Hydrogen Peroxide Derived From Beating Heart Mediates Coronary Microvascular Dilation During Tachycardia

Yasunori Kokusho, Tatsuya Komaru, Satoru Takeda, Katsuaki Takahashi, Ryoji Koshida, Kunio Shirato, Hiroaki Shimokawa

Objective—Coronary flow is closely correlated to the myocardial metabolic demand. We tested the hypothesis that hydrogen peroxide (H_2O_2) derived from beating hearts mediates metabolic coronary microvascular dilation.

Methods and Results—We used a bioassay method in which an isolated microvessel is placed on a beating heart to detect myocardium-derived vasoactive mediators. A rabbit coronary arterial microvessel (detector vessel [DV], n=25) was pressurized and placed on a canine beating heart. After intrinsic tone of DV had developed, we observed DV at rest (heart rate, 120 bpm) and during tachypacing (heart rate, 240 bpm) using an intravital microscope equipped with a floating objective. The tachypacing produced DV dilation by 8.2% ($P<0.01$ versus baseline), and the dilation was abolished by cell-impermeable catalase (a H_2O_2 scavenger, 500 U/mL). We performed myocardial biopsy at rest and tachypacing. The biopsy specimens were loaded with 2′,7′-dichlorodihydrofluorescein diacetate (10 μmol/L) to visualize H_2O_2, and observed with confocal microscopy. Dichlorofluorescein fluorescence was diffusely identified in the myocardium and the tachypacing increased the fluorescence intensity ($P<0.01$). Exogenous H_2O_2 caused vasodilation of arterial microvessels in vitro in a concentration-dependent manner that was abolished by catalase.

Conclusions—H_2O_2 derived from the beating heart mediates tachypacing-induced metabolic coronary vasodilation in vivo. (***Arterioscler Thromb Vasc Biol*. 2007;27:1057-1063.)**

Key Words: coronary circulation ■ myocardium ■ reactive oxygen species ■ tachycardia ■ vasodilation

Coronary blood flow is linearly correlated to the myocardial oxygen consumption.[1,2] The tight coupling between the cardiac metabolism and the flow conductance underlies the widely accepted assumption that the myocardium-derived vasoactive factor, so-called metabolic factor, rapidly regulates the vascular tone of coronary microvessels for matching the coronary flow to the cardiac metabolic state. However, the metabolic factor that is transmitted from the myocardium to the coronary microvessels has not been identified yet. Although there are many candidates for the mediators such as adenosine, prostanoids, autacoids, nitric oxide (NO), factors activating potassium channels, and so on, no substance solely explains the metabolic microvascular dilation.[3,4]

Although excessive reactive oxygen species (ROS) are produced in various pathological conditions such as ischemia/reperfusion and cardiac failure[5,6] and are often hazardous for living organisms, increasing evidence has shown that ROS also play important roles as biological signals that mediate physiological phenomena at low concentrations.[7,8] In the field of vascular biology, hydrogen peroxide (H_2O_2) has been known as one of the possible endothelium-derived hyperpolarizing factors.[9] The cardiac myocyte is another major source of ROS.[5,10] Superoxide ($O_2^{-\cdot}$) and H_2O_2 are produced as byproducts of electron transfer reactions during normal aerobic metabolism. $O_2^{-\cdot}$ produced in mitochondria accounts for 1% to 2% of total consumed oxygen,[11] and it is reduced to H_2O_2 by mitochondrial SOD. The steady-state levels of H_2O_2 inside the cardiac myocyte may allow a portion to diffuse out of the cell into the interstitial space and subsequently to the blood vessels because of its lipophilic property. It is possible that the diffused H_2O_2 is a signal of cardiac metabolism for the coronary microvessels. Furthermore, H_2O_2 potently dilates coronary arterial microvessels in endothelium-dependent and endothelium-independent fashion.[12] Accordingly, we hypothesized that H_2O_2 derived from the beating heart mediates the dilation of the coronary microvessels during tachypacing.

We tested the hypothesis using a bioassay method developed in our laboratory,[13–15] in which the isolated coronary vessel is placed on the beating heart, because the system enables us to separately control the beating heart and the coronary microvessels, and it is advantageous for detecting the myocardium-derived vasoactive signals.

Materials and Methods

The present study was approved by institutional ethical committee for animal experiments and conformed to the *Guide for the Care and Use of Laboratory Animals*, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

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From the Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan.

