PRECLINICAL STUDIES

Important Role of Endogenous Hydrogen Peroxide in Pacing-Induced Metabolic Coronary Vasodilation in Dogs In Vivo

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Objectives	We examined whether endogenous hydrogen peroxide (H_2O_2) is involved in pacing-induced metabolic vasodilation in vivo.
Background	We have previously demonstrated that endothelium-derived H_2O_2 is an endothelium-derived hyperpolarizing factor in canine coronary microcirculation in vivo. However, the role of endogenous H_2O_2 in metabolic coronary vasodilation in vivo remains to be examined.
Methods	Canine subepicardial small coronary arteries ($\geq 100\ \mu m$) and arterioles ($< 100\ \mu m$) were continuously observed by a microscope under cyclooxygenase blockade (ibuprofen, 12.5 mg/kg intravenous [IV]) (n = 60). Experiments were performed during paired right ventricular pacing under the following 7 conditions: control, nitric oxide (NO) synthase inhibitor (N^G-monomethyl-L-arginine [L-NMMA], 2 μmol/min for 20 min intracoronary [IC]), catalase (a decomposer of H_2O_2, 40,000 U/kg IV and 240,000 U/kg/min for 10 min IC), 8-sulfophenyltheophylline (SPT) (an adenosine receptor blocker, 25 μg/kg/min for 5 min IC), L-NMMA+catalase, L-NMMA+tetraethylammonium (TEA) (K_{Ca}-channel blocker, 10 μg/kg/min for 10 min IC), and L-NMMA+catalase+8-SPT.
Results	Cardiac tachypacing (60 to 120 beats/min) caused coronary vasodilation in both-sized arteries under control conditions in response to the increase in myocardial oxygen consumption. The metabolic coronary vasodilation was decreased after L-NMMA in subepicardial small arteries with an increased fluorescent H_2O_2 production compared with catalase group, whereas catalase decreased the vasodilation of arterioles with an increased fluorescent NO production compared with the L-NMMA group, and 8-SPT also decreased the vasodilation of arterioles. Furthermore, the metabolic coronary vasodilation was markedly attenuated after L-NMMA+catalase, L-NMMA+TEA, and L-NMMA+catalase+8-SPT in both-sized arteries.
Conclusions	These results indicate that endogenous H_2O_2 plays an important role in pacing-induced metabolic coronary vasodilation in vivo. (J Am Coll Cardiol 2007;50:1272–8) © 2007 by the American College of Cardiology Foundation

Cardiac tachycardia by pacing or exercise increases myocardial oxygen consumption (MVO_2) and increases coronary blood flow by several mechanisms (1–3). Shear stress plays a crucial role in modulating vascular tone by endothelium-derived releasing factors (EDRFs), including nitric oxide (NO), prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factor (EDHF) (4,5). Flow-induced vasodilation is mediated by either NO (6,7), PGI_2 (8), both of them

(9), or EDHF (10). Matoba et al. have previously identified that endothelium-derived hydrogen peroxide (H_2O_2) is a

primary EDHF in mesenteric arteries of mice and humans (11,12). Morikawa et al. (13,14) subsequently confirmed

that endothelial Cu,Zn-superoxide dismutase (SOD) plays an important role as an EDHF synthase in mice and humans. Miura et al. (15) demonstrated that endothelium-derived H_2O_2 is involved as an EDHF in the flow-induced vasodilation of isolated human coronary arterioles in vitro. We have recently confirmed that endogenous H_2O_2 plays an important compensatory role during coronary autoregulation (16) and reperfusion injury in vivo (17) through the interactions with NO and adenosine.

It is known that vascular α-adrenergic receptor is modulated by the endothelium in dogs (18), whereas cardiac β-adrenergic receptor is modulated by K_{Ca} channels in pigs (19) and H_2O_2 in mice (20). However, the role of endogenous H_2O_2 in metabolic coronary vasodilation in vivo remains largely unknown. In the present study, we thus examined whether H_2O_2 is involved in pacing-induced metabolic coronary vasodilation in canine coronary microcirculation in vivo.

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 Institutes of Health.

Animal preparation. Anesthetized mongrel dogs of either gender (15 to 25 kg in body weight, n = 60) were ventilated with a ventilator (Model VS600, IDC, Pittsburgh, Pennsylvania). We continuously monitored aortic pressure and left ventricular pressure (LVP) with a catheter (SPC-784A, Millar, Houston, Texas) and blood flow of the left anterior descending coronary artery (LAD) with a transonic flow probe (T206, Transonic Systems, Ithaca, New York).

