Erythropoietin enhances hydrogen peroxide-mediated dilatation of canine coronary collateral arterioles during myocardial ischemia in dogs in vivo

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[1]*Department of Medical Engineering and Systems Cardiology, Kawasaki Medical School, Kurashiki;* [2]*Division of Nephrology and Rheumatology, Department of Internal Medicine, Kawasaki Medical School, Kurashiki; and* [3]*Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan*

Yada T, Shimokawa H, Hiramatsu O, Satoh M, Kashihara N, Takaki A, Goto M, Ogasawara Y, Kajiya F. Erythropoietin enhances hydrogen peroxide-mediated dilatation of canine coronary collateral arterioles during myocardial ischemia in dogs in vivo. *Am J Physiol Heart Circ Physiol* 299: H1928–H1935, 2010. First published September 24, 2010; doi:10.1152/ajpheart.00331.2010.—We have previously demonstrated that endothelium-derived hydrogen peroxide (H_2O_2) plays an important role in the canine coronary microcirculation as an endothelium-derived hyperpolarizing factor in vivo. However, it remains to be examined whether endogenous H_2O_2 is involved in the dilatation of coronary collaterals during myocardial ischemia in vivo and, if so, whether erythropoietin (EPO) enhances the responses. Canine subepicardial native collateral small arteries (CSA; ≥ 100 μm) and arterioles (CA; <100 μm) were observed using an intravital microscope. Experiments were performed after left anterior descending coronary artery ischemia (90 min) under the following eight conditions ($n = 5$ each): control, EPO, EPO+catalase, EPO+*N*-monomethyl-L-arginine (L-NMMA), EPO+L-NMMA+catalase, EPO+L-NMMA+iberiotoxin [Ca^{2+}-activated K^+ (K_{Ca}) channel blocker], EPO+L-NMMA+apamin+charybdotoxin (K_{Ca} channel blocker), and EPO+wortmannin (phosphatidylinositol 3-kinase inhibitor). Myocardial ischemia caused significant vasodilatation in CA but not in CSA under control conditions, which was significantly decreased by catalase in CA. After EPO, the vasodilatation was significantly increased in both sizes of arteries and was significantly decreased by catalase. The enhancing effect of EPO was reduced by L-NMMA but not by catalase in CSA and was reduced by L-NMMA+catalase in CA, where the greater inhibitory effects were noted with L-NMMA+catalase, L-NMMA+iberiotoxin, L-NMMA+apamin+charybdotoxin, or wortmannin. EPO significantly ameliorated ischemia-induced impairment of myocardial Akt phosphorylation, which was abolished by L-NMMA+catalase or wortmannin. EPO also ameliorated oxidative stress and myocardial injury, as assessed by plasma 8-hydroxydeoxyguanosine and troponin-T, respectively. These results indicate that EPO enhances H_2O_2-mediated dilatation of coronary collateral arterioles during myocardial ischemia in dogs in vivo.

coronary microcirculation; endothelium-derived hyperpolarizing factors

RECENT STUDIES SUGGEST that endothelium-derived hyperpolarizing factor (EDHF) plays an important role in microcirculation. Several candidates for EDHF have been proposed, including cytochrome *P*-450 metabolites (2, 5), endothelium-derived K^+ (8), and electrical communications through gap junctions between endothelial cells and vascular smooth muscle cells (38). It was reported that endothelium-derived hydrogen peroxide (H_2O_2) is a primary EDHF in mesenteric arteries of mice and humans (15, 16), where endothelial Cu,Zn-SOD plays an important role as an EDHF synthase (21, 22). EDHF/H_2O_2-mediated responses are critically dependent on the endothelial nitric oxide synthase (eNOS) system in mouse mesenteric arteries (37), and H_2O_2 acts as an EDHF through activation of Ca^{2+}-activated K^+ (K_{Ca}) channels (18, 19). We subsequently confirmed that endothelium-derived H_2O_2 plays an important regulatory role in microcirculation in vivo, including coronary autoregulation (48) and metabolic coronary vasodilatation (50) in canine coronary microcirculation and reactive hyperemia in mouse mesenteric microcirculation (51).

Coronary collateral microcirculation plays an important role under both physiological and pathological conditions. The dilation of native collaterals after coronary artery occlusion is mediated by activation of ATP-sensitive K^+ (K_{ATP}) channels (13). Endothelial dysfunction has been implicated in the reduced dilatation of coronary collateral microcirculation (9, 30), and exercise training has been shown to improve vasodilatation of coronary collateral vessels mediated by H_2O_2 (39). H_2O_2 also may be involved at the collateral level (39). However, it remains to be examined whether H_2O_2 also plays an important role in vasodilatation of coronary collateral microvessels during myocardial ischemia in vivo.

