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Cardioprotective role of endogenous hydrogen peroxide during ischemia-reperfusion injury in canine coronary microcirculation in vivo

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Yada, Toyotaka, Hiroaki Shimokawa, Osamu Hiramatsu, Yoshisuke Haruna, Yoshitaka Morita, Naoki Kashihara, Yoshiro Shinozaki, Hidezo Mori, Masami Goto, Yasuo Ogasawara, and Fumihiko Kajiya. Cardioprotective role of endogenous hydrogen peroxide during ischemia-reperfusion injury in canine coronary microcirculation in vivo. *Am J Physiol Heart Circ Physiol* 291: H1138–H1146, 2006. First published April 28, 2006; doi:10.1152/ajpheart.00187.2006.—We have recently demonstrated that endogenous H_2O_2 plays an important role in coronary autoregulation in vivo. However, the role of H_2O_2 during coronary ischemia-reperfusion (I/R) injury remains to be examined. In this study, we examined whether endogenous H_2O_2 also plays a protective role in coronary I/R injury in dogs in vivo. Canine subepicardial small coronary arteries (≥100 μm) and arterioles (<100 μm) were continuously observed by an intravital microscope during coronary I/R (90/60 min) under cyclooxygenase blockade ($n = 50$). Coronary vascular responses to endothelium-dependent vasodilators (ACh) were examined before and after I/R under the following seven conditions: control, nitric oxide (NO) synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA), catalase (a decomposer of H_2O_2), 8-sulfophenyltheophylline (8-SPT, an adenosine receptor blocker), L-NMMA + catalase, L-NMMA + tetraethylammonium (TEA, an inhibitor of large-conductance Ca^{2+}-sensitive potassium channels), and L-NMMA + catalase + 8-SPT. Coronary I/R significantly impaired the coronary vasodilatation to ACh in both sized arteries (both $P < 0.01$); L-NMMA reduced the small arterial vasodilatation (both $P < 0.01$), whereas it increased ($P < 0.05$) the ACh-induced coronary arteriolar vasodilatation associated with fluorescent H_2O_2 production after I/R. Catalase increased the small arterial vasodilatation ($P < 0.01$) associated with fluorescent NO production and increased endothelial NOS expression, whereas it decreased the arteriolar response after I/R ($P < 0.01$). L-NMMA + catalase, L-NMMA + TEA, or L-NMMA + catalase + 8-SPT further decreased the coronary vasodilatation in both sized arteries (both, $P < 0.01$). L-NMMA + catalase, L-NMMA + TEA, and L-NMMA + catalase + 8-SPT significantly increased myocardial infarct area compared with the other four groups (control, L-NMMA, catalase, and 8-SPT; all, $P < 0.01$). These results indicate that endogenous H_2O_2, in cooperation with NO, plays an important cardioprotective role in coronary I/R injury in vivo.

endothelium-derived relaxing factor; myocardial infarction; vascular endothelial function

VASCULAR ENDOTHELIAL CELLS play an important role in maintaining vascular homeostasis by synthesizing and releasing endothelium-derived relaxing factors (EDRFs), including prostacyclin (PGI_2), nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF) (6, 9, 26). Endothelial dysfunction is thus characterized by a reduction in the activity of PGI_2, NO, and EDHF, thereby enhancing vasoconstrictor responses mediated by endothelin, serotonin, and thrombin (26). Endothelial injury secondary to myocardial ischemia-reperfusion (I/R) decreases the production and activity of EDRFs in acute myocardial infarction (18).

Among the three different EDRFs, the roles of PGI_2 and NO have been extensively investigated (6, 9, 26). Regarding EDHF, since the first reports on its existence (6, 9), several candidates for EDHF have been proposed, including cytochrome *P*-450 metabolites (2, 4), endothelium-derived K^+ (7), and electrical communications through gap junctions between endothelial cells and vascular smooth muscle cells (29). Matoba et al. (16, 17) have previously identified that endothelium-derived H_2O_2 is a primary EDHF in mesenteric arteries of mice and humans. Morikawa et al. (21) have recently confirmed that endothelial Cu,Zn-SOD plays an important role as an EDHF synthase in mice. We have subsequently confirmed the importance of H_2O_2 in canine coronary microcirculation during coronary autoregulation with reduced coronary perfusion pressure in vivo (35).

However, it remains to be examined whether H_2O_2 also exerts cardioprotective effects during I/R in the coronary microcirculation in vivo, and if so, whether such effects of H_2O_2 compensate the impaired NO-mediated responses due to I/R injury in vivo. In this study, we tested our hypothesis that H_2O_2 plays an important cardioprotective and compensatory role during coronary I/R injury in dogs in vivo.

METHODS

This study conformed to the Guideline on Animal Experiments of Kawasaki Medical School, and approved by an independent review committee from the same institution, and the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health.

Animal preparation. Anesthetized mongrel dogs (15–25 kg in body wt, $n = 50$) of either sex were ventilated with a ventilator (model VS600, IDC, Pittsburgh, PA). Aortic pressure and left ventricular (LV) pressure were continuously monitored with a catheter (SPC-784A, Millar, TX). The blood flow of the left anterior descending coronary artery (LAD) was continuously measured by a transonic flow probe (T206, Transonic Systems, Ithaca, NY).

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Measurements of coronary diameter by intravital microscope. We continuously monitored coronary vascular responses by an intravital microscope (VMS 1210, Nihon-Kohden, Tokyo) with a needle probe in vivo as previously described (32). We gently placed the needle probe on subepicardial microvessels. When a clear vascular image was obtained, end-diastolic vascular images were taken with 30 pictures/s (32).

Measurements of regional myocardial blood flow. Regional myocardial blood flow was measured by the nonradioactive microsphere (Sekisui Plastic, Tokyo) technique, as previously described (20). Briefly, the microsphere suspension was injected into the left atrium 85 min after the onset of coronary occlusion. Myocardial collateral flow in the apex during suturing of the collateral vessels from the left circumflex artery (LCX) was calculated according to the formula: time flow = tissue counts × (reference flow/reference counts) and was expressed in milliliters per gram per minute (20).

Detection of H_2O_2 and NO production. 2′,7′-Dichlorodihydrofluorescein diacetate (DCF, Molecular Probes, Eugene, OR) and diaminorhodamine-4M AM (DAR, Daiichi Pure Chemicals, Tokyo) were used to detect H_2O_2 and NO production in coronary microvessels without a different NO scavenger (e.g., methylene blue), respectively, as previously described (21). Briefly, fresh and unfixed heart tissue was cut into several blocks and frozen in optimal cutting temperature compound (Tissue-Tek, Sakura Fine Chemical, Tokyo) within a few hours. Fluorescent images of the microvessels were obtained 3 min after application of ACh by using a fluorescence microscope (Olympus BX51, Tokyo) (21). We used different animals for the fluorescent treatment (DCF and DAR) and the 2,3,5-triphenyltetrazolium chloride (TTC) treatment.