Correspondence to Tatsuya Komaru, MD, PhD, Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Seiryo-machi, Aoba-ku, Sendai, 980-8574, Japan. E-mail komaru@cardio.med.tohoku.ac.jp

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Bioassay Method

The bioassay system consists of a pressurized isolated rabbit coronary microvessel for detection of vasoactive signals and a beating canine heart as previously described.[13–15]

For the preparation of the detector vessel (DV), male Japanese white rabbits (n=25, body weight 2.0±0.0 kg; Japan SLC, Hamamatsu) were anesthetized and anticoagulated with pentobarbital (50 mg/kg) and heparin (1000 U). After the rabbits were euthanized by bleeding from the carotid artery, the hearts were excised and immersed in chilled Krebs solution. A coronary arterial microvessel of the left ventricle (100 to 300 μm) was carefully isolated and used as DV. One end of the vessel was cannulated to a micropipette and the other end was ligated. DV was incubated in warm (38°C) Krebs solution until use. In 6 DVs, the endothelium was removed as previously described.[16]

Beagle dogs of either gender (n=25; body weight, 5.8±0.2 kg; NARC, Chiba, Japan) were used and prepared as described in our previous studies.[13–15] Briefly, the dogs were anesthetized with α-chloralose (60 mg/kg, intravenous; Wako Pure Chemicals, Osaka, Japan) and artificially ventilated. The heart was exposed and paced at 120 bpm by left atrial pacing after the suppression of the sinoatrial node with formaldehyde injection.

The incubated DV was gently placed on the beating left ventricle of the dog and pressurized to 80 cm H_2O to produce intrinsic tone. DV and the heart surface were kept moist throughout the experiment by continuous suffusion of Krebs solution (38°C) at a rate of 1 to 1.5 mL/min.

For visualization of DV placed on the beating left ventricle, an intravital microscope system equipped with a floating objective and a charge-coupled device camera (HV-D28S; Hitachi, Tokyo) was used. This optical system was originally developed in our laboratory[17] for the visualization of epimyocardial coronary microvessels in vivo. The spatial resolution of this optical system was 2 μm. Epi-illumination with a mercury lamp was applied to obtain the DV images. Obtained images were captured on a personal computer monitor, and the inner diameters were measured with Scion Image (version β4.0.2; Scion, Frederick, Md). The detailed description of the bioassay method is shown in online data supplement (please see http://atvb.ahajournals.org).

Experimental Protocols

Experiments were performed ≈1 hour after DV was pressurized to 80 cm H_2O on the beating heart, when all hemodynamic variables and blood gas parameters had become stable and the intrinsic tone of DV had developed.

Protocol 1 (vehicle group, n=11)

After the stable intrinsic tone of DV (with endothelium, n=5; without endothelium, n=6) was established at the resting condition (heart rate=120 bpm), the images of DV were collected. Thereafter, the heart rate was increased to 240 bpm and the images of DV were collected 10 minutes after the tachypacing. The efficacy of endothelial denudation was verified by the lack of vasodilation to 1 μmol/L acetylcholine (Sigma, St. Louis, Mo). At the end of the protocol, sodium nitroprusside (100 μmol/L; Wako) was suffused for 5 minutes to induce the maximal dilation and DV images were again collected.

Protocol 2 (n=14)

To investigate the involvement of H_2O_2, NO, K_{ATP} channels, and adenosine, we suffused cell-impermeable catalase (500 U/mL; Sigma; catalase group, n=6), N^{ω}-nitro-L-arginine ([LNNA] NO synthase inhibitor, 100 μmol/L; Sigma) with glibenclamide ([GC] a K_{ATP} channel inhibitor, 5 μmol/L; Wako) (LNNA+GC group, n=4), and 8-phenyltheophylline ([8-PT] a selective adenosine receptor antagonist, 10 μmol/L; Sigma; 8-PT group, n=4) onto DV throughout the experiments. Ten minutes (catalase) or 30 minutes (LNNA+GC and 8-PT) after the pretreatment, the images of DV were collected before and after the tachypacing as in protocol 1. In protocol 2, all DVs were with endothelium.