Measurements of coronary diameter by intravital microscope. We continuously monitored coronary vascular responses by an intravital microscope (VMS 1210, Nihon Kohden, Tokyo, Japan) with a needle-probe in vivo, as previously described (21). 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 (21).

Measurements of regional myocardial blood flow. Regional myocardial blood flow was measured by the non-radioactive microsphere (Sekisui Plastic Co. Ltd., Tokyo, Japan) technique, as previously described (22). Briefly, the microspheres suspension was injected into the left atrium 3 min after tachypacing. Myocardial flow in the LAD area was calculated according to the formula "time flow = tissue counts × (reference flow/reference counts)" and was expressed in ml/g/min (22).

Detection of H_2O_2 and NO production in coronary microvessels. 2′,7′-dichlorodihydrofluorescein diacetate (DCF) (Molecular Probes, Eugene, Oregon) and diaminorhodamine-4M AM (DAR) (Daiichi Pure Chemicals, Tokyo, Japan) were used to detect H_2O_2 and NO production in coronary microvessels, respectively, as previ-

ously described (17). Briefly, fresh and unfixed heart tissues were cut into several blocks and immediately frozen in optimal cutting temperature compound (Tissue-Tek, Sakura Fine Chemical, Tokyo, Japan). Fluorescent images of the microvessels were obtained 3 min after application of acetylcholine (ACh) by using a fluorescence microscope (OLYMPUS BX51, Tokyo, Japan) (17).

Experimental protocols. After the surgical procedure and instrumentation, at least 30 min were allowed for stabilization while monitoring hemodynamic variables. Coronary vasodilator responses were examined before and after cardiac tachypacing (60 to 120 beats/min) under the following 7 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 without any inhibitor; 2) L-NMMA alone (2 μmol/min intracoronary [IC] for 20 min); 3) catalase alone (40,000 U/kg intravenous [IV] and 240,000 U/kg/min IC for 10 min, an enzyme that dismutates

Abbreviations and Acronyms

CBF = coronary blood flow

DAR = diaminorhodamine-4M AM

DCF = 2′,7′-dichlorodihydrofluorescein diacetate

EDHF = endothelium-derived hyperpolarizing factor

H_2O_2 = hydrogen peroxide

L-NMMA = N^G-monomethyl-L-arginine

LAD = left anterior descending coronary artery

MVO_2 = myocardial oxygen consumption

NO = nitric oxide

PGI_2 = prostacyclin

SPT = sulfophenyltheophylline

TEA = tetraethylammonium
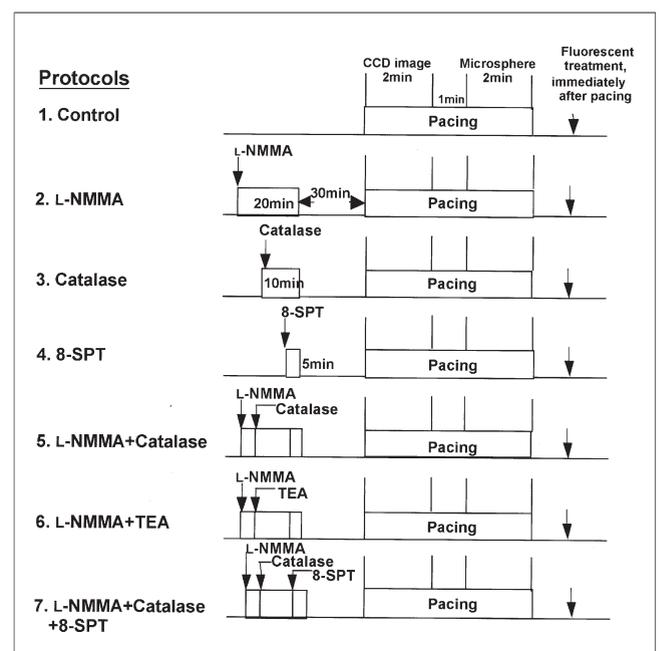
Figure 1   **Experimental Protocols**