Recombinant human erythropoietin (EPO) exerts anti-inflammatory (27) and antioxidative stress effects (14) in rat and mouse hearts, respectively, and enhances eNOS activity and Akt phosphorylation in the rabbit cerebral artery (28). Furthermore, it has been reported that phosphatidylinositol 3-kinase (PI3-kinase) activity is required for EPO-mediated recovery of myocardial contractile dysfunction and inhibition of myocardial apoptosis caused by myocardial ischemia-reperfusion ex vivo (3). Inhibition of both sarcolemmal and mitochondrial K_{Ca} channels completely abrogates the protective effects of EPO in isolated rabbit hearts (32), whereas H_2O_2 causes endothelial NO release mediated through PI3-kinase/Akt-dependent eNOS phosphorylation at serine 1179 (40). EPO enhances EDHF-mediated vasodilatation with *N*-monomethyl-L-arginine (L-NAME) and indomethacin in rat middle cerebral arteries (31). The role of H_2O_2 as an intermediate factor between EPO and activation of K_{Ca} channels and PI3-kinase/Akt-mediated eNOS activation remains to be examined.

However, it remains to be examined whether EPO acutely improves coronary collateral vasodilatation during myocardial ischemia via PI3-kinase/Akt-dependent pathway in vivo, and if so, whether such beneficial effects of EPO are mediated by endogenous H_2O_2. Thus the present study was designed to

examine *1*) the role of H_2O_2 in coronary collateral dilatation during myocardial ischemia, *2*) the effects of EPO as a dose of clinical use on the ischemia-induced dilatation of coronary collaterals, and *3*) the involvement of PI3-kinase/Akt-mediated eNOS activation and K_{Ca} channels in the responses of coronary collateral circulation.

MATERIALS AND METHODS

Experimental protocols were reviewed and approved by the Animal Research Committee of Kawasaki Medical School and conducted according to the "Guide for the Care and Use of Laboratory Animals" of Kawasaki Medical School.

Animal Preparation

Mongrel dogs (*n* = 70, 15–25 kg) of either sex were anesthetized with ketamine (10 mg/kg im) and pentobarbital sodium (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 left ventricular pressure (LVP) were continuously monitored with a catheter (SPC-784A; Millar, Houston, TX). Blood flow of the left anterior descending coronary artery (LAD) was continuously monitored by a transonic flow probe (T206; Transonic Systems, Ithaca, NY).

Measurements of Coronary Diameter by Intravital Microscope

We continuously monitored coronary vascular responses using an intravital microscope (VMS 1210; Nihon Kohden, Tokyo, Japan) with a needle-probe (magnification, ×200–300) in vivo, as previously described (47). Briefly, we gently placed the needle-probe on subepicardial native collateral microvessels. Native coronary collateral small arteries (CSA; ≥100 μm) and arterioles (CA; <100 μm) were visually traced between LAD and left circumflex coronary arteries (LCX) with an injection of indocyanine green. Native collaterals were identified only when there was a continuous segment of vessel visible between the LAD and LCX (13). When a clear vascular image was obtained, end-diastolic vascular images were taken at a rate of 30 pictures/s (47).

Plasma Levels of 8-Hydroxydeoxyguanosine

Blood samples were obtained at baseline and 5 h of reperfusion from the catheterized coronary sinus and the ascending aorta. Measurement of plasma 8-hydroxydeoxyguanosine (8-OHdG) levels, as a marker of oxidative stress (14), was performed with high-performance liquid chromatography (HPLC) coupled to an electrochemical detector (ECD-700; Eicom, Kyoto, Japan), as previously described (12). After centrifugation at 15,000 *g* for 10 min, an aliquot of the supernatant was injected into the HPLC-ECD system. The voltage of the guard cell was set at 550 mV. The analytical column was a EIKOMPAK (CA-50DS; Eicom) equipped with a guard column, and the mobile phase was sodium phosphate buffer (0.1 mol/l, pH 6.0, 97%), including methanol (3%) and sodium L-octanesulfonate (100 mg/l). The flow rate was 0.23 ml/min, and the column temperature was set at 30°C. Quantification of 8-OHdG was performed by comparison of the peak area with that of authentic 8-OHdG (12).

Western Blot Analysis

Total proteins from myocardial samples were extracted, and samples (40 μg/lane) were subjected to immunoblotting analysis using antibodies against eNOS (Abcam, Tokyo, Japan), total Akt, phospho-Akt (p-Akt; Cell Signaling Technology, Tokyo, Japan), p-Akt/total Akt as a marker of Akt activity (29), and actin (Sigma Aldrich Japan, Tokyo, Japan). Signals were detected using the enhanced chemiluminescence system (Amersham Biosciences). The integrated density (density 3 area) of the bands was quantified using NIH Image analysis software (29).

Measurements of Troponin-T Levels

Measurement for plasma levels of troponin-T was performed using the ECLIA method (SRL, Tokyo, Japan). Blood samples were obtained at baseline and 5 h of reperfusion from the catheterized coronary sinus.

Experimental Protocols

After the surgical procedure and instrumentation, at least 30 min were allowed for stabilization while hemodynamic variables were continuously monitored. The following protocols were examined.

Protocol 1: Effects of H_2O_2 as an EDHF on native coronary collateral vasodilatation during myocardial ischemia. To evaluate the role of H_2O_2, we examined vasodilating responses of native coronary collaterals before and after myocardial ischemia (15 and 85 min) by proximal LAD occlusion under the following four conditions (*n* = 5 each) with cyclooxygenase blockade (ibuprofen, 12.5 mg/kg iv) before the onset of the ischemia: *1*) control condition, *2*) L-NMMA (a NOS inhibitor; 2 μmol/min ic for 20 min), *3*) catalase (an enzyme that dismutates H_2O_2 into water and oxygen; 40,000 U/kg iv and 240,000 units·kg^{-1}·min^{-1} ic for 10 min), and *4*) L-NMMA+catalase.