Western blotting. Portions of myocardial samples were homogenized in lysis buffer. After centrifugation, the supernatants were used for Western immunoblotting. The proteins were transferred by semidry electroblotting to polyvinylidene difluoride membranes. The blots were then blocked and incubated with horseradish peroxidase-conjugated rabbit anti-endothelial NO synthase (eNOS, dimer form) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-actin antibody (Santa Cruz Biotechnology). The antibody was visualized by using an enhanced chemiluminescence method (ECL; Amersham Biosciences, Tokyo). The integrated density of the bands was quantified by using NIH Image analysis, and the protein expression level of eNOS was normalized to that of actin (24).

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

Coronary vascular responses to endothelium-dependent [ACh, 0.5 and 1.0 μg/kg intracoronary (ic)] and -independent [sodium nitroprusside (SNP), 40 and 80 μg/min ic] vasodilators were examined before ischemia (90 min)-reperfusion (60 min) (I/R). ACh and SNP were continuously and retrogradely infused into the diagonal branch of the LAD by using a syringe pump (STC 525, Terumo, Tokyo). The coronary vascular responses to ACh and SNP were examined for 2 min, and the image of maximal vasodilatation was taken at 2 min of infusion of ACh or SNP.

Coronary vasodilator responses to ACh and SNP were examined before and after coronary ischemia (90 min)-reperfusion (60 min) by proximal LAD occlusion under the following seven conditions with cyclooxygenase blockade (ibuprofen, 12.5 mg/kg iv) to evaluate the role of H_2O_2 and NO without PGI_2 in a different set of animals (Fig. 1); *1*) control conditions, *2*) N^G-monomethyl-L-arginine (L-NMMA) alone (2 μmol/min ic for 20 min), *3*) catalase alone (40,000 U/kg iv and 240,000 $U \cdot kg^{-1} \cdot min^{-1}$ ic for 10 min, an enzyme that dismutates H_2O_2 into water and oxygen), *4*) adenosine receptor blockade alone [8-sulfophenyltheophylline (8-SPT), 25 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ic for 5 min], *5*) catalase plus L-NMMA, *6*) catalase plus tetraethylammonium [TEA, 10 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ic for 10 min, an inhibitor of large-

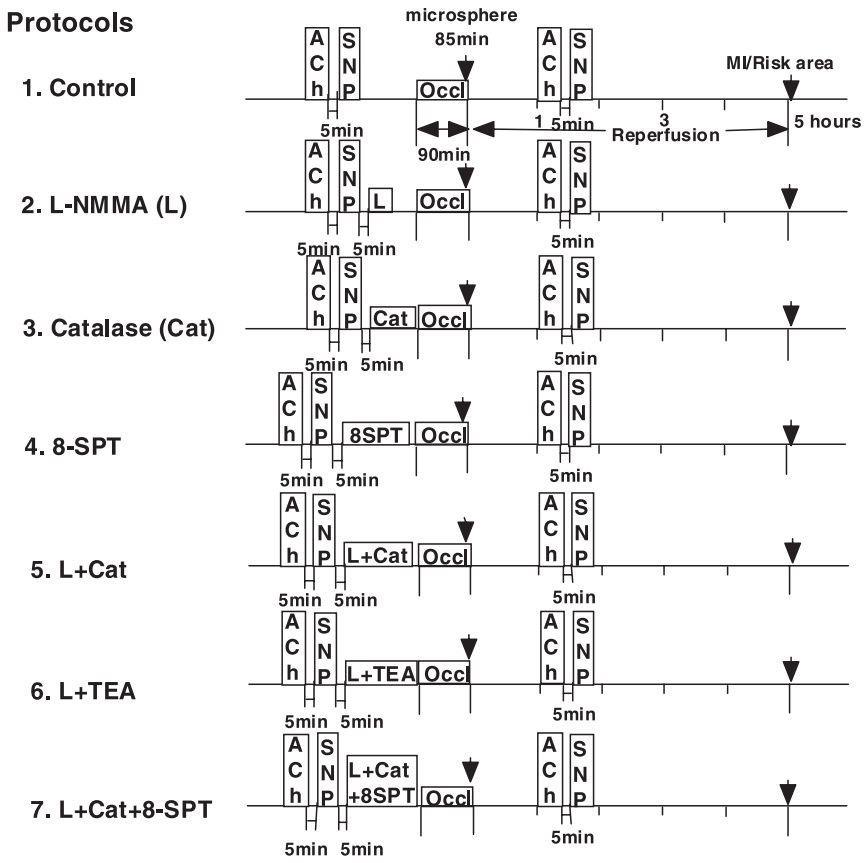

Fig. 1. Experimental protocols. TEA, tetraethylammonium; 8-SPT, 8-sulfophenyltheophylline; ACh, acetylcholine; SNP, sodium nitroprusside; Occl, coronary occlusion; Cat, catalase; L-NMMA (L), N^G-monomethyl-L-arginine; MI, myocardial infarction.

Table 1. *Hemodynamics during coronary ischemia-reperfusion injury in dogs*

		Before I/R				After I/R		
	n	Baseline	ACh	SNP	Ischemia (85 min)	Baseline	ACh	SNP
MBP, mmHg								
Control	5	92±4	91±6	92±5	93±14	92±4	91±5	92±6
L-NMMA	5	97±8	98±7	94±9	92±10	97±7	98±8	95±8
Cat	5	96±8	92±8	94±9	92±9	96±7	96±8	98±6
L-NMMA + Cat	5	94±4	93±9	97±9	95±11	95±8	98±5	94±5
L-NMMA + TEA	5	95±12	93±13	95±14	94±10	91±14	93±15	98±10
L-NMMA + Cat + 8-SPT	5	95±3	96±4	95±3	93±11	96±3	97±4	95±3
Heart rate, beats/min								
Control	5	152±5	155±3	154±3	156±7	156±5	154±5	153±5
L-NMMA	5	157±5	156±5	157±6	158±6	153±5	153±5	153±5
Cat	5	155±4	159±6	158±5	157±6	151±7	155±8	154±8
L-NMMA + Cat	5	156±12	158±13	158±13	154±5	156±13	156±14	159±13
L-NMMA + TEA	5	153±13	154±12	155±11	155±5	150±10	151±11	152±10
L-NMMA + Cat + 8-SPT	5	152±7	155±9	153±3	153±5	152±7	151±6	153±7