H_2O_2 Detection

In 5 anesthetized open-chest beagle dogs, epimyocardial biopsy of the left ventricle was performed at the heart rates of 120, 160, 200, and 240 bpm. The obtained specimens were loaded with 10 μmol/L 2′,7′-dichlorodihydrofluorescein diacetate (Sigma) to visualize H_2O_2 as 2′,7′-dichlorofluorescein (DCF) fluorescence.[18] The fluorescent images were acquired using a laser scanning confocal microscope (C1; Nikon) and the fluorescence intensity was quantitated (AxioVision v4.5.0.0; Carl Zeiss, Jena, Germany). Detailed description of the method is in online data supplement.

Immunohistochemistry

We performed immunohistochemistry for sarcomeric α-actinin to stain the myocardium to examine the existence of DCF signal in the myocardium. The frozen myocardial sections (thickness, 5 μm) were incubated with anti-sarcomeric α-actinin antibody (1:250 mouse monoclonal IgG1; Sigma). The sections were incubated with Alexa 546-conjugated goat anti-mouse IgG1 secondary antibody (1:250; Molecular Probes, Eugene, Ore). Anti-sarcomeric α-actinin is specific for α-skeletal and α-cardiac muscle actinin, and does not bind to nonsarcomeric muscle elements such as the connective tissue and smooth muscle.[19] The fluorescence image was acquired using a laser scanning confocal microscope (LSM5 PASCAL; Carl Zeiss). The detailed description of the method is shown in online data supplement.

In Vitro Studies

Coronary arterial microvessels (n=16, 198±8 μm, at maximal diameter) were isolated from rabbit hearts (n=9; body weight, 2.1±0.1 kg) and cannulated with dual glass micropipettes in a vessel chamber (CH/2/M; Living Systems Instrumentation, Burlington, Vt) containing Krebs solution (stop flow, 60 cm H_2O of distending pressure, 38°C).

After the development of the spontaneous tone, the responses of coronary arterial microvessels to H_2O_2 (Wako) and sodium nitroprusside were examined, and the effect of extraluminal application of cell-impermeable catalase (500 U/mL) and the endothelial denudation were evaluated. Detailed description of the method is in the online data supplement.

Drugs

Cell-impermeable catalase (with ≤0.01 mg/mL of thymol as impurity), PEG-catalase, acetylcholine, LNNA, sodium nitroprusside, and H_2O_2 were freshly dissolved in physiological salt solution to the desired concentrations. Glibenclamide was dissolved with dimethyl sulfoxide at first, and then the target concentration was obtained by dilution with Krebs solution. The final concentration of dimethyl sulfoxide was 0.005 vol%. 8-PT was dissolved in 80% pure methanol and 20% 0.2 mol/L NaOH. This solution was diluted to 10 μmol/L. A 10 mmol/L stock solution of 2′,7′-dichlorodihydrofluorescein diacetate was prepared in ethanol on every experimental day and freshly diluted to 10 μmol/L with phosphate-buffered saline for the experiments.

Data Analysis

All variables are expressed as mean±SEM. In the bioassay studies, the vascular diameters were normalized to the maximal diameters defined as the vascular diameter in the presence of 100 μmol/L nitroprusside. When the spontaneous tone of DV did not develop (baseline diameter >90% of the passive diameter), the vessel was discarded. The diameter changes of DV and the differences in hemodynamic variables between baseline and tachypacing were compared by paired *t* test. Differences in the fluorescence intensity among each heart rate were statistically analyzed by 1-way ANOVA for repeated measures and Bonferroni corrections were applied. Differences in the maximal DV diameters, basal tone, and the percent change in the DV diameter from baseline among each group were compared by 1-way ANOVA. In the in vitro study, the vascular responses were expressed with the percentage of the maximal dilation caused by 100 μmol/L of nitroprusside, and the diameter

TABLE 1. Hemodynamic and Blood Gas Data of Dogs in Each Group

	Mean BP (mm Hg)	Rate-Pressure Product (10^2 mm Hg·bpm)	pO_2 (mm Hg)	pCO_2 (mm Hg)	pH
Vehicle group (n=11)					
HR: 120 bpm	87±8	135±8	96±5	36±1	7.38±0.01
HR: 240 bpm	86±8	261±19			
Catalase group (n=6)					
HR: 120 bpm	117±8	163±9	103±6	36±2	7.38±0.01
HR: 240 bpm	119±9	334±18			
LNNA+GC group (n=4)					
HR: 120 bpm	108±12	150±9	91±2	34±3	7.41±0.01
HR: 240 bpm	103±13	284±19			
8-PT group (n=4)					
HR: 120 bpm	114±4	160±6	97±16	32±1	7.35±0.00
HR: 240 bpm	111±6	297±14			

Values are means±SEM.
BP indicates blood pressure; bpm, beats per minute; HR, heart rate.
Vehicle group includes experiments detector vessels with (n=5) and without endothelium (n=6).

changes were statistically analyzed with 2-way ANOVA for repeated measures. Post hoc analysis with Bonferroni corrections was applied to detect the concentrations of the significant difference. $P<0.05$ was considered statistically significant.