Protocol 2: Effects of two doses of EPO on native coronary collateral vasodilatation during myocardial ischemia. To test the vasodilating effects of EPO, we intravenously administered a low-dose (L-EPO; 100 IU/kg, *n* = 5) and a high-dose of EPO (H-EPO; 1,000 IU/kg, *n* = 5) as a single injection. The vasodilating responses of native coronary collaterals were examined during myocardial ischemia (15 and 85 min) by proximal LAD occlusion.

Protocol 3: Effects of EPO on vasodilatation of native coronary collaterals during myocardial ischemia. The vasodilating responses of native coronary collaterals were examined before and during myocardial ischemia (15 and 85 min) by proximal LAD occlusion under the following eight conditions (*n* = 5 each) with cyclooxygenase blockade (ibuprofen intravenously) before the onset of myocardial ischemia to evaluate the role of EPO in combination of H_2O_2 and NO without PGI$_2$ in a different set of animals: *1*) control condition, *2*) EPO (1,000 IU/kg iv), *3*) EPO+catalase, *4*) EPO+L-NMMA, *5*) EPO+L-NMMA+catalase, *6*) EPO+L-NMMA+iberiotoxin (IBTX, a blocker of large-conductance K_{Ca} channels; 1.0 g·kg^{-1}·min^{-1} ic for 20 min), *7*) EPO+L-NMMA+apamin (a blocker of small-conductance K_{Ca} channels; 1 μmol/min)+charybdotoxin (CTX, a blocker of large- and intermediate-conductance K_{Ca} channels; 100 nmol/min ic for 20 min), and *8*) EPO+wortmannin (an inhibitor of PI3-kinase; 1.5 μg·kg^{-1}·min^{-1} ic for 30 min).

The inhibitors were given at 30 min before induction of myocardial ischemia. We administered EPO before the ischemia as a single intravenous injection. After administration of EPO, inhibitor solution (catalase, L-NMMA, or wortmannin) was infused into the LAD at a rate of 0.5 ml/min. In the case of combined infusion (L-NMMA+catalase, L-NMMA+IBTX, or L-NMMA+apamin+CTX) after administration of EPO, L-NMMA infusion was first started, followed by infusion of catalase, IBTX, or apamin+CTX for 5 min after initiation of L-NMMA infusion. Blood samples were obtained at baseline and 5 h of reperfusion from the catheterized coronary sinus (Fig. 1).

Drugs

All drugs were obtained from Sigma Chemical and were diluted in a physiological saline immediately before use. EPO was provided by Chugai Pharmaceutical (Tokyo, Japan).

Statistical Analysis

Results are means ± SE. Vascular responses of coronary collaterals were analyzed using one-way analysis of variance followed by Scheffé's post hoc test for multiple comparisons (see Figs. 2 and 3). Differences in the vasodilatation of subepicardial coronary microvessels before and during myocardial ischemia were examined by multiple regres-
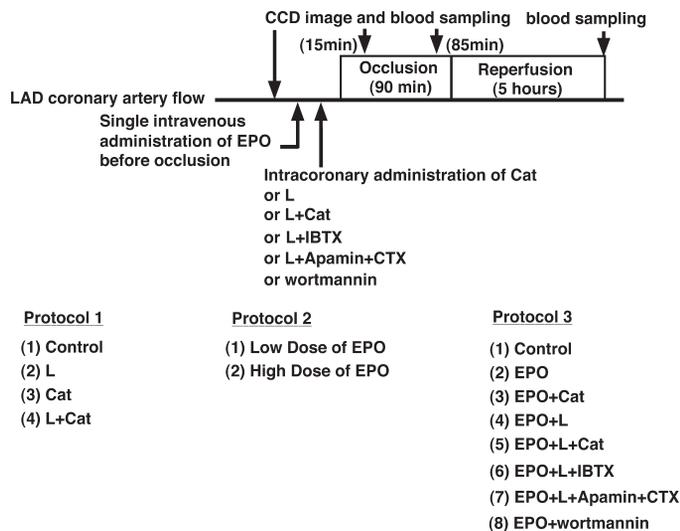
Fig. 1. Experimental protocols. CCD, charge-coupled device; LAD, left anterior descending coronary artery; EPO, erythropoietin; Cat, catalase; L, *N*-monomethyl-L-arginine (L-NMMA); IBTX, iberiotoxin; CTX, charybdotoxin.

sion analysis using a model in which the change in coronary diameter was set as a dependent variable (*y*) and vascular size as an explanatory variable (*x*), while the statuses of control and other inhibitors were set as dummy variables (*D1*, *D2*) in the following equation: $y = a0 + a1x + a2D1 + a3D2$, where *a0* through *a3* are partial regression coefficients (48). Significance tests were made as simultaneous tests for slope and intercept differences. Pairwise comparisons against control were made

without adjustment for multiple comparisons. The vessel was the unit of analysis without correction for correlated observations. 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 at $P < 0.05$.