CCD = charge-coupled device; L-NMMA = N^G-monomethyl-L-arginine; SPT = sulfophenyltheophylline; TEA = tetraethylammonium.
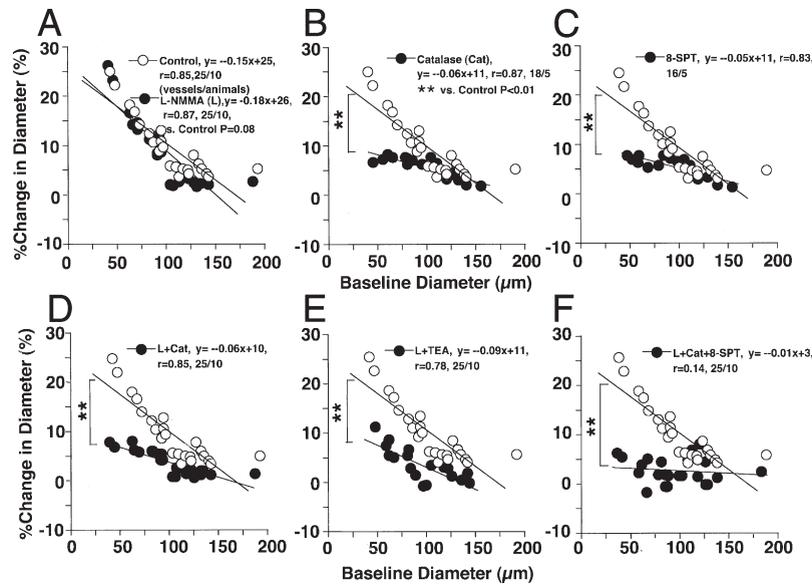
Figure 2   **Coronary Vascular Responses to Cardiac Pacing**

The coronary vasodilating responses of both-sized coronary arteries were significantly
inhibited in all experimental conditions except L-NMMA alone. **p < 0.01. Abbreviations as in Figure 1.

H_2O_2 into water and oxygen); 4) adenosine receptor blockade alone (8-sulfophenyltheophylline [8-SPT], 25 μg/kg/min IC for 5 min); 5) catalase plus L-NMMA; 6) catalase plus tetraethylammonium (TEA) (10 μg/kg/min IC for 10 min, an inhibitor of large conductance K_{Ca} channels to inhibit EDHF-mediated responses) (23); and 7) catalase plus L-NMMA with 8-SPT (16). These inhibitors were given at 30 min before cardiac tachypacing (Fig. 1). The basal coronary diameter was defined as that before pacing. We continuously observed the diameter change in subepicardial small coronary arteries (≥100 μm) and arterioles (<100 μm) with an intravital microscope before and at 2 min after pacing. Microspheres were administered at 3 min after the pacing was started (Fig. 1). In the combined infusion protocol (L-NMMA+catalase+8-SPT), L-NMMA infusion was first started, followed by catalase infusion, and then 8-SPT was added at 15 min after the initiation of L-NMMA infusion (Fig. 1). Then, fresh and unfixed heart tissues were cut into several blocks and immediately frozen in optimal cutting temperature compound after the pacing. The flow and MVO₂ were measured as full-thickness values.

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

Statistical analysis. Results are expressed as means ± SEM. Differences in the vasodilation of subepicardial coronary microvessels before and after pacing (Fig. 2) were examined by a multiple regression 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 inhibi-

tors were set as dummy variables (D1, D2) in the following equation: y = a0 + a1x + a2D1 + a3D2, where a0 through a3 are partial regression coefficients (16). 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. Vascular fluorescent responses (Figs. 3 and 4) were analyzed by one-way analysis of variance followed by Scheffe's post hoc test for multiple comparisons. The criterion for statistical significance was at p < 0.05.