Results

Hemodynamic and Blood Gas Data of Dogs

Although baseline mean aortic pressure was lower in vehicle group compared with other groups, it did not significantly change by tachypacing, and rate-pressure products (heart rate × systolic blood pressure) were doubled by tachypacing in each group (Table 1). The blood gases and pH were kept within physiological ranges during experiments.

Detector Vessel Responses to Cardiac Tachypacing

Vessel sizes and the basal tone of the detector vessels in the experimental groups are shown in Table 2. There were no differences in the vessel sizes of DV among 5 groups. There were no statistical difference in the developed basal tone between vehicle group (with endothelium) and any other groups. Endothelium denudation did not affect the basal tone.

Tachypacing significantly increased DV diameter in the vehicle group (Figure 1A). When the endothelium was removed, cardiac tachypacing again produced DV dilation (Figure 1B). In contrast, when cell-impermeable catalase was suffused onto DV, tachypacing-induced dilation was abolished (Figure 1C). In the presence of LNNA+GC or 8-PT, tachypacing resulted in DV dilation (Figure 1D, 1E). In the vehicle group, the percent change of DV by tachypacing was 8.2±0.8% with endothelium and 6.6±2.1% without endothelium, and there was no statistical difference between them (Figure 1F). When catalase was applied, the diameter change by tachypacing was −2.0±3.1% ($P<0.05$ versus vehicle groups). Neither LNNA+GC nor 8-PT inhibited the tachypacing-induced dilation (12.5±4.0% and 12.5±3.1%, respectively).

H_2O_2 Detection in the Myocardium

DCF fluorescence was increased by tachypacing (Figure 2A, 2B) and the fluorescence intensity increased in the heart rate-dependent manner (Figure 2C). The preincubation of the myocardium with PEG-catalase abolished the tachypacing-induced increase in the DCF fluorescence (Figure 2D), indicating the specificity of DCF signal for the H_2O_2 detection. In contrast, the cell-impermeable catalase, which was used in the bioassay experiments, did not decrease the myocardial DCF signal (Figure 2E).

Sarcomeric α-actinin and H_2O_2 staining were performed for the same section (Figure 3A, 3B), and the superimposed image (Figure 3C) revealed that H_2O_2 was colocalized with sarcomeric α-actinin, indicating that H_2O_2 was located in the myocardium.

TABLE 2. Vessel Sizes and Basal Tone of the Detector Vessels

	Vehicle With Endothelium (n=5)	Vehicle Without Endothelium (n=6)	Catalase (n=6)	LNNA+GC (n=4)	8-PT (n=4)
Maximal diameter (μm)	218±18	207±27	202±23	250±44	212±8
Baseline diameter (μm)	160±13	140±13	148±16	194±25	130±11
Basal tone (%)	73.9±1.9	70.3±1.7	73.3±2.2	79.7±5.3	61.0±3.3

Values are means±SEM.
Maximal diameter is detector vessel diameters in the presence of 100 μmol/L of sodium nitroprusside at 80 cm H_2O intraluminal pressure. Basal tone is detector vessel diameter normalized to the maximal diameters at heart rate of 120 bpm.