RESULTS

Hemodynamics and Blood Gases during Myocardial Ischemia

Throughout the experiments, mean aortic pressure and heart rate at baseline were constant and comparable, and P_{O_2}, P_{CO_2}, and pH were maintained within the physiological ranges (P_{O_2} >70 mmHg, P_{CO_2}, 25–40 mmHg, and pH 7.35–7.45). Hemodynamic variables at baseline did not differ significantly before and after myocardial ischemia (Table 1).

Effects of H₂O₂ as an EDHF on Vasodilatation of Native Coronary Collaterals During Myocardial Ischemia

Under control conditions, ischemia induced a dilatation of CA but not of CSA at 15 and 85 min (Fig. 2, *A–D*). L-NMMA and L-NMMA+catalase, but not catalase alone, constricted CSA, and catalase and L-NMMA+catalase reduced the dilatation of CA (Fig. 2, *A–D*).

Effects of EPO on Coronary Native Collateral Vasodilatation During Myocardial Ischemia

H-EPO (1,000 IU/kg iv) but not L-EPO (100 IU/kg iv) enhanced the vasodilatation of both sizes of arteries (CSA at 15

Table 1. *Hemodynamics during coronary ischemia in dogs*

	n	Baseline	Ischemia 15	Ischemia 85
Heart rate, beats/min				
Control	5	142 ± 8	140 ± 10	144 ± 10
EPO	5	150 ± 5	150 ± 6	153 ± 5
EPO+Cat	5	136 ± 12	135 ± 10	136 ± 10
EPO+L	5	159 ± 9	158 ± 8	157 ± 7
EPO+L+Cat	5	136 ± 12	135 ± 10	136 ± 10
EPO+L+IBTX	5	135 ± 15	135 ± 10	133 ± 9
EPO+L+apamin+CTX	5	135 ± 15	136 ± 10	135 ± 11
EPO+Wort	5	138 ± 10	135 ± 10	133 ± 10
Mean blood pressure, mmHg				
Control	5	89 ± 5	90 ± 5	93 ± 5
EPO	5	102 ± 6	100 ± 10	100 ± 10
EPO+Cat	5	105 ± 7	100 ± 9	101 ± 10
EPO+L	5	108 ± 7	105 ± 10	104 ± 12
EPO+L+Cat	5	100 ± 8	94 ± 11	90 ± 12
EPO+L+IBTX	5	95 ± 5	105 ± 5	103 ± 5
EPO+L+apamin+CTX	5	105 ± 5	104 ± 5	99 ± 5
EPO+Wort	5	96 ± 6	101 ± 7	105 ± 6
Coronary blood flow, ml/min				
Control	5	25 ± 4		
EPO	5	23 ± 2		
EPO+Cat	5	18 ± 4		
EPO+L	5	24 ± 4		
EPO+L+Cat	5	18 ± 1		
EPO+L+IBTX	5	21 ± 3		
EPO+L+apamin+CTX	5	25 ± 2		
EPO+Wort	5	20 ± 2		

Values are means ± SE during coronary ischemia at 15 (Ischemia 15) and 85 min (Ischemia 85); *n* = no. of animals. EPO, erythropoietin; Cat, catalase; L, N-monomethyl-l-arginine; IBTX, iberiotoxin; CTX, charybdotoxin; Wort, wortmannin.
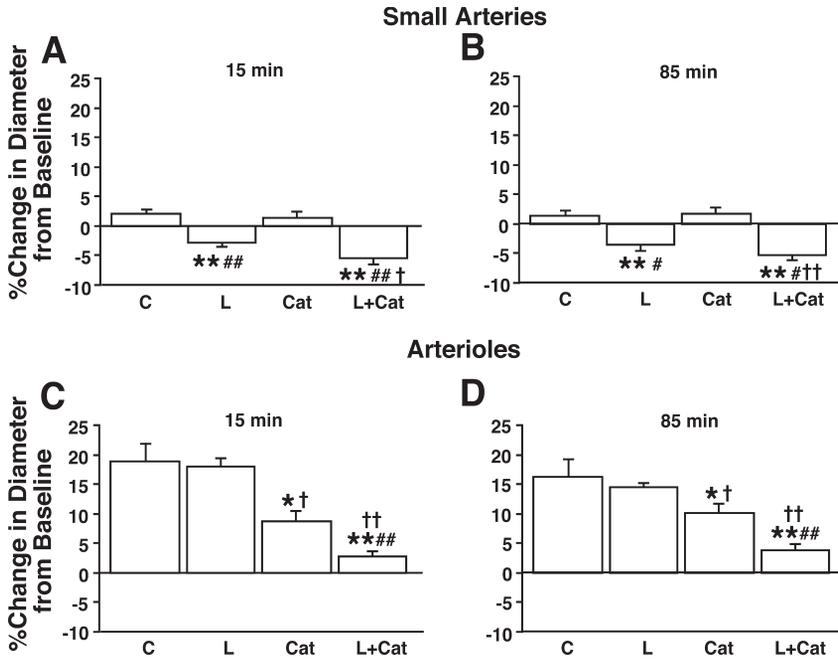
Small Arteries

