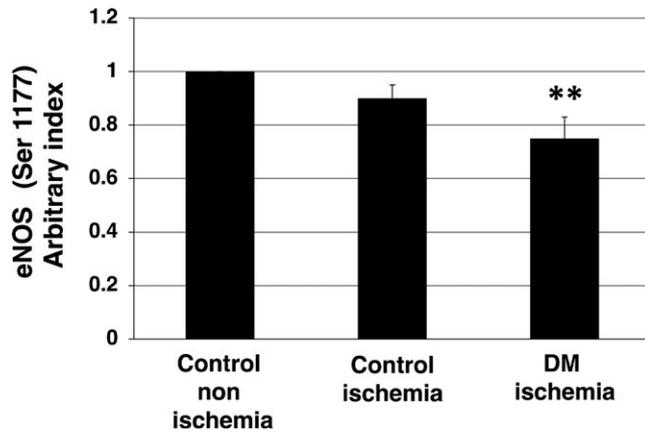
### 4.1 | Roles of caveolin-1, EDH, and NO during myocardial ischemia and DM in vasodilatation of coronary collateral arteries and arterioles

The extent of Cav-1 was significantly greater in mesenteric artery than in the aorta and that EDH-mediated relaxations and hyperpolarizations were inhibited in mesenteric arteries of Cav-1<sup>-/-</sup> mice.<sup>10</sup> Excessive endothelium-derived NO with reduced EDH impairs cardiovascular homeostasis in Cav-1<sup>-/-</sup> and eNOS-Tg mice in vivo and that endothelial Cav-1 plays crucial roles for EDH production in mouse mesenteric arteries.<sup>11</sup> In the present study, Cav-1 levels were greater in coronary microvessels than in coronary conduit arteries in the control group, a consistent finding with the previous studies.<sup>10,11,21</sup> We have previously demonstrated that the importance of EDH increases as the vessel size decreases,<sup>12-14</sup> which could be explained by the present finding on the distribution of Cav-1.

The extent of eNOS phosphorylation at Ser1177 was significantly reduced in mesenteric arteries than in the aorta in accordance with the vascular Cav-1 level.<sup>10</sup> The amount of Cav-1 was increased, while eNOS phosphorylations and plasma NO levels were decreased



**FIGURE 3** Endothelium-dependent coronary vasodilatation to bradykinin before and after coronary I/R injury in dogs in vivo. A, In the small arteries ( $\geq 100$   $\mu$ m), endothelium-dependent vasodilations to bradykinin were significantly decreased after occlusion of control, while they did not increase before occlusion (Oc 15 and 85) of DM and decreased after occlusion of DM and DM+L-NMMA+Apm+CTx compared with before occlusion of control. B, In the arterioles ( $< 100$   $\mu$ m), the responses were restored by compensation of EDH (Oc 15 and 85 of control and DM) and the remaining vasodilations were diminished in B, Oc 15 and 85 of DM+L-NMMA+Apm+CTx. Number of coronary native collateral arterioles per animals used was 6 of 6 for each group.  $*P < .05$  and  $**P < .01$  vs B of control;  $\dagger P < .05$  vs B of DM. B, before occlusion; Oc<sub>15</sub>, 15 min after occlusion; Oc<sub>85</sub>, 85 min after occlusion; Apm, CTx



**FIGURE 4** Extent of phosphorylation of eNOS (Ser 1177) in the myocardium after ischemia. The extent of eNOS phosphorylation at Ser 1177 was significantly suppressed in DM (ischemia) compared with control (nonischemia). Number of animals used was 5 for each group. \*\* $P < .01$  vs control (nonischemia)

in DM rats fed with high-salt diet.<sup>22</sup> In the present study, eNOS activity, as evaluated by the extent of phosphorylation at an important stimulatory site, was low, while Cav-1 level was maintained in coronary arterioles after ischemia in DM. Thus, the present findings support the notion for the compensatory interactions between eNOS and Cav-1.