Results

Hemodynamic status and blood gases during pacing. Throughout the experiments, mean aortic pressure was constant and comparable (Table 1), and pO₂, pCO₂, and pH were maintained within the physiological ranges (pO₂ >70 mm Hg, pCO₂ 25 to 40 mm Hg, and pH 7.35 to 7.45). Baseline coronary diameter was comparable in the absence and presence of inhibitors under the 7 different experimental conditions (Table 1). Cardiac tachypacing increased coronary blood flow and MVO₂ from the baseline values (Table 2, both p < 0.01). Combined infusion of L-NMMA+catalase+8-SPT significantly decreased coronary blood flow (CBF) and MVO₂ as compared with control, L-NMMA alone (both p < 0.01), catalase alone (both p < 0.01), 8-SPT alone (both p < 0.01), L-NMMA+catalase (both p < 0.05), L-NMMA+TEA (both p < 0.05). Com-
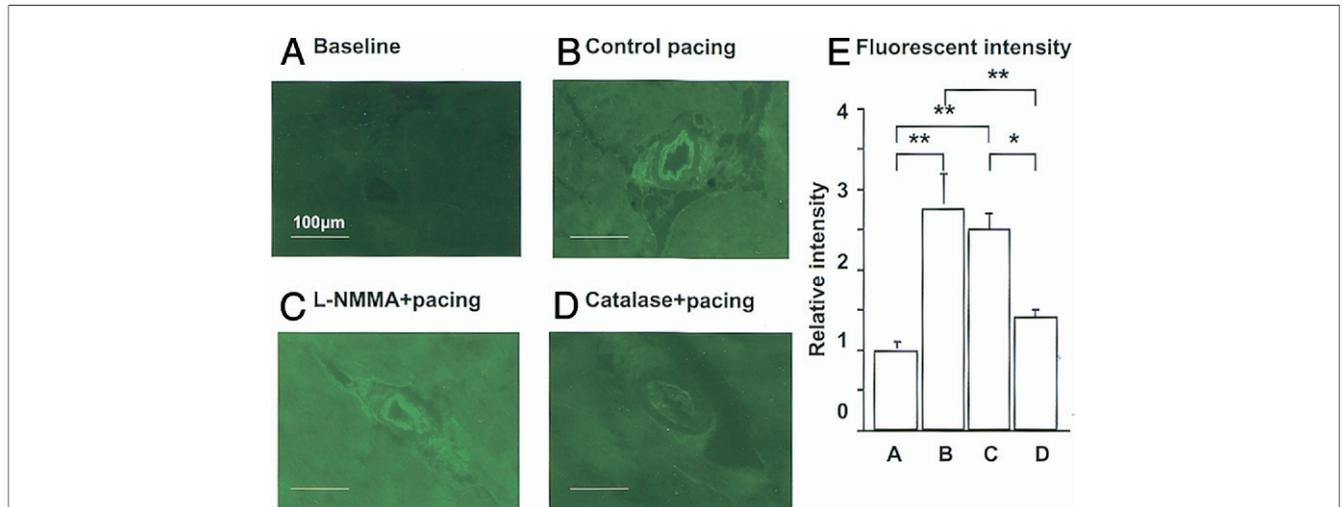
Figure 3   Detection of H$_2$O$_2$ Production With DCF Fluorescent Method

Hydrogen peroxide (H$_2$O$_2$) production was unaltered after N^G-monomethyl-L-arginine (L-NMMA) but was markedly suppressed by catalase. Number of arterioles/animals used was 5/5 for each group. *p < 0.05, **p < 0.01. DCF = 2′,7′-dichlorodihydrofluorescein diacetate.

bined infusion of L-NMMA+catalase or L-NMMA+ TEA significantly decreased CBF (both p < 0.05) and MVO$_2$ (both p < 0.05) as compared with control after the pacing.

Coronary vasodilation before and after cardiac tachypacing. Cardiac tachypacing caused coronary vasodilation in both-sized arteries under control conditions (small coronary arteries, 5 ± 1%; arterioles, 14 ± 2%) (Fig. 2A) with decreased coronary venous pO$_2$ (Table 2). The metabolic coronary vasodilation was significantly decreased after L-NMMA in small coronary arteries (3 ± 1%) but not in arterioles (14 ± 2%), whereas catalase and 8-SPT decreased