Fig. 2. Effects of nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF)/H2O2 on coronary native collateral vasodilatation in small arteries (*A* and *B*) and arterioles (*C* and *D*) during ischemia at 15 (*A* and *C*) and 85 min (*B* and *D*). The number of coronary native collateral arterioles per animals used was 5/5 for each group. C, control. ★*P* < 0.05; ★★*P* < 0.01 vs. control. #*P* < 0.05; ##*P* < 0.01 vs. catalase. ††*P* < 0.01 vs. L-NMMA.

min: L-EPO, 3.9 ± 1% vs. H-EPO, 7.8 ± 1%, *P* < 0.01; CSA at 85 min: L-EPO, 3.0 ± 1% vs. H-EPO, 6.0 ± 1%, *P* < 0.01; CA at 15 min: L-EPO, 19.8 ± 3% vs. H-EPO, 26.5 ± 5%, *P* < 0.01; CA at 85 min: L-EPO, 18.3 ± 3 vs. H-EPO, 22.0 ± 4%, *P* < 0.05). EPO (1,000 IU/kg iv) significantly enhanced the vasodilatation compared with control in both sizes of arteries (Fig. 3, *A–D*). Catalase after EPO administration did not affect the vasodilatation of CSA but significantly decreased the response of CA (Fig. 3, *A–D*). In CA, L-NMMA significantly reduced the dilatation after EPO administration at 85 min but not at 15 min. L-NMMA after EPO administration constricted coronary collateral vessels compared with EPO in CSA, whereas residual vasodilatation was noted in CA (Fig. 3, *A–D*). L-NMMA+catalase after EPO further decreased the arteriolar vasodilatation and constricted CSA compared with control or EPO (Fig. 3, *A–D*). The effect of the combination of EPO+L-NMMA+catalase, EPO+L-NMMA+IBTX, EPO+L-NMMA+apamin+CTX, or EPO+wortmannin was greater than that of EPO+L-NMMA in CSA or that of EPO+catalase in CA.
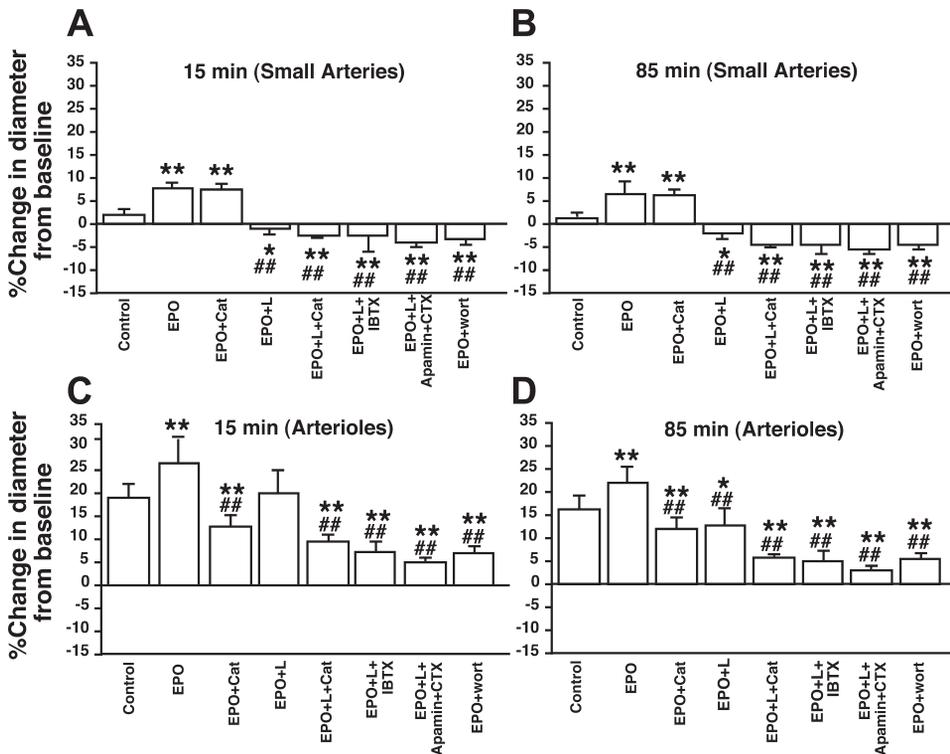