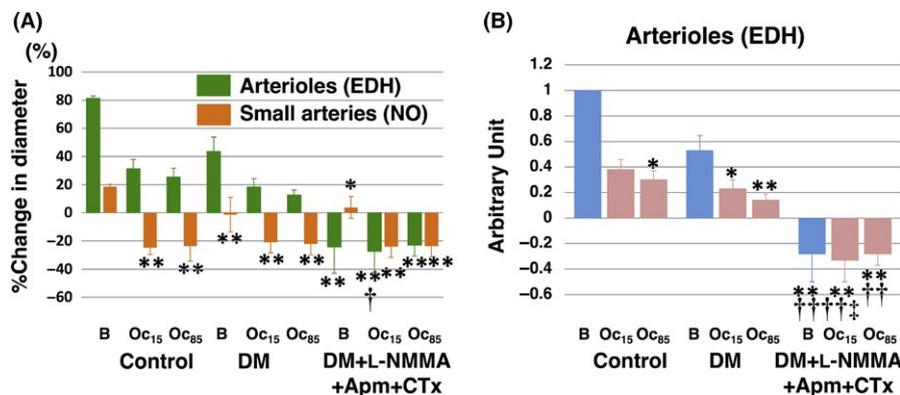
In the present study, although apoptosis level increased in ischemia under control conditions and DM compared with baseline under control conditions, there was no significant difference in the apoptosis level between the control and DM groups. Endothelium-derived  $H_2O_2$  plays an important compensatory role in the presence of impaired NO-mediated vasodilatation after MI.<sup>13,14</sup> Apoptosis was

further increased in diabetic MI mice compared with nondiabetic MI mice.<sup>23</sup> This discrepancy may be due to the difference in vascular beds examined (small arteries vs arterioles) and/or animal species used (dogs vs mice).

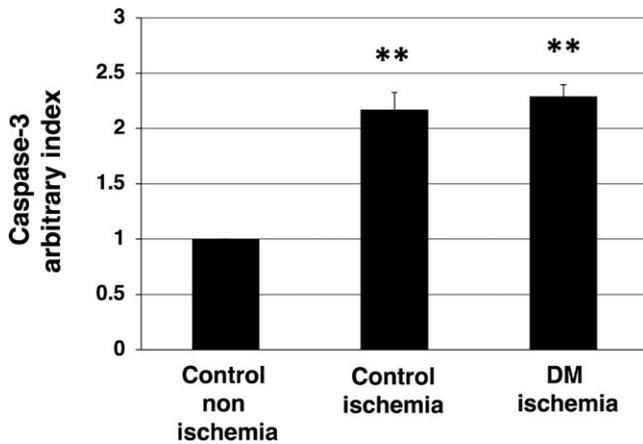
Several studies demonstrated that  $BH_4$ -dependent eNOS uncoupling may be an important mechanism for endothelial dysfunction and increased superoxide production in cardiovascular diseases with coronary risk factors.<sup>24,25</sup> Endothelial production of superoxide anions from eNOS is noted under physiological conditions in the absence of  $BH_4$  deficiency.<sup>26</sup> In the present study, plasma levels of  $BH_4$  did not differ between the control and DM groups. These results suggest that pathological eNOS uncoupling is not involved in the production of superoxide anions in normal and DM in the present study.

#### 4.2 | Relative contribution of the endothelium to enhanced EDH-mediated responses in coronary collateral microvessels

Coronary artery tone is regulated by the interactions among several vasodilators, including NO and EDH.<sup>12,13</sup> EDH-mediated relaxations to acetylcholine were reduced in streptozotocin-induced diabetic mice.<sup>16</sup> NO-mediated, endothelium-dependent coronary vasodilation is significantly reduced in type 2 diabetic (db/db) mice and EDH-mediated vasodilation compensated the loss of NO, a consistent finding with the previous report that EDH plays an important role diabetes-induced coronary endothelial dysfunction.<sup>27</sup> In the present study, endothelium-dependent arteriolar vasodilatation to bradykinin in the control and DM groups before ischemia was maintained and was restored arteriolar vasodilatation during myocardial ischemia, while small arteries constricted in response