the vasodilation of arterioles (both 4 ± 1%) but not in small coronary arteries (both 7 ± 1%) (Figs. 2B and 2C). Furthermore, the metabolic coronary vasodilation was markedly attenuated after L-NMMA+catalase and L-NMMA+TEA in small coronary arteries (both 2 ± 1%), and L-NMMA+catalase+8-SPT almost abolished the vasodilating responses in both-sized arteries (small coronary arteries, −1 ± 1%; arterioles, 1 ± 1%) (Figs. 2D to 2F). When expressed in a linear regression analysis, the coronary vasodilating responses of both-sized coronary arteries were significantly inhibited in all experimental conditions except L-NMMA alone (Fig. 2A).
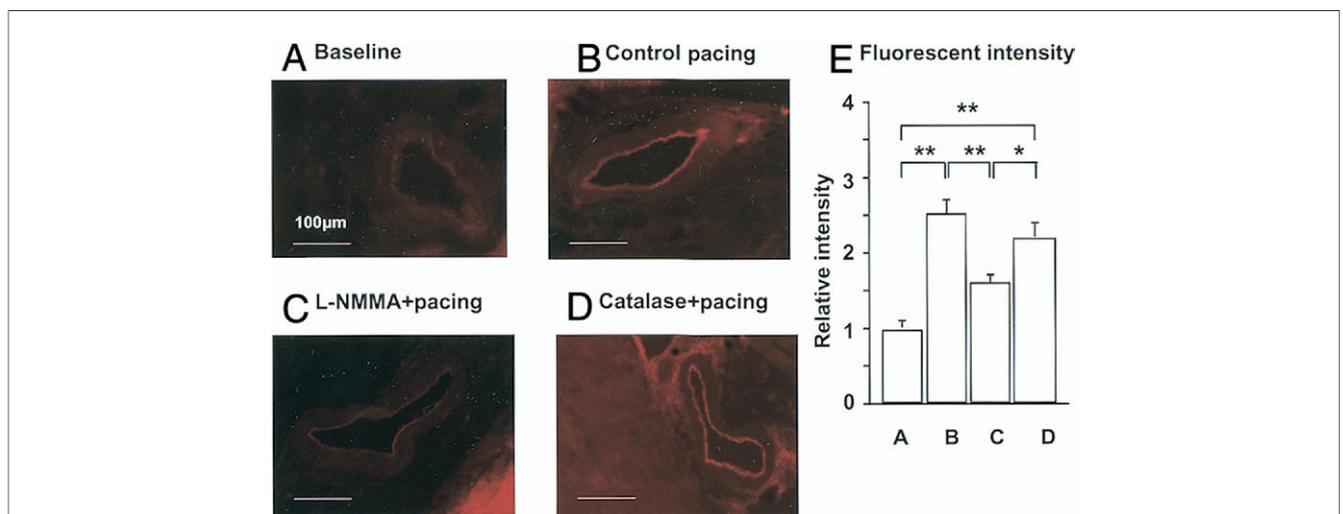
Figure 4   Detection of NO Production With DAR Fluorescent Method

Nitric oxide (NO) production was unaltered after catalase but was markedly suppressed by N^G-monomethyl-L-arginine (L-NMMA). Number of arterioles/animals used was 5/5 for each group. *p < 0.05, **p < 0.01. DAR = diaminorhodamine-4M AM.

Table 1   The Small Artery and Arteriolar Diameter Measurements at Rest and During Cardiac Pacing

	Control	L-NMMA (L)	Catalase (Cat)	8-SPT	L+Cat	L+TEA	L+Cat+8-SPT
Small artery							
n (vessels/dogs)	12/10	12/10	9/5	7/5	12/10	12/10	12/10
Rest (μm)	127 ± 7	125 ± 6	127 ± 5	126 ± 6	125 ± 7	123 ± 6	124 ± 7
Cardiac pacing (μm)	134 ± 7*	129 ± 7†	132 ± 5*	131 ± 6*	127 ± 7	124 ± 6	123 ± 6
Arteriole							
n (vessels/dogs)	12/10	12/10	9/5	9/5	12/10	12/10	12/10
Rest (μm)	75 ± 5	73 ± 5	71 ± 5	71 ± 5	72 ± 5	74 ± 5	72 ± 6
Cardiac pacing (μm)	85 ± 5*	82 ± 5*	77 ± 6†	77 ± 6†	77 ± 5†	77 ± 5	73 ± 5

Results are expressed as mean ± SEM. *p < 0.01, †p < 0.05 versus rest.
L-NMMA = N^G-monomethyl-L-arginine; SPT = sulfophenyltheophylline; TEA = tetraethylammonium.

Detection of H₂O₂ and NO production. Fluorescent microscopy with DCF showed that cardiac tachypacing increased coronary H₂O₂ production compared with baseline conditions in arterioles (Fig. 3). The pacing-induced H₂O₂ production as assessed by DCF fluorescent intensity was unaltered after L-NMMA but was markedly suppressed by catalase (Fig. 3). By contrast, in small coronary arteries, vascular NO production as assessed by DAR fluorescent intensity was significantly increased in response to the pacing compared with baseline conditions (Fig. 4). The pacing-induced NO production was unaltered after catalase but was markedly suppressed by L-NMMA (Fig. 4). Pacing caused no significant increase in H₂O₂ production in small coronary arteries or NO production in arterioles (data not shown).