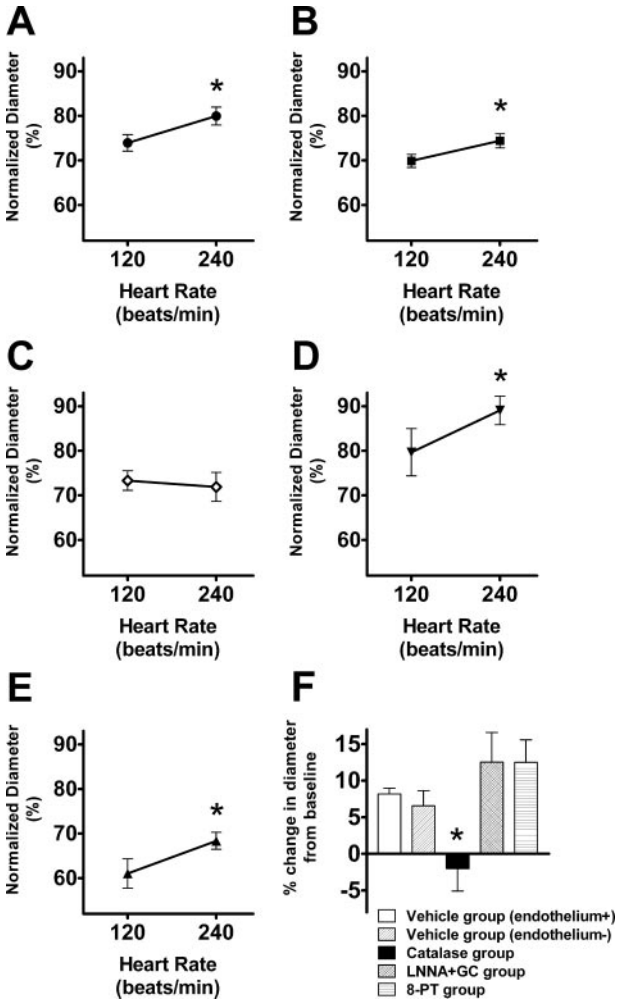

Figure 1. The tachypacing-induced detector vessel diameter changes in the vehicle group with endothelium-intact (A; n=5), with endothelium-denuded vessels (B; n=6), catalase group (C; n=6), LNNA+GC group (D; n=4), and 8-PT group (E; n=4). F: The percent change in diameter from baseline of the 5 groups. *$P<0.05$ vs heart rate=120 bpm (A, B, D, E) or between catalase group and the other groups (F).

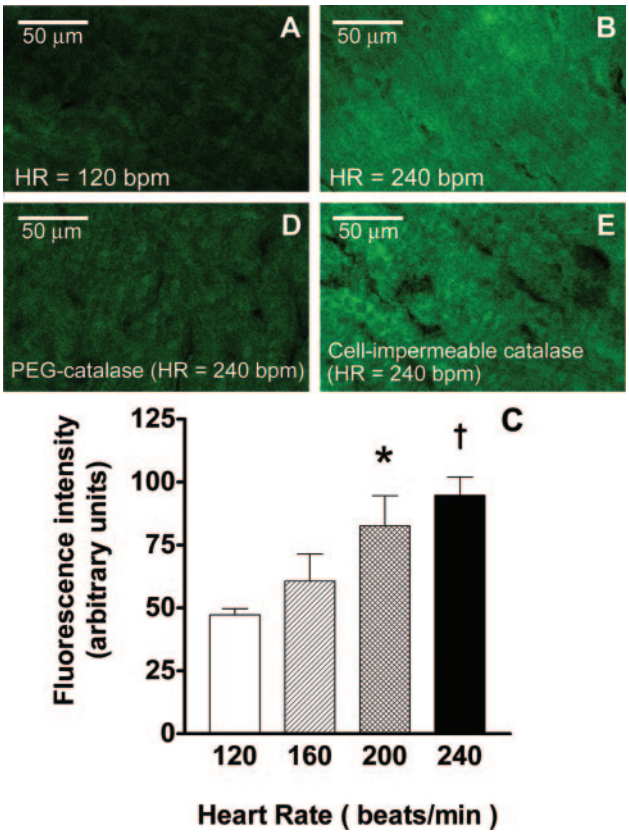

Figure 2. Tachypacing increased DCF fluorescence (A, B), and the increase was heart rate-dependent (C). The DCF fluorescence was abolished by PEG-catalase (D) but not by cell-impermeable catalase (E). *$P<0.05$ vs heart rate=120 bpm. †$P<0.01$ vs heart rate=120 bpm.

In Vitro Studies

The spontaneous vascular tone was developed in the isolated coronary arterial microvessels without any pharmacological preconstriction (66±2% of their maximal diameter). The microvessels dilated in response to the extraluminal application of H_2O_2 in a concentration-dependent manner and the dilation was abolished in the presence of cell-impermeable catalase (Figure 4A). Sodium nitroprusside caused dose-dependent dilation, and cell-impermeable catalase was without effect on the dilator responses (Figure 4B). We confirmed that the time-controlled microvascular responses to H_2O_2 in the absence of catalase were identical (data not shown). Endothelial denudation did not affect the vasodilation in response to H_2O_2 (Figure 4C).