**FIGURE 5** Quantitative analysis of the relative contribution of NO and EDH to the endothelium-dependent relaxations to bradykinin. A, The ratio of vasodilatation in arterioles to those in small arteries in the control and the DM groups was significantly greater in arterioles than in small arteries. The ratio is each % change in diameter to bradykinin divided by arteriolar (EDH) + small arterial (NO) vasodilatation in baseline. B, The ratio of EDH-mediated arteriolar vasodilations before coronary occlusion in the control group was significantly greater than that after occlusion in the control (Oc<sub>85</sub>) and DM group and the before and after coronary occlusion in the DM+L-NMMA+Apm+CTx group. The ratio of EDH-mediated vasodilations before coronary occlusion in the DM group was significantly greater than that after occlusion in the DM+L-NMMA+Apm+CTx group. Compared with the control group, vasodilatation at 15 min after coronary occlusion (Oc<sub>15</sub>) was significantly decreased in the DM+L-NMMA+Apm+CTx group. Arbitrary unit is vs arteriolar vasodilatation to bradykinin in baseline. Number of small arteries and arterioles per animals was 6 of 6 for each group. \* $P < .05$ , \*\* $P < .01$  vs B of control; † $P < .05$ , †† $P < .01$  vs B of DM; ‡ $P < .05$  vs Oc<sub>15</sub> of control. B, before occlusion; Oc<sub>15</sub>, 15 min after occlusion; Oc<sub>85</sub>, 85 min after occlusion; C, Apm, CTx.



**FIGURE 6** Effects of myocardial caspase-3 after myocardial ischemia. The ratio of caspase-3 in control (ischemia) and DM (ischemia) was significantly greater than control (nonischemia). \*\* $P < .01$  vs control (nonischemia)

to bradykinin after ischemia in the control group and did so before and after ischemia in the DM group, and the remaining arteriolar dilation was diminished by  $K_{Ca}$  channel blockade. These present findings further support our notion that NO and EDH play an important compensatory role in coronary autoregulation,<sup>12</sup> myocardial ischemia,<sup>13</sup> and collateral microcirculation<sup>14</sup> in vivo. In the present study, we examined for the first time the relative contribution of the endothelium and vascular smooth muscle cell to the enhanced EDH-mediated responses in canine coronary collateral arterioles in DM.

Endothelium-dependent vasodilations are different depending on agonists (eg, acetylcholine vs bradykinin). EDH plays a dominant role in endothelium-dependent vasodilatation to bradykinin compared with that to acetylcholine using cytochrome P-450 antagonist, miconazole, in vivo in the canine coronary microcirculation.<sup>20,28</sup> In the present study, remaining endothelium-dependent arteriolar vasodilatation to bradykinin before ischemia in the Occlusion<sub>15</sub> of control and DM was diminished by  $K_{Ca}$  channel blockade (apamine+charybdotoxin), suggesting that EDH plays an important compensatory role during the early phase of ischemia. Thus, we consider that EDH plays an important role in endothelium-dependent vasodilatation to bradykinin.

#### 4.3 | Study limitations

Several limitations should be mentioned for the present study. First, in the present study, the duration of elevated blood glucose level was only 1 week. This was because we aimed to maintain the conditions of dogs, as the compensation of endothelial functions by EDH may decrease with progression of DM. Second, in the present study, we did not assess the infarct size of the heart by TTC staining with a ligation of coronary collaterals. Instead, we evaluated caspase-3 in the myocardium as one of an indices of apoptosis to evaluate bradykinin-induced coronary vasodilatation during LAD occlusion without ligation of collateral from LCX.

#### 4.4 | Clinical implications

Coronary endothelial dysfunction with various risk factors is important factor in the pathogenesis of coronary artery disease. The synthesis and action of endothelium-derived NO are impaired under various pathological conditions, such as hypertension, hyperlipidemia, and DM.<sup>18,29</sup> In DM, EDH activities are increased in a compensatory manner with reduced NO activity in pacing-induced metabolic coronary vasodilatation in dogs in vivo.<sup>30</sup> The present results suggest that NO and EDH compensate each other to maintain coronary vasodilatation during myocardial ischemia in DM in vivo.

### 5 | PERSPECTIVE

Present study provides evidence that EDH, in cooperation with NO and Cav-1, plays an important cardioprotective role in DM-induced canine coronary collateral microcirculation. The importance of EDH increases as the vessel size decreases, which could be explained by the present findings on the distribution of Cav-1, and the present findings support the notion for the compensatory interactions among EDH, eNOS, and Cav-1. These findings may have important clinical implications as EDH-mediated mechanisms substantially contribute to endothelium-dependent vasodilatation during myocardial ischemia in vivo.

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