Fig. 3. Effects of EPO on coronary native collateral vasodilatation in small arteries (*A* and *B*) and arterioles (*C* and *D*) during ischemia at 15 (*A* and *C*) and 85 min (*B* and *D*). The number of small arteries and arterioles per animals used was 5/5 for each group. Wort, wortmannin. ★*P* < 0.05; ★★*P* < 0.01 vs. control. ##*P* < 0.01 vs. EPO.
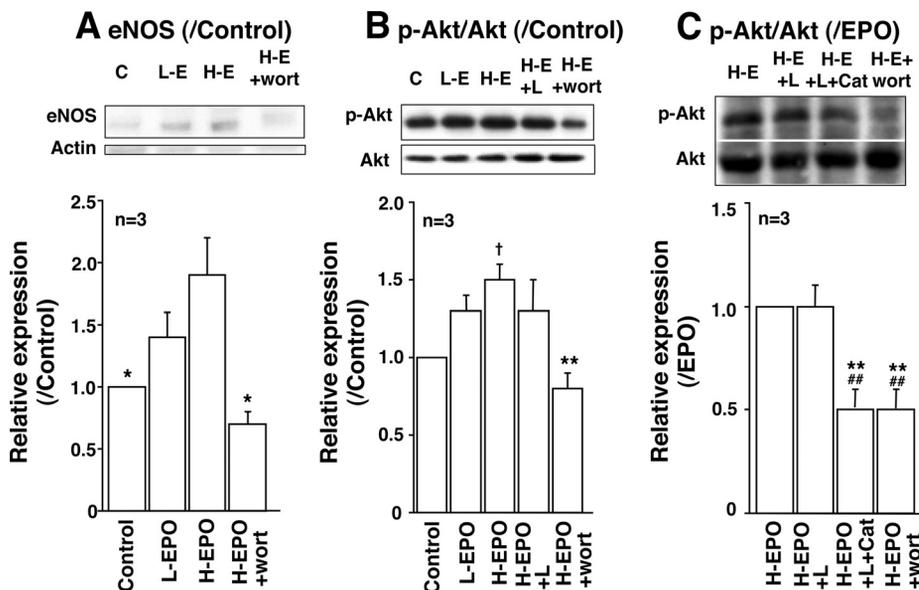
Fig. 4. Effects of EPO on myocardial endothelial NO synthase (eNOS) and Akt phosphorylation relative to control (*A* and *B*, respectively) and Akt activity relative to eNOS (*C*) after myocardial ischemia. The number of animals used was 3 for eNOS and p-Akt. L-E, low-dose EPO (L-EPO), H-E, high-dose EPO (H-EPO). †*P* < 0.05 vs. control. ★*P* < 0.05; ★★*P* < 0.01 vs. H-EPO. ##*P* < 0.01 vs. H-EPO+L-NMMA.

Effects of EPO on eNOS and Akt Activity After Myocardial Ischemia

Myocardial expression of eNOS and Akt activity (as expressed by the ratio phospho-Akt/total Akt) in the myocardium of the ischemic LAD area of the control group were increased by EPO (Fig. 4, *A* and *B*). These beneficial effects of EPO were abolished by wortmannin (Fig. 4, *A* and *B*). L-NMMA after EPO had no effects on myocardial Akt activity, whereas addition of catalase after L-NMMA or wortmannin significantly reduced the Akt activity (Fig. 4*C*).

Effects of EPO on Plasma Levels of 8-OHdG After Myocardial Ischemia

In the control group, plasma levels of 8-OHdG in the coronary sinus were significantly increased at 5 h after myocardial ischemia-reperfusion compared with baseline (nonischemia), and the increase in oxidative stress responses was sig-

nificantly ameliorated in the EPO groups, whereas it was increased by catalase or L-NMMA alone and further deteriorated by L-NMMA+catalase, L-NMMA+IBTX, L-NMMA+apamin+CTX, or wortmannin alone in EPO (Fig. 5). In contrast, there was no significant difference in the plasma levels of 8-OHdG in the ascending aorta among the groups (data not shown).

Effect of EPO on Ischemia-Induced Troponin-T in Coronary Sinus

The plasma level of troponin-T, a specific marker of myocardial injury, at 5 h after reperfusion was ~1 ng/ml (Fig. 6). EPO significantly decreased myocardial troponin-T release, and this beneficial effect of EPO on myocardial injury was abolished by catalase or L-NMMA alone and further deteriorated by L-NMMA+catalase, L-NMMA+IBTX, L-NMMA+apamin+CTX, or wortmannin alone in EPO (Fig. 6).
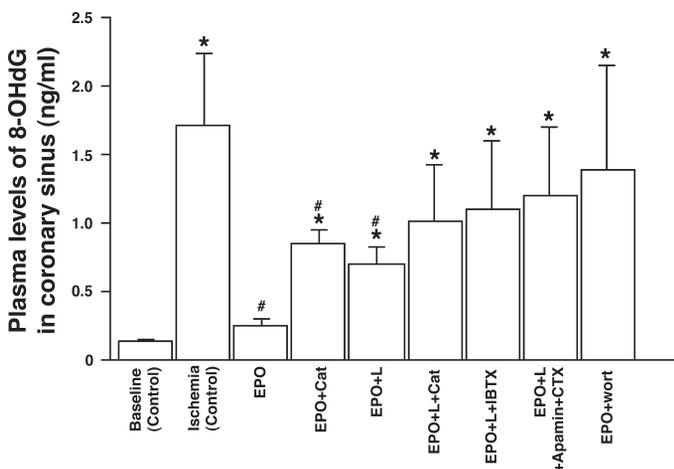