Discussion

Methodological Considerations

The metabolic microvascular regulation is a complicated phenomenon because the flow increase by the metabolic factor increases shear stress in upstream vessels resulting in the enhanced release of the endothelium-derived relaxing factors, and the consequent endothelium-derived relaxing factor-induced dilation increases the distending pressure of the downstream microvessels, which could also affect microvascular tone.[20] Furthermore, the enhanced metabolism of the heart is often accompanied by the neurohumoral activation such as the sympathetic nervous system. All of those changes modulate the microvascular diameter changes and make it difficult to characterize the metabolic factor from the myocardium during the increased metabolism in vivo. However, the metabolic coronary dilation can be observed only in the in vivo setting. Our bioassay method resolves this dilemma because we can separately control the beating heart and DV. That is, the distending pressure and flow state (stop flow) of DV were kept constant throughout the experiment and DV was separated from the bloodstream and nervous controls. Thus, the present bioassay system is advantageous for the detection of the beating heart-derived metabolic factor.

H_2O_2 as a Vasoactive Mediator for Metabolic Dilation

H_2O_2 might have derived from DV in response to another mediator released from the beating heart. If it was the case, the vascular endothelium or vascular smooth muscle must have been responsible for the H_2O_2 release. However, the

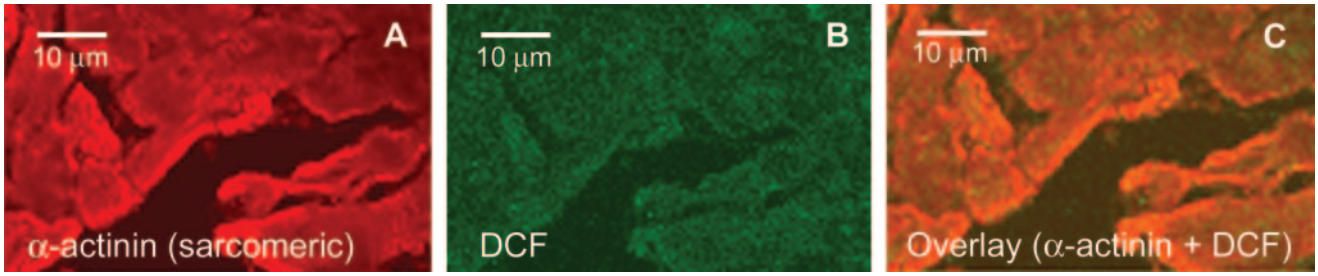

Figure 3. A, The immunohistochemical staining of sarcomeric α-actinin. B, DCF fluorescence of the same section. C, The superimposed image of (A) and (B).

former is unlikely because endothelial denudation did not have any effect on the detector vessel dilation. The latter is also unlikely, because we used cell-impermeable catalase in the bioassay. If H_2O_2 produced in the vascular smooth muscle itself caused dilation, cell-impermeable catalase should not have abolished the dilation. Accordingly, the beating heart, not DV, is responsible for the H_2O_2 release.

DCF fluorescence study showed that H_2O_2 in the beating heart is increased as the heart rate increases. Double staining of DCF fluorescence and sarcomeric α-actinin strongly suggests that the enhanced H_2O_2 release takes place in the myocardium, because the anti-sarcomeric α-actinin does not bind to the vascular smooth muscle.[19] Although we did not quantitate the exact tissue concentration of H_2O_2, Saito et al[10] have recently measured the H_2O_2 concentration in canine hearts using H_2O_2 electrode, reporting that it was ≈50 μmol/L at baseline (heart rate=140 bpm). In vitro studies in the present studies indicate the vasodilator effect of H_2O_2 on the coronary microvessels and the concentration-diameter curve for H_2O_2 demonstrates that 10 to 100 μmol/L of H_2O_2 produce coronary microvascular dilation. The magnitude of the microvascular dilation to the tachypacing was consistent with previous in vivo observations for the size class of microvessels we used (≈200 μm).[21] All of these data support the concept that H_2O_2 released from beating myocardium is a physiological mediator of metabolic coronary microvascular dilation. However, the possibility that H_2O_2 produced in the vascular endothelium of the beating heart takes part in the DV dilation during tachypacing cannot be ruled out because earlier studies showed that the increased shear stress stimulates H_2O_2 production from the vascular endothelium.[22]