Results are expressed as means ± SE; *n* = no. of dogs. I/R, ischemia-reperfusion; MBP, mean blood pressure; Cat, catalase; SNP, sodium nitroprusside; TEA, tetraethylammonium; 8-SPT, sulfophenyltheophylline; L-NMMA, N^G-monomethyl-L-arginine.

conductance Ca^{2+}-sensitive potassium (K_{Ca}) channels], and *7*) catalase plus L-NMMA with 8-SPT (35). These inhibitors were given at 30 min before I/R. An interval between each treatment was 5 min. The basal coronary diameter was defined as that before administration of ACh or SNP either before or after I/R. L-NMMA, catalase, TEA, and 8-SPT were administered alone at 5 min after administration of ACh or SNP. Microspheres were administered at 85 min after the initiation of coronary occlusion. In the combined infusion (L-NMMA + catalase + 8-SPT), catalase solution was infused into the LAD at a rate of 0.5 ml/min at 5 min after infusion of L-NMMA, and then 8-SPT was added into the LAD at 15 min after the initiation of L-NMMA.

After 1 h of reperfusion, coronary vasodilator responses to ACh and SNP were examined.

After 5 h of reperfusion, we reoccluded the LAD and injected Evans blue dye into a systemic vein. Then, myocardial slices (5 μm thick) were incubated in 1% TTC (Sigma) solution to detect the infarct area (36). Different animals were used for fluorescent treatment (DCF and DAR) and TTC treatment.

Drugs. All drugs were obtained from Sigma Chemical and were diluted in a physiological saline immediately before use.

Statistical analysis. Results are expressed as means ± SE. Vascular responses (see Figs. 3*C*, 5*F*, 6*F*, 7, and 9*A*) were analyzed by one-way ANOVA followed by Scheffé's post hoc test for multiple comparisons. Difference in the effects of ACh and SNP on subepicardial coronary microvessels before and after I/R (see Figs. 3, *A* and *B*, 4, and 8, *A* and *B*), and difference between infarct size/risk area and transmural collateral flow in control and other inhibitors (see Fig. 9*B*) were examined by a multiple regression analysis by 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 (D_1, D_2) in the following equation: $y = a_0 + a_1x + a_2D_1 + a_3D_2$, where a_0 through a_3 are partial regression coefficients (36). The criterion for statistical significance was at $P < 0.05$.

RESULTS

Hemodynamics and blood gases during I/R injury. Immediately after reperfusion, coronary blood flow was increased and some arrhythmias occurred; however, those changes returned to the control levels 1 h after reperfusion when we repeated the measurements. Thus, 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 (pH 7.35–7.45, $P_{O_2} > 70$ mmHg, and P_{CO_2} 25–40 mmHg,). Hemodynamic variables at baseline did not significantly change after I/R compared with those before I/R (Tables 1 and 2).

Dose responses to ACh and SNP. ACh (0.5 and 1.0 μg/kg ic) and SNP (40 and 80 μg/min ic) caused coronary vasodilatation in a dose-dependent manner at both small arteries and arterioles (Fig. 2). Then we chose the maximal dose of the vasodilators (ACh, 1.0 μg/kg ic, and SNP, 80 μg/min ic) in the following experiments.

Endothelium-dependent coronary vasodilatation before and after I/R. There was no significant difference in baseline diameter after ACh before I/R among the groups. All inhibitors did not affect resting coronary artery diameter or coronary

Table 2. *Baseline vascular diameter before I/R in response to ACh*

	Small Artery	Arteriole
Control	104–150 μm (120±7, *n* = 7)	37–96 μm (70±6, *n* = 12)
L-NMMA	106–164 μm (131±7, *n* = 8)	36–95 μm (63±5, *n* = 16)
Cat	100–147 μm (121±5, *n* = 10)	28–89 μm (61±6, *n* = 12)
8-SPT	114–162 μm (130±8, *n* = 6)	30–88 μm (60±10, *n* = 5)
L-NMMA + Cat	102–141 μm (118±5, *n* = 8)	34–95 μm (77±4, *n* = 10)
L-NMMA + TEA	105–142 μm (123±6, *n* = 5)	34–95 μm (62±9, *n* = 8)
L-NMMA + Cat + 8-SPT	110–145 μm (128±6, *n* = 5)	38–87 μm (67±7, *n* = 7)

Results are expressed as range (means ± SE); *n* = no. of blood vessels.

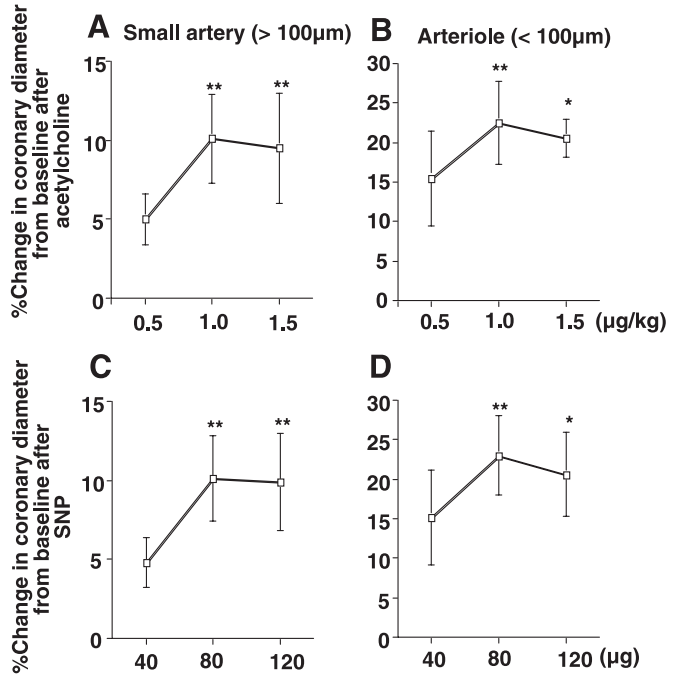

Fig. 2. Dose responses to ACh (*A* and *B*) and SNP (*C* and *D*) before ischemia-reperfusion (I/R). Number of small arteries (*A* and *C*) and arterioles (*B* and *D*) per animals used was 5/5 for each group. $*P < 0.05$, $**P < 0.01$ vs. ACh (0.5 μg/kg) and SNP (40 μg).