Fig. 5. Effects of EPO on plasma levels of 8-hydroxydeoxyguanosine (8-OHdG) in the coronary sinus after myocardial ischemia. The number of animals used was 5 for each group. ★*P* < 0.05 vs. baseline (control). #*P* < 0.05 vs. ischemia (control).
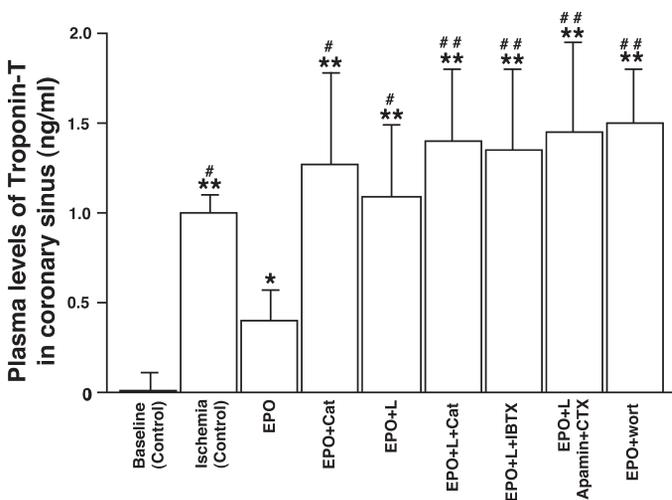

Fig. 6. Effects of EPO on plasma levels of troponin-T release after myocardial ischemia. The number of animals used was 5 for each group. ★*P* < 0.05; ★★*P* < 0.01 vs. baseline (control). # *P* < 0.05; ## *P* < 0.01 vs. EPO.

DISCUSSION

The major findings of the present study are that acute coronary occlusion causes a significant vasodilatation of native canine coronary collateral arterioles under control conditions in vivo, where endogenous H_2O_2 is substantially involved, and that exogenous EPO, when administered just before acute coronary occlusion, further enhances the ischemia-induced coronary collateral vasodilatation via PI3-kinase/Akt-dependent pathway in vivo. After EPO administration, the plasma levels of 8-OHdG (a marker of oxidative stress) and troponin-T (a marker of myocardial injury) in the coronary sinus were significantly decreased in the coronary circulation, suggesting acute anti-inflammatory and cardioprotective effects of EPO. To the best of our knowledge, this is the first report that demonstrates the important protective roles of endogenous H_2O_2 and EPO against myocardial ischemia in vivo.

Validations of Experimental Model and Methodology

Based on previous reports (11, 23, 36, 48), we chose the present doses of EPO, L-NMMA, catalase, IBTX, apamin, CTX, and wortmannin to examine their effects on coronary collateral vasodilatation during myocardial ischemia. We have previously confirmed the validity of the methods that we used in the present study (47).

Role of Endogenous H₂O₂ During Myocardial Ischemia in Vivo

Previous studies have indicated that H_2O_2 as an endogenous EDHF is crucial in the mechanism of vasodilatation in vascular adaptation to chronic coronary occlusion. These include the involvement of H_2O_2 in the training-induced restoration of endothelium-dependent dilatation of collateral vessels in dogs (39) and increased plasma H_2O_2 levels during exercise (45) and in cases of impaired NO-mediated dilation of coronary microvessels in patients with coronary artery disease (20). In the present study, acute coronary occlusion caused a significant vasodilatation of native coronary collaterals under control conditions that was suppressed by catalase in CA but not in CSA, that was also suppressed by L-NMMA in CSA but not in CA, and that was further inhibited by L-NMMA+catalase in both sizes of arteries. Only CA dilated during myocardial ischemia, for which H_2O_2 and NO are the key mediators. It was reported that coronary arteriolar dilatation during exercise was less sensitive to L-NAME but was highly sensitive to catalase (39). These results indicate that endothelium-derived H_2O_2 plays an important compensatory role in the presence of impaired NO-mediated vasodilatation.

EDHF/H_2O_2 causes K_{Ca} channel-mediated vasodilatation, especially at microvessels, for which several mechanisms have been proposed, including cellular acidosis (33), increase in intracellular Ca^{2+} concentration after ischemia (34), and H_2O_2 production by inflammatory cells (6). Recently, Takaki et al. (36) demonstrated that endothelial oxidases other than NOS are not involved in EDHF/H_2O_2 responses in mice, suggesting a specific link between eNOS systems and EDHF responses under physiological conditions (36). H_2O_2 is a relatively stable reactive oxygen intermediate and plays an important role in coronary autoregulation (48) and protection against myocardial ischemia-reperfusion injury (49).

Role of EPO During Myocardial Ischemia In Vivo

In the present study, EPO enhanced the dilatation of native coronary collaterals during myocardial ischemia, and this effect was associated with a beneficial reduction in myocardial injury and oxidative stress as evidenced by the decrease in plasma levels of troponin-T and coronary sinus levels of 8-OHdG. It was recently demonstrated that EPO reduces troponin-T and oxidative stress (8-OHdG, glutathione, GSH) in rats (17) and mice (14). Furthermore, EPO is known to exert protective effects against myocardial ischemia-reperfusion injury in terms of infarct size (11, 26), LV enlargement and myocyte apoptosis (41), and LV remodeling and capillary density (42). In patients with acute coronary syndrome, circulating EPO levels were significantly decreased during the hyperacute phase (6 h) (1). Thus, during myocardial ischemia, EPO may have inhibitory effects on functional myocardial loss.