The dilation of DV without endothelium was comparable with that of DV with endothelium, demonstrating that the metabolic microvascular dilation is endothelium-independent. The contribution of the endothelium to the H_2O_2-induced dilation is controversial. Thengchaisri et al[12] have recently demonstrated that H_2O_2 induces endothelium-dependent and endothelium-independent coronary arteriolar dilation in pigs, and suggested that the endothelial cyclooxygenase-1 partly mediates the H_2O_2 dilation. However, Rogers et al[23] reported that denuding the endothelium of canine coronary arteries or arterioles did not affect the H_2O_2 dilation and that 4-aminopyridine sensitive K_V channels had a role in the coronary arteriolar dilation to H_2O_2. The mechanism of H_2O_2-induced dilation may be species-dependent or vessel size-dependent.

We previously showed that pertussis toxin-sensitive G protein (G_{PTX}) in the microvascular wall plays a role in the coronary microvascular dilation in response to the increased cardiac metabolism in vivo.[21] It is possible that H_2O_2 activates a signaling pathway which involves G_{PTX}, although how H_2O_2 links to G_{PTX} is not clear at this point. There is a report that H_2O_2 acutely upregulates sphingosine-1-phosphate receptors, which activate G_{PTX}.[24] Further studies are needed to determine the signaling mechanisms of H_2O_2 derived from the myocardium.

Catalase we used in the bioassay and in vitro studies was cell-impermeable catalase. We validated that it does not decrease the intracellular H_2O_2, whereas PEG catalase does by performing DCF staining (Figure 2D, 2E). Therefore, it is

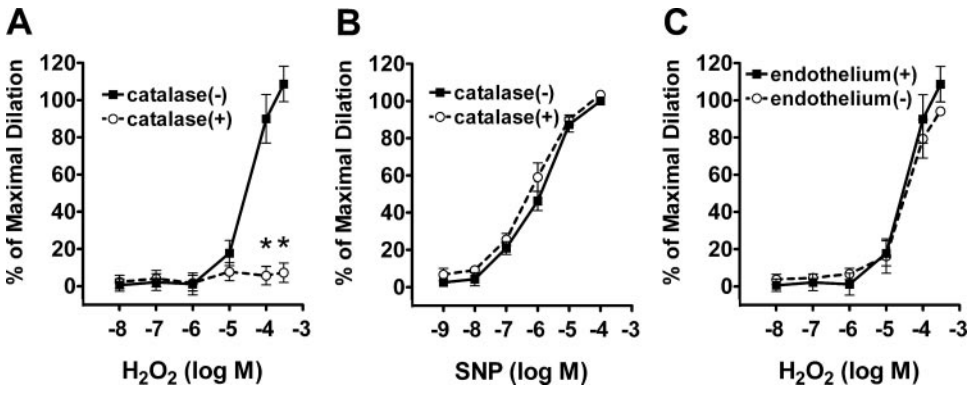

Figure 4. Effect of cell-impermeable catalase on the dose-response curves of exogenous H_2O_2 (n=5; A) and nitroprusside (n=6; B) in isolated rabbit coronary microvessels. Catalase (500 U/mL) abolished the H_2O_2-induced but not nitroprusside-induced vascular responses. C, Endothelial denudation did not alter the vasodilation to H_2O_2 (n=5 for each). *$P<0.05$ vs without catalase.

plausible that the cell-impermeable catalase quenched H_2O_2 in the space between the beating heart and DV. These results are in concert with the concept that H_2O_2 is a transferable vasoactive mediator released from the beating heart to the coronary microvessels.