blood flow. Under control conditions (before I/R), intracoronary administration of ACh caused a significantly greater coronary vasodilatation in arterioles than in small arteries (Fig. 3, *A* and *B*). Coronary I/R significantly impaired the coronary vasodilatation to ACh in both sized arteries (Figs. 3*A* and 4*A*), and L-NMMA reduced the vasodilatation in small arteries (Figs. 3*A* and 4*B*) but rather increased the response in arterioles compared with control (Figs. 3*B* and 4*A*) after I/R. Catalase and 8-SPT increased the ACh-induced vasodilatation in small arteries (Figs. 3*A* and 4, *C* and *D*) but decreased the response in arterioles (Fig. 3*B*) after I/R. There was no significant difference in coronary blood flow before and after I/R among the control, the L-NMMA, and the catalase group (Fig. 3*C*). L-NMMA + catalase (Figs. 3, *A* and *B*, and 4*E*) or L-NMMA + TEA (Figs. 3, *A* and *B*, and 4*F*) decreased the vasodilatation in both sized arteries (Fig. 3, *A* and *B*) with decrement of coronary blood flow (Fig. 3*C*), and L-NMMA plus catalase with 8-SPT further decreased the vasodilatation in both sized arteries (Figs. 3, *A* and *B*, and 4*G*) compared with other groups (Fig. 3, *A*–*C*).

Detection of H_2O_2 and NO production. Fluorescent microscopy with DCF showed that I/R increased the vascular H_2O_2 production in control LCX (Fig. 5, *B* and *F*) compared with baseline conditions (Fig. 5, *A* and *F*) and decreased the H_2O_2 production in control LAD (Figs. 5, *C* and *F*), which was enhanced by L-NMMA (Fig. 5, *D* and *F*) and was abolished by catalase (Fig. 5, *E* and *F*) in arterioles. By contrast, the production of NO as assessed with DAR fluorescence was increased in control LCX (Fig. 6, *B* and *F*) compared with baseline LCX (Fig. 6, *A* and *F*) after I/R, decreased in control LAD (Fig. 6, *C* and *F*), inhibited by L-NMMA (Fig. 6, *D* and *F*), and was enhanced by catalase (Fig. 6, *E* and *F*) in small arteries.

Western blotting of eNOS protein expression in myocardium. In the control group, expression of eNOS protein in the ischemic LAD area was significantly decreased compared with the nonischemic LCX area (Fig. 7). In the catalase group, this decrease in the eNOS protein expression was inhibited by catalase (Fig. 7).

Endothelium-independent coronary vasodilatation. Coronary vasodilator responses to SNP were comparable under all conditions in both sized arteries (Fig. 8). Those coronary vasodilator responses were resistant to the blockade of NO synthesis with L-NMMA (Fig. 8).

Effect of H_2O_2 on I/R-induced myocardial infarct size. I/R injury caused myocardial infarction, the size of which was ~40% of the LV risk area (Fig. 9*A*). Intracoronary L-NMMA, catalase, or 8-SPT alone did not further increase the I/R-induced infarct size (Fig. 9*A*). By contrast, intracoronary L-NMMA plus catalase or TEA markedly increased the infarct size, and L-NMMA plus catalase with 8-SPT further increased

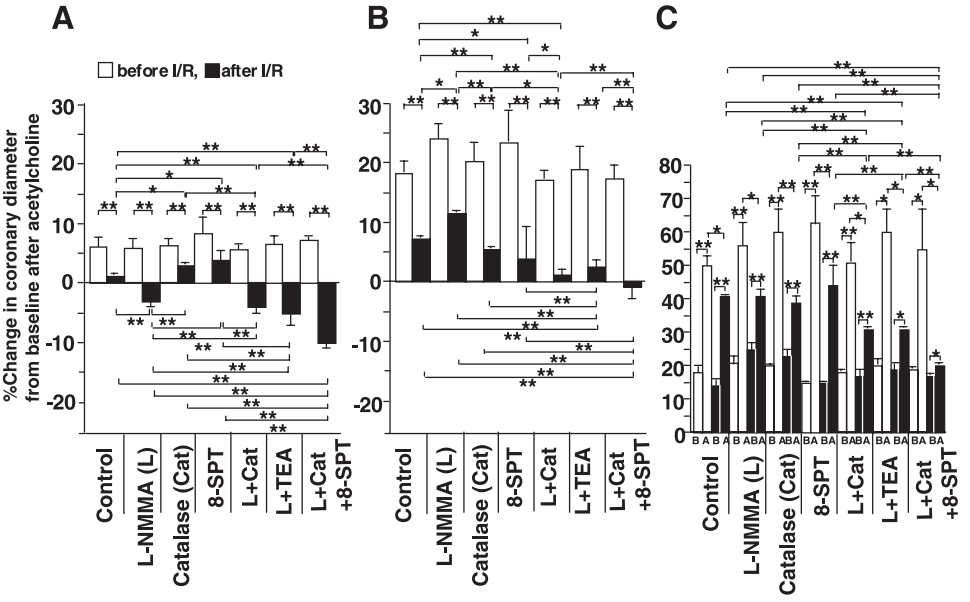

Fig. 3. Endothelium-dependent coronary vasodilatation to ACh before and after coronary I/R injury in dogs in vivo. *A*: small artery (≥100 μm). *B*: arteriole (<100 μm). *C*: coronary blood flow (CBF). No. of small arteries or arterioles per animals (*n*/*n*) used was 7/5 for control, 8/5 for L-NMMA, 10/5 for catalase, 6/5 for 8-SPT, 8/5 for L-NMMA plus catalase, 5/5 for L-NMMA plus TEA, and 5/5 for L-NMMA plus catalase plus 8-SPT in small arteries; and 12/5 for control, 16/5 for L-NMMA, 12/5 for catalase, 5/5 for 8-SPT, 10/5 for L-NMMA plus catalase, 8/5 for L-NMMA plus TEA, and 7/5 for L-NMMA plus catalase plus 8-SPT in arterioles. No. of animals during the measuring CBF used was 5 for each group. B, before ACh; A, after ACh. $*P < 0.05$, $**P < 0.01$.