Discussion

The major finding of the present study is that endogenous H₂O₂ plays an important role in pacing-induced metabolic coronary dilation as a compensatory mechanism for NO in vivo. We demonstrated the important role of endogenous H₂O₂ in the mechanisms for metabolic coronary dilation in vivo. **Validations of experimental model and methodology.** We chose, on the basis of our previous reports (16,17), the adequate dose of L-NMMA, catalase, TEA, and 8-SPT in order to inhibit NO synthesis, H₂O₂, K_{Ca} channels, and the adenosine receptor, respectively. The TEA at low doses is fairly specific for K_{Ca} channel, but at higher doses it might block a number of other K channels. Because several K_{Ca} channels might be involved in H₂O₂-mediated responses (5), we selected nonselective K_{Ca} inhibitor, TEA, to inhibit all K_{Ca} channels (23). We have previously confirmed the validity of our present methods (21).

Role of NO and H₂O₂ after cardiac pacing. Matoba et al. have demonstrated that endothelium-derived H₂O₂ is an EDHF in mouse (11) and human (12) mesenteric arteries and pig coronary microvessels (24). Morikawa et al. also

Table 2   Hemodynamic Status at Rest and During Cardiac Pacing

	Control	L-NMMA (L)	Catalase (Cat)	8-SPT	L+Cat	L+TEA	L+Cat+8-SPT
n (dogs)	10	10	5	5	10	10	10
SBP							
Rest (mm Hg)	135 ± 14	135 ± 14	114 ± 9	123 ± 5	98 ± 9	99 ± 9	96 ± 8
Cardiac pacing	137 ± 14	136 ± 14	125 ± 12	130 ± 7	100 ± 9	100 ± 8	103 ± 9
MBP							
Rest (mm Hg)	117 ± 10	117 ± 10	98 ± 8	99 ± 5	89 ± 10	90 ± 10	87 ± 9
Cardiac pacing	124 ± 9	120 ± 13	107 ± 10	110 ± 7	91 ± 10	92 ± 10	92 ± 10
DP							
Rest	8,100 ± 845	8,100 ± 845	6,855 ± 527	7,350 ± 312	5,880 ± 537	5,910 ± 527	5,730 ± 478
Cardiac pacing	16,440 ± 1,718*	16,320 ± 1,680*	15,000 ± 1,423*	15,630 ± 778*	11,940 ± 11,029*	12,000 ± 1,011*	12,300 ± 1,078*
CVPO₂							
Rest (mm Hg)	20 ± 1	17 ± 1	16 ± 1	17 ± 1	15 ± 1†	15 ± 1†	14 ± 1†
Cardiac pacing	14 ± 1*	11 ± 1*	11 ± 1*	12 ± 1*	10 ± 1*†	10 ± 1*†	9 ± 1*†
MVO₂							
Rest (μIO₂/min/g)	70 ± 2	66 ± 2	67 ± 2	73 ± 5	62 ± 5	61 ± 5	60 ± 5
Cardiac pacing	171 ± 4‡	168 ± 2‡	158 ± 12‡	168 ± 13‡	133 ± 4†‡	130 ± 18†‡	95 ± 9*§
CBF							
Rest (ml/min/g)	0.66 ± 0.06	0.63 ± 0.06	0.66 ± 0.03	0.66 ± 0.01	0.59 ± 0.06	0.62 ± 0.05	0.51 ± 0.04
Cardiac pacing	1.48 ± 0.32‡	1.46 ± 0.06‡	1.36 ± 0.02‡	1.40 ± 0.01‡	1.22 ± 0.01†‡	1.24 ± 0.12†‡	0.96 ± 0.07‡§

Results are expressed as mean ± SEM. *p < 0.05 versus at rest. †p < 0.05 versus corresponding control measurements. ‡p < 0.01 versus rest. §p < 0.01 versus corresponding control measurements.
CBF = coronary blood flow; CVPO₂ = coronary venous pO₂; DP = double product; MBP = mean blood pressure; MVO₂ = myocardial oxygen consumption; SBP = systolic blood pressure; other abbreviations as in Table 2.