Mechanism For EPO-mediated Enhancement of Coronary Collateral Vasodilatation

In the present study, we further examined the mechanisms for the EPO-mediated enhancement of coronary collateral vasodilatation. We noted the importance of K_{Ca} channels given that paxilline, a blocker of both sarcolemmal and mitochondrial K_{Ca} channels, completely abolished the protective effects of EPO in the isolated rabbit heart and that glibenclamide, a blocker of K_{ATP} channels, also abolished the responses, suggesting an involvement of K_{ATP} channel activation (32). Indeed, activation of sarcolemmal K_{Ca} and K_{ATP} channels may act to reduce calcium influx into the cell during ischemia (32), and activation of mitochondrial K_{Ca} channels exerts cardioprotective effects by improving myocardial energetics (46). It was recently reported that EPO enhances EDHF-mediated vasodilation by uridine 5′-triphosphate (an endothelium-dependent vasodilator) with L-NAME and indomethacin in rat middle cerebral arteries (31). In the present study, L-NMMA alone or catalase alone after EPO did not completely abolish the arteriolar vasodilatation, whereas L-NMMA+catalase, L-NMMA+IBTX, or L-NMMA+apamin+CTX after EPO markedly attenuated the residual vasodilatation in vivo, as did wortmannin, indicating that H_2O_2 exerts important vasodilating effects during myocardial ischemia with EPO administration in the canine coronary microcirculation in vivo. This finding is consistent with our previous findings that NO and H_2O_2 play an important compensatory role in coronary autoregulation and protection against ischemia-reperfusion injury in the canine coronary microcirculation in vivo (48, 49).

Table 2. *Baseline coronary collateral diameter before coronary ischemia*

	Small Artery, μm	Arteriole, μm
Control	115 ± 5	78 ± 5
EPO	116 ± 6	75 ± 8
EPO+Cat	114 ± 6	76 ± 6
EPO+L	122 ± 8	78 ± 4
EPO+L+Cat	116 ± 4	67 ± 6
EPO+L+IBTX	123 ± 7	72 ± 6
EPO+L+apamin+CTX	116 ± 8	79 ± 4
EPO+Wort	104 ± 2	74 ± 9

Values are means ± SE; n = no. of blood vessels (n = 5 each).

In the present study, L-NMMA had no effects at 15 min of ischemia, whereas wortmannin showed significant inhibitory effects. Thus it is conceivable that the initial enhancement of arteriolar dilatation by EPO is mediated by H_2O_2/EDHF, whereas the later persistent dilatation is mediated by eNOS activation by H_2O_2 through a PI3-kinase/Akt-dependent mechanism. The effect of the combination of L-NMMA+catalase (or +IBTX, +apamin+CTX, or +wortmannin) was higher than that of L-NMMA in CSA or that of catalase alone in CA. These findings indicate the involvement of the two parallel pathways (K_{Ca} channels and PI3-kinase/eNOS). It was reported that PI3-kinase activity is required for EPO to improve contractile dysfunction and inhibit myocyte apoptosis induced in a rat model of myocardial ischemia-reperfusion (3). On the other hand, H_2O_2 caused endothelial NO release from bovine and porcine aortic endothelial cells mediated in a PI3-kinase/Akt-dependent manner with eNOS phosphorylation at serine 1179 (40). In the present study, eNOS protein expression and Akt phosphorylation in the ischemic LAD area were significantly decreased in the control group compared with the EPO group. Although L-NMMA alone had no effects, L-NMMA+catalase significantly decreased Akt phosphorylation to the same level with wortmannin. These results indicate that EPO improves eNOS protein expression and Akt phosphorylation, at least in part, through activation of PI3-kinase/Akt and EDHF/H_2O_2 pathways.

Limitations of the Study

Several limitations should be mentioned for the present study. First, we only measured the diameter of native coronary collaterals on the subepicardial surface, and the responses of intramyocardial coronary microvessels may be different (13). We have previously demonstrated that vascular pulsation is greater in subendocardial arterioles compared with subepicardial arterioles (47); however, we cannot maintain the intravital microscope probe for a long time. Thus the response of subepicardial collaterals may not represent the response of intramyocardial microvascular collaterals. Second, in the present study, we did not assess the infarct size of the heart by triphenyltetrazolium chloride staining with a ligation of coronary collaterals. Instead, we evaluated troponin-T in the coronary sinus as an index of myocardial injury to evaluate collateral vasodilatation during LAD occlusion without ligation of collateral.

Clinical Implications and Conclusions

Myocardial ischemia impairs endothelial vasodilator function (10, 25, 43). The hematopoietic cytokine EPO and its receptor are present not only in blood vessels but also in the heart (4), especially in cardiomyocytes and endothelial cells (7, 44). After myocardial infarction, exogenous EPO improves cardiac function and reduces LV remodeling in rabbits, mice, and humans (24, 26, 35). It has been suggested that a high dose of EPO (1,000 IU/kg), the same dose used in the present study, increases capillary density and regional myocardial blood flow in the ischemic region in dogs in vivo (11). In the present study, the EPO treatment during myocardial ischemia acutely exerted various cardioprotective effects in terms of coronary collateral vasodilatation, oxidative stress, and Akt phosphorylation. Thus the present study indicates that H_2O_2 is an endog-

enous mediator for dilatation of canine coronary collateral arterioles during myocardial ischemia and that exogenous EPO exerts its beneficial effect against myocardial ischemia by enhancing this mechanism. In conclusion, the present study indicates that EPO enhances H_2O_2-mediated dilatation of coronary collateral arterioles during myocardial ischemia in dogs in vivo. (Table 2)

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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