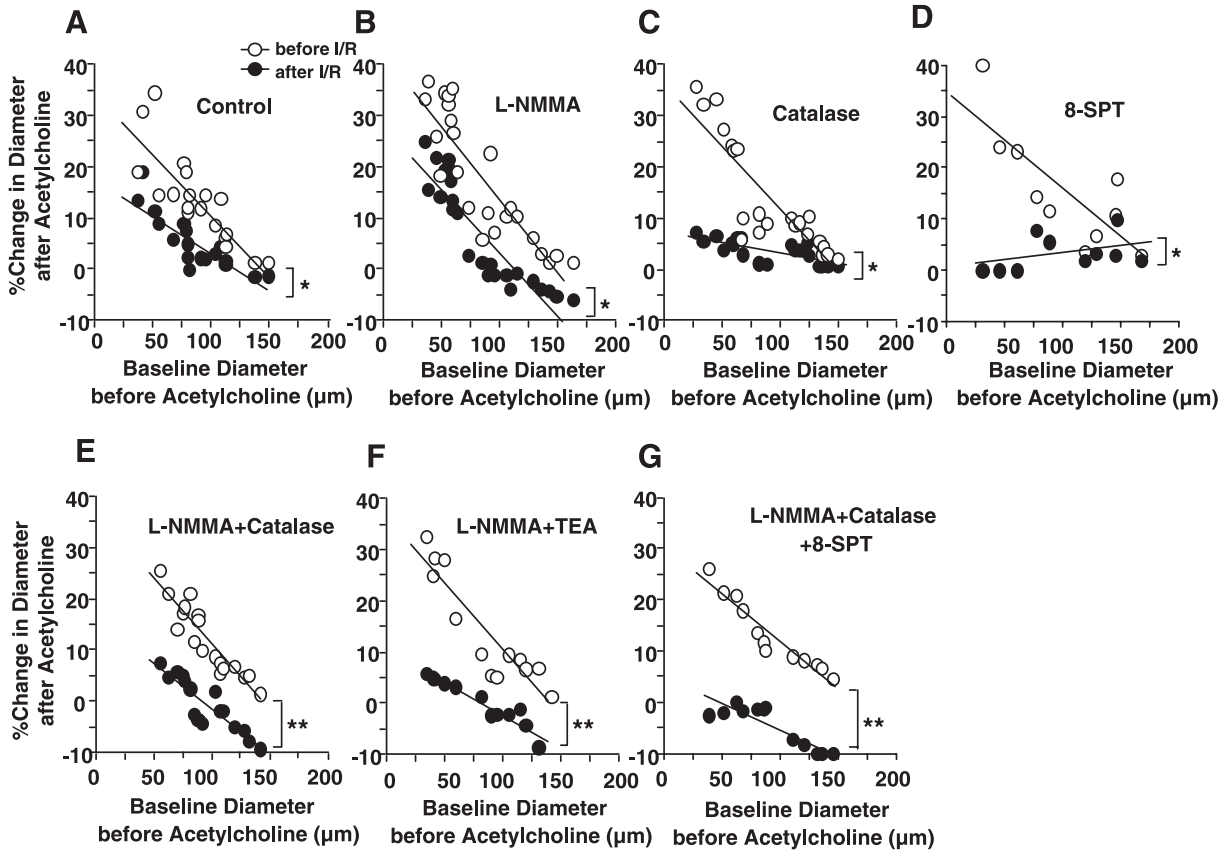

Fig. 4. Percent change in diameter after ACh before and after coronary I/R injury in dogs in vivo. No. of small arteries and arterioles per animals used was 7/5 for control (*A*), 8/5 for L-NMMA (*B*), 10/5 for catalase (*C*), 6/5 for 8-SPT (*D*), 8/5 for L-NMMA plus catalase (*E*), 5/5 for L-NMMA plus TEA (*F*), and 5/5 for L-NMMA plus catalase plus 8-SPT in small arteries (*G*); and 12/5 for control (*A*), 16/5 for L-NMMA (*B*), 12/5 for catalase (*C*), 5/5 for 8-SPT (*D*), 10/5 for L-NMMA plus catalase (*E*), 8/5 for L-NMMA plus TEA (*F*), and 7/5 for L-NMMA plus catalase plus 8-SPT in arterioles (*G*). $*P < 0.05$, $**P < 0.01$.

the infarct size (Fig. 9*A*). In the control group, there was an inverse relation between the infarct size and transmural collateral blood flow measured by microsphere technique ($r = 0.90$, $P < 0.01$). There was no significant difference in the relationship among the control, L-NMMA, and catalase treatment (Fig. 9*B*). L-NMMA plus catalase or TEA significantly shifted the regression line upward compared with the control group (both $P < 0.01$), and L-NMMA plus catalase with 8-SPT further shifted the regression line upward compared with L-NMMA plus catalase or TEA (Fig. 9*B*, both $P < 0.01$).

DISCUSSION

The major finding of the present study is that endogenous H_2O_2, in cooperation with NO, plays an important cardioprotective role during coronary I/R injury as a compensatory mechanism for NO in vivo. To the best of our knowledge, this is the first report that demonstrates the important protective role of endogenous H_2O_2, in cooperation with NO, against coronary I/R injury in vivo.

Validations of experimental model and methodology. On the basis of the previous reports (22, 31), we chose the adequate dose of ACh, SNP, L-NMMA, catalase, TEA, and 8-SPT to examine the effects of endothelium-dependent and -independent coronary vasodilator responses and inhibition of NO synthesis, H_2O_2, K_{Ca} channels, and adenosine receptor, respectively. In addition, on the basis of previous studies and our own (31, 35), we choose the doses of ACh and SNP that cause maximal coronary vasodilatation in dogs in vivo. TEA at low doses is fairly specific for K_{Ca} channel, but at higher doses it may block a number of other K channels. Because several K_{Ca} channels are involved in H_2O_2-mediated responses (26), we selected the nonselective K_{Ca} inhibitor TEA to inhibit all K_{Ca} channels (15). We have previously confirmed the validity of the methods that we used in the present study (32). After 60–90 min of ischemia, ultrastructural damage of coronary endothelium was observed particularly in the subendocardium in the present study, a finding consistent with the previous study (8).

H_2O_2 during coronary I/R in vivo. It was previously reported that relaxations of isolated large canine coronary arteries to exogenous H_2O_2 were partially endothelium dependent (23). Recently, Matoba et al. (16, 17) identified that endothelium-derived H_2O_2 is an EDHF in mouse and human mesenteric microvessels. Subsequently, we (35) and others (19) have confirmed that endogenous H_2O_2 exerts important vasodilator effects in canine coronary microcirculation in vivo and in isolated human coronary microvessels, respectively. It is conceivable that H_2O_2 is produced from superoxide anions derived from several sources in endothelial cells, including eNOS, cyclooxygenase, lipoxygenase, cytochrome *P*-450 enzymes, and NAD(P)H oxidases (16). In the present study, L-NMMA or catalase alone did not com-