have demonstrated that endothelial Cu,Zn-SOD plays an important role as an H_2O_2/EDHF synthase in mouse (13) and human (14) mesenteric arteries. Subsequently, we (16,17) and others (15) confirmed that endogenous H_2O_2 exerts important vasodilator effects in canine coronary microcirculation in vivo and in isolated human coronary microvessels, respectively. In the present study, the pacing-induced metabolic coronary vasodilation was significantly decreased after L-NMMA in small coronary arteries but not in arterioles, whereas catalase decreased the vasodilation of arterioles but not that of small arteries, and the coronary vasodilation was markedly attenuated after L-NMMA+catalase (Fig. 2). These findings indicate that NO and H_2O_2 compensate for each other to maintain coronary vasodilation in response to increased myocardial oxygen demand. Coronary venous pO_2 tended to be lower after L-NMMA+catalase, suggesting that NO and H_2O_2 coordinately cause coronary vasodilation during cardiac tachypacing.

Saitoh et al. (25) suggested that the production of H_2O_2, which stems from the dismutation of $\cdot O_2^-$ that is formed during mitochondrial electron transport, is seminal in the coupling between oxygen metabolism and blood flow in the heart. Thus, the contribution of H_2O_2 production in response to the change in metabolism cannot be excluded.

Endothelial Cu,Zn-SOD plays an important role in the synthesis of H_2O_2 as an EDHF synthase in mouse (13) and human (14) mesenteric arteries, and exercise training enhances expression of Cu,Zn-SOD in normal pigs (26). It remains to be examined whether exercise-induced up-regulation of Cu,Zn-SOD enhances metabolic coronary vasodilation mediated by endogenous H_2O_2.

Compensatory vasodilator mechanism among H_2O_2, NO, and adenosine. The EDHF acts as a partial compensatory mechanism to maintain endothelium-dependent vasodilation in the forearm microcirculation of patients with essential hypertension, where NO activity is impaired owing to oxidative stress (27). We have recently demonstrated in the fluorescent microscopy study that coronary vascular production of H_2O_2 and NO is enhanced after myocardial ischemia/reperfusion in small coronary arteries and arterioles, respectively (17). In the present study, the DCF fluorescent intensity was comparable between control and L-NMMA, and that of DAR was also comparable between control and catalase (Figs. 3 and 4). Although the exact source of vascular production of H_2O_2 and NO remains to be elucidated, it is highly possible that endothelium-derived NO and H_2O_2 compensate for each other to maintain coronary vasodilation in response to increased MVO_2.

In the dog, blockade of any vasodilator mechanisms fails to blunt the increase in coronary blood flow in response to exercise, indicating that adenosine, K^+_{ATP}-channel opening, prostanoids, or NO might not be mandatory for exercise-induced coronary vasodilation, or that these redundant vasodilator mechanisms compensate for each other when one mechanism is blocked (28). In the present study,

adenosine blockade with 8-SPT alone inhibited the pacing-induced vasodilation of arteriole but not that of small artery, whereas combined administration of L-NMMA+catalase+8-SPT almost abolished the pacing-induced coronary vasodilation of both-sized arteries with an increase in coronary blood flow (Fig. 2). The discrepancy between the diameter and flow responses is likely due to the metabolic autoregulation of smaller arterioles. These results indicate that adenosine also plays an important role to maintain metabolic coronary vasodilation in cooperation with NO and H_2O_2, a finding consistent with our previous study on coronary autoregulatory mechanisms (15).

Study limitations. Several limitations should be mentioned for the present study. First, although we were able to demonstrate the production of H_2O_2 with fluorescent microscopy with DCF, we were unable to quantify the endothelial H_2O_2 production, because DCF reacts with H_2O_2, peroxynitrite, and hypochlorous acid (13). Second, we were unable to find smaller arterioles, owing to the limited spatial resolution of our charge-coupled device intravital microscope. With an intravital camera with higher resolution, we would be able to observe coronary vasodilation of smaller arterioles. Third, we were unable to determine whether H_2O_2 is produced by shear stress or cardiac metabolism. This point remains to be elucidated in a future study.

Conclusions

We were able to demonstrate that endogenous H_2O_2 plays an important role in pacing-induced metabolic coronary vasodilation in canine coronary microcirculation in vivo and that there are substantial compensatory interactions among NO, H_2O_2, and adenosine to maintain metabolic coronary vasodilation, which is one of the most important mechanisms for cardiovascular homeostasis in vivo.

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