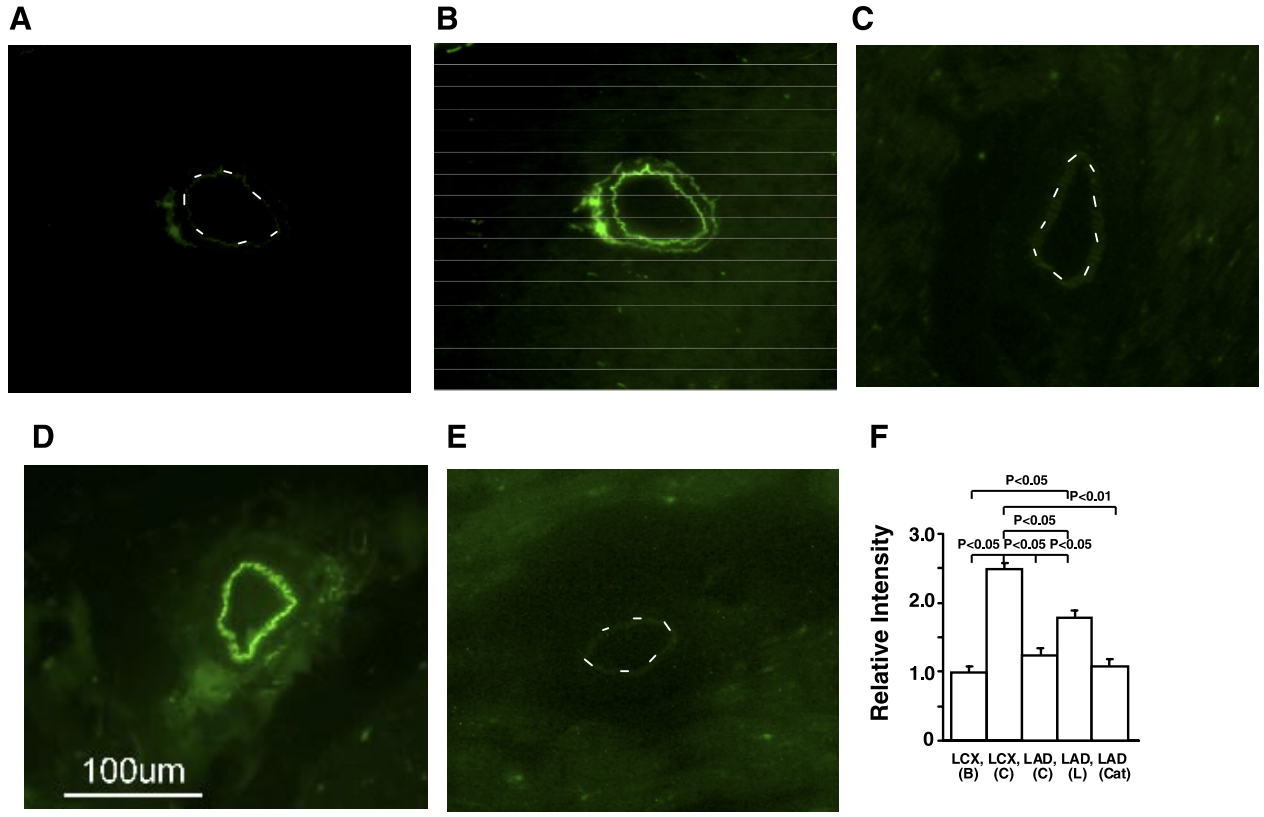

Fig. 5. Detection of H_2O_2 production. *A*: left circumflex artery (LCX; baseline without ACh). *B*: LCX (control). *C*: left anterior descending coronary artery (LAD; control). *D*: LAD (L-NMMA). *E*: LAD (catalase). *F*: fluorescent intensity (B, baseline without ACh; C, control, L, L-NMMA; Cat, catalase). No. of arterioles per animals used was 5/5 for each group. Dashed line, outline of vessels. Bar, 100 μm.

pletely abolish the ACh-induced vasodilatation in both sized arteries, whereas L-NMMA plus catalase markedly attenuated the residual vasodilatation in vivo as did TEA, indicating that H_2O_2 exerts important vasodilator effects during I/R injury in canine coronary microcirculation in vivo (Figs. 3 and 4). Furthermore, in the present study, endogenous H_2O_2-mediated coronary vasodilatation was noted to a greater extent in arterioles than in small arteries (Figs. 3 and 4), confirming the predominant role of H_2O_2 in microvessels and that of NO in relatively large arteries in vivo (25).

Compensatory vasodilator mechanism among H_2O_2, NO, and adenosine. It is well known that coronary vascular tone is regulated by the interactions among several endogenous vasodilators, including NO, H_2O_2, and adenosine (33). These vasodilators play an important role in compensatory vasodilatation of coronary microvessels during myocardial ischemia (35). In the present study (Figs. 3 and 4), endothelium-dependent arteriolar vasodilatation to ACh during coronary I/R was significantly increased by L-NMMA while small arterial vasodilatation to ACh was increased by catalase and 8-SPT, and the residual arteriolar dilation was further inhibited by both of them (L-NMMA plus catalase or TEA). Furthermore, fluorescent microscopy with DCF and DAR, respectively, showed that H_2O_2 and NO production after I/R were enhanced in small coronary arteries and arterioles by L-NMMA [fluorescent intensity (FI) 1.8] and catalase (FI 1.9) compared with those in the LAD of control group (Figs. 5 and 6, FI: DAR 1.2 and DCF 1.1). The residual small arteriolar dilatation after combined administration of L-NMMA + catalase was completely blocked by 8-SPT, an adenosine receptor blocker, indicating that adenosine also compensated for the loss of action of NO and H_2O_2. Taken together, these results indicate the compensatory vasodilator effects among NO, H_2O_2, and adenosine to maintain coronary blood flow during coronary I/R injury in vivo. H_2O_2 and NO were mutually compensatory in both small arteries and arterioles, and in the presence of their inhibitors (catalase and L-NMMA), adenosine also caused arteriolar vasodilatation, as we reported previously (35). This finding is consistent with our finding that NO, H_2O_2, and adenosine play an important compensatory role in coronary autoregulation in canine coronary microcirculation in vivo (35). It was reported that TEA inhibited adenosine-induced vasodilatation of canine subepicardial coronary arteries in vitro (3). Furthermore, H_2O_2 stimulates protein kinase C, phospholipase A_2, and arachidonic acid release and increases intracellular cAMP levels (10). These findings suggest that cAMP-mediated pathway is involved, at least in part, during coronary vasodilatation through K_{Ca} channels after I/R injury.

Role of H_2O_2 during coronary I/R. It is known that K_{Ca} channels substantially contribute to coronary vasodilatation in myocardial ischemia (22) and that H_2O_2 also activates K_{Ca} channels (11). However, it remains to be examined whether H_2O_2 contributes to coronary vasodilatation during I/R in vivo. The present results demonstrate that H_2O_2

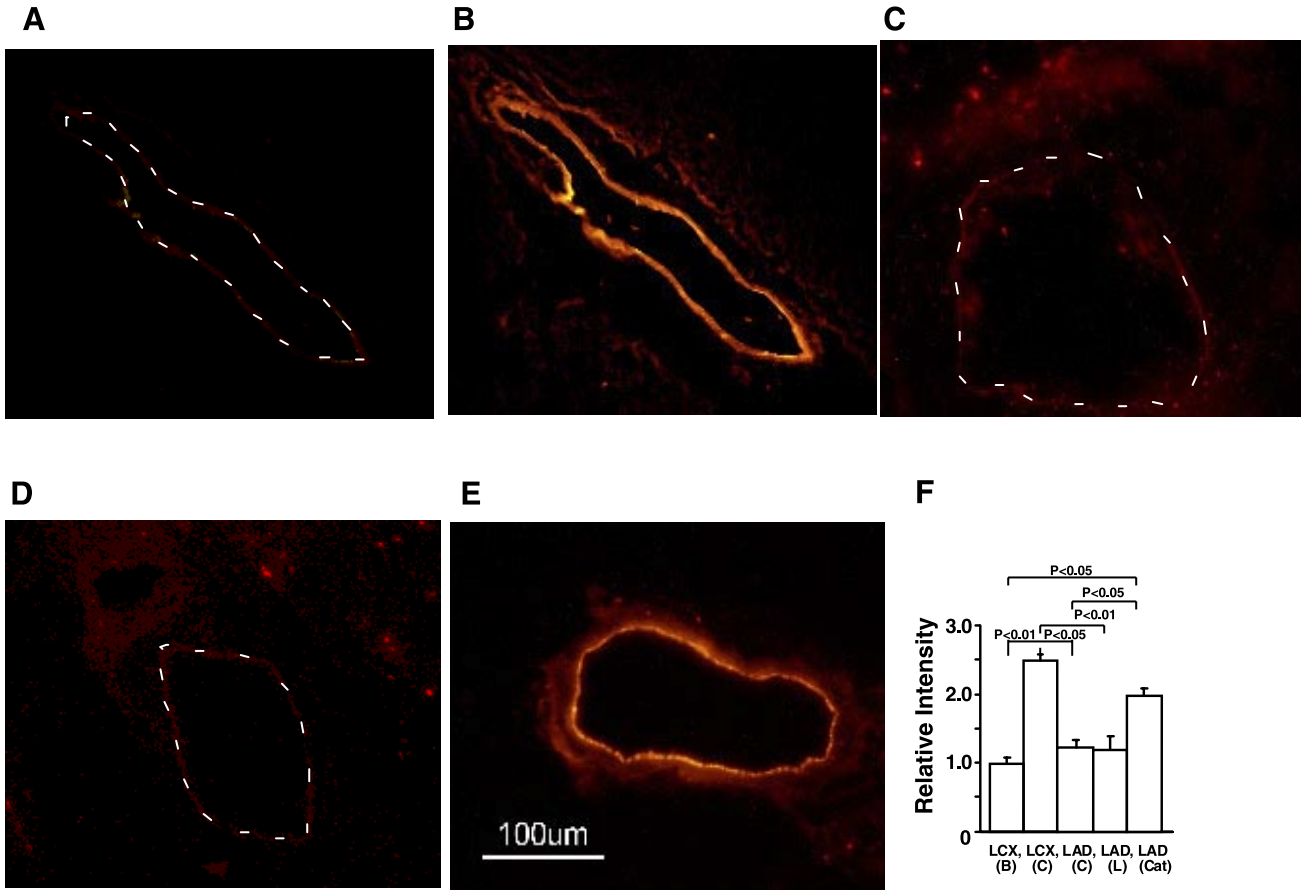

Fig. 6. Detection of nitric oxide (NO) production. *A*: LCX (baseline without ACh). *B*: LCX (control). *C*: LAD (control). *D*: LAD (L-NMMA). *E*: LAD (catalase). *F*: fluorescent intensity (B, baseline without ACh; C, control, L, L-NMMA; Cat, catalase). No. of small arteries per animals used was 5/5 for each group. Dashed line, outline of vessels.

substantially contributes to coronary vasodilatation during I/R in vivo as a compensatory mechanism for the loss of NO. Several mechanisms have been proposed for K_{Ca} channel opening during coronary I/R, including cellular acidosis

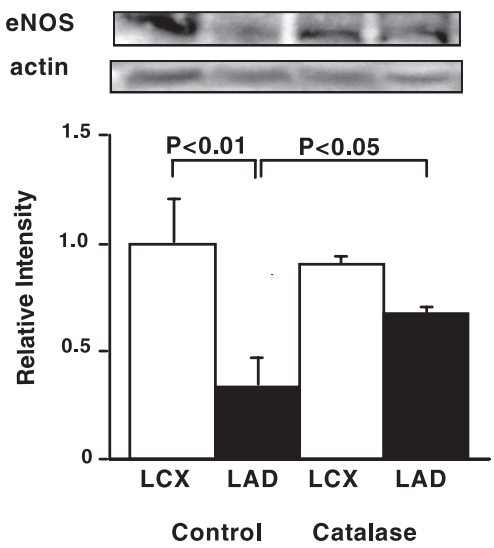

Fig. 7. Western blotting showing the effects of catalase on endothelial nitric oxide synthase (eNOS) protein expression in the myocardium of LAD and LCX. No. of animals used was 3 for each group.

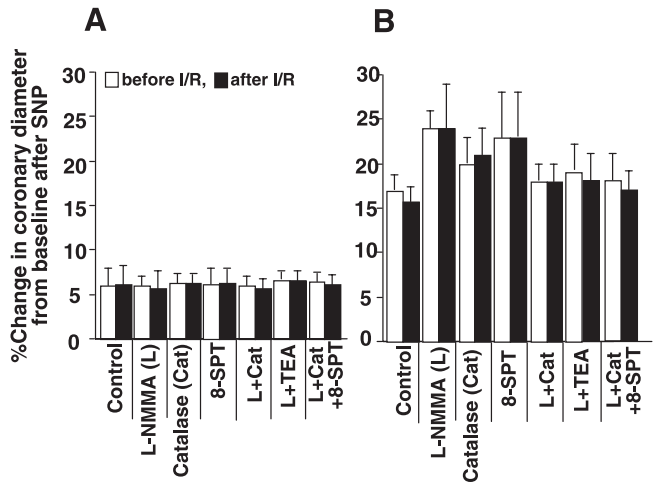

Fig. 8. Endothelium-independent coronary vasodilatation before and after coronary I/R injury in dogs in vivo. *A*: small artery (≥100 μm). *B*: arteriole (<100 μm). No. of small arteries and arterioles per animals used (*n*/*n*) was 7/5 for control, 8/5 for L-NMMA, 10/5 for catalase, 6/5 for 8-SPT, 8/5 for L-NMMA plus catalase, 5/5 for L-NMMA plus TEA, and 5/5 for L-NMMA plus catalase plus 8-SPT in small arteries; and 12/5 for control, 16/5 for L-NMMA, 12/5 for catalase, 5/5 for 8-SPT, 10/5 for L-NMMA plus catalase, 8/5 for L-NMMA plus TEA, and 7/5 for L-NMMA plus catalase plus 8-SPT in arterioles.

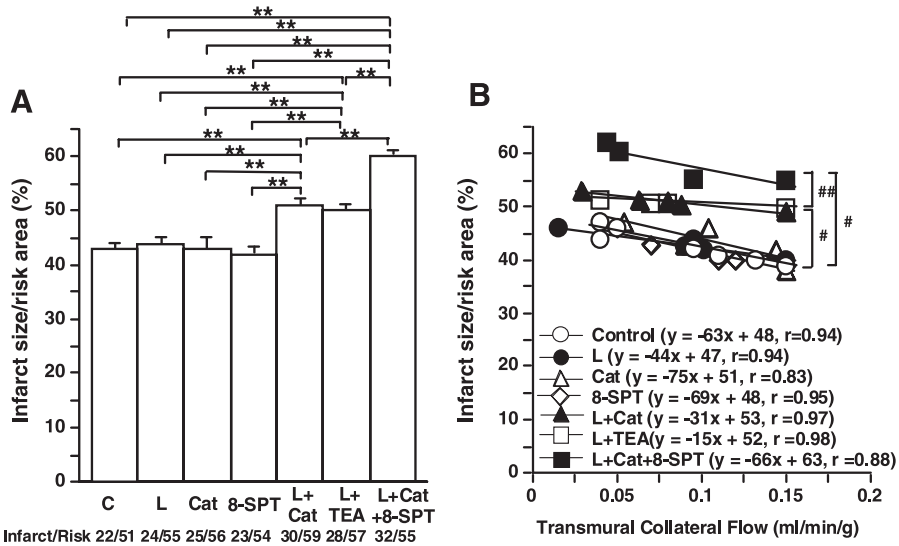

Fig. 9. Effects of H_2O_2, NO, and adenosine on I/R-induced MI in dogs in vivo. *A*: I/R-induced left ventricular infarct size in dogs in vivo. C, control. *B*: plot of infarct size expressed as a percentage of the risk area and regional collateral flow during I/R. $^{**}P < 0.01$, $\#P < 0.05$ vs. L-NMMA (L) or Cat or 8-SPT; $\#\#P < 0.01$ vs. L + TEA or L + Cat.

(27), increase in intracellular Ca^{2+} concentration after ischemia (28), and H_2O_2 production by inflammatory cells (5). Furthermore, an inhibitor of NO synthesis [N^G-nitro-L-arginine methyl ester (L-NAME)] or that of K_{Ca} channels (charybdotoxin) partly inhibits the protective effect on myocardial infarct size (22). Liu et al. (14) demonstrated that peroxynitrite inhibits K_{Ca} channel activity in human coronary arterioles during I/R. This mechanism might contribute to impaired H_2O_2-mediated dilation in I/R where NO synthase activity is increased in the presence of excess of O_2^-. In the present study, inhibition of H_2O_2 or NO alone did not significantly increase myocardial infarct size compared with control conditions (Fig. 9). These results suggest that H_2O_2 and NO exert cardioprotective effects against the development of myocardial infarction in a compensatory manner.

Recently, we have demonstrated that the expression of eNOS protein is decreased in the ischemic myocardium, which is improved by a selective Rho-kinase inhibitor, hydroxyfasudil, during coronary I/R injury in dogs in vivo (36). Furthermore, a physiological concentration (2 μmol/l) of H_2O_2 improved the recovery of both cardiac contractile function and energy metabolism after I/R in perfused rat heart (37). In the present study, the expression of eNOS protein was decreased in the ischemic myocardium, which was increased by catalase during I/R injury (Fig. 7). All these mechanisms may be involved in the beneficial effects of H_2O_2 on the I/R-induced myocardial injury. It also is conceivable that I/R reduces endothelial tetrahydrobiopterin levels in coronary vessels and impairs eNOS function (30).

Limitations of the study. Several limitations should be mentioned for the present study. First, we did not examine coronary vasodilatation in response to SOD/SOD mimetic (e.g., Tempol) or peroxynitrite inhibitor (e.g., ebselen) after I/R. However, because of the complex interactions among the oxygen species, we consider that both Tempol and ebselen also affect H_2O_2 metabolism by scavenging superoxide anions and peroxynitrite, respectively. Second, in addition to catalase, endogenous glutathione peroxidase (GSH) also plays an important role in removing H_2O_2, and NO also could be a substrate for endogenous catalase (1). However, in the present study, we used exogenous catalase to remove H_2O_2 to examine the role of the reactive oxygen species. Third, the exact source of vascular H_2O_2 production remains to be elucidated (e.g., the endothelium, smooth muscle, or cardiomyocytes). Fourth, while we were able to demonstrate the production of H_2O_2 using fluorescent microscopy with DCF, we were unable to quantitatively measure the H_2O_2 production because DCF detects H_2O_2, $ONOO^-$, and HOCl as well. Fifth, we were unable to find smaller arterioles because of the limited spatial resolution of our CCD intravital microscope. If we had an intravital camera with higher resolution, we would be able to observe coronary vasodilator responses of smaller arterioles.

Clinical implications and conclusions. During coronary I/R, microemboli of atherosclerosis debris and platelet plugs are released into the coronary microcirculation, particularly at revascularization with thrombolysis and/or percutaneous coronary intervention. Thus preexisting coronary endothelial dysfunction with various risk factors may be an important determinant for I/R injury in acute myocardial infarction. The synthesis and/or action of endothelium-derived NO are impaired under various pathological conditions, such as hypertension, hyperlipidemia, and diabetes mellitus (26, 34). In hypertension, K channel activities are increased in a compensatory manner with reduced NO activity (13). The present results suggest that NO and H_2O_2 compensate each other to cause coronary vasodilatation during I/R injury in vivo.

In conclusion, we were able to demonstrate that endogenous H_2O_2, in cooperation with NO, plays an important cardioprotective role in coronary I/R injury in vivo. The present findings may have important clinical implications because H_2O_2-mediated mechanisms substantially contribute to endothelium-dependent vasodilatation in coronary I/R in vivo.

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