Other Mechanisms

In the present experiments, none of the inhibitors of NO, K_{ATP} channels, or adenosine abolished the tachypacing-induced DV dilation. Our results are consistent with earlier studies. It has been shown that NO inhibitors do not impair the coronary flow increase during exercises[25,26] or tachypacing.[27] It is conceivable that NO does not play a major role in the metabolic flow increase, whereas it modulates redistribution of the coronary resistance from large microvessels to small ones during the increased cardiac metabolism.[27] Our previous study showed that K_{ATP} channels are likely to play an important role in determining coronary microvascular basal tone but not in dilating coronary microvessels during tachypacing.[28] Other researchers have also provided evidence that K_{ATP} channel blocker does not result in the impairment of flow response to increased cardiac metabolism.[3] Furthermore, adenosine is unlikely to mediate the metabolic coronary vasodilation under the normal perfusion, whereas it plays a role during impaired coronary flow state.[3]

Study Limitation

We suffused warm Krebs solution throughout the experiments at the rate of 1 to 1.5 mL/min to keep the bioassay system wet and warm. The space between the floating objective and the heart surface was always filled with the solution, which could dilute the vasoactive signals. Therefore, it is possible that the transferable vasomotor signals evaluated in our bioassay method may have been underestimated to some extent.

Because of the technical limitation, the size of DV was ≈150 μm in inner diameter with spontaneous tone. It is greater in size for the coronary microvessels that is most sensitive to metabolic factors.[4] The vascular wall of the smaller vessels was too thin to be detected on the beating heart. However, the microvessels ≈150 μm bear significant resistance;[29] therefore, we believe that the present results well demonstrate the physiological significance of myocardium-derived metabolic factor on coronary resistance regulation.

Clinical Implication

In the present study, we have demonstrated that H_2O_2, one of the ROS, is involved in the physiological regulation of coronary microvascular tone during enhanced cardiac metabolism. Redox homeostasis is delicately balanced by a network of ROS production systems and many scavenging systems. It has been recognized that ROS play pathological roles in many diseases. In the failing myocardium, oxidative stress is increased and appears to play a role in the pathogenesis.[30,31] The imbalanced redox homeostasis in the myocardium may affect the production and diffusion of H_2O_2 in the myocardium, leading to the impairment of the coupling between the myocardial metabolic state and coronary flow regulation.

Conclusion

We have demonstrated that the metabolically stimulated heart releases H_2O_2 that mediates the coronary microvascular dilation. H_2O_2 may be one of metabolic factors that links the myocardial metabolic state to coronary flow regulation. The concept that ROS plays an important role as a physiological signal for coronary flow regulation may provide a new insight for the understanding of the coronary regulation and the pathophysiology of various diseased conditions.

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Disclosure

During the preparation of this manuscript, the study elucidating the role of H_2O_2 in metabolic coronary dilation was published.[10]

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Arteriosclerosis, Thrombosis, and Vascular Biology

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Hydrogen Peroxide Derived From Beating Heart Mediates Coronary Microvascular Dilation During Tachycardia

Yasunori Kokusho, Tatsuya Komaru, Satoru Takeda, Katsuaki Takahashi, Ryoji Koshida, Kunio Shirato and Hiroaki Shimokawa

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Bioassay method

For the preparation of the detector vessel (DV), male Japanese white rabbits (n=25, body weight 2.0±0.0 kg) were anesthetized and anticoagulated with an intravenous administration of pentobarbital sodium (50 mg/kg) and heparin (1,000U), respectively, via the marginal ear vein. After the rabbits were sacrificed by bleeding from the carotid artery, the hearts were quickly excised and immersed in chilled Krebs solution (in mmol/L: NaCl 120.0, KCl 4.7, $CaCl_2$ 2.0, $MgSO_4$ 1.17, $NaHPO_4$ 1.20, calcium disodium EDTA 0.02, $NaHCO_3$ 25.0, glucose 5.0, pH adjusted to 7.40 by bubbling with 95% O_2 and 5% CO_2). A coronary arterial microvessel of the left ventricle (100-300 μm) was carefully isolated and used as DV. One end of the vessel was cannulated to a polyethylene micropipette filled with filtered Krebs solution, the tip of which was tapered (<100 μm), and tied with a monofilament of silk suture thread. The micropipette was connected to a polyethylene tube and to a pressure reservoir. The other end of the vessel was completely ligated. Vessels with any leakage were not used for further experiments. DV was incubated in warm (38°C) Krebs solution until use.

In 6 DVs, the endothelium was removed as previously described (1). Briefly, 2 mL of air was injected into the lumen of the vessel via the micropipette, and the vessels were

slowly perfused with the Krebs solution at 40 cmH_2O for 10 min to clear the debris before the ligation.

Beagle dogs of either sex (n=25, body weight: 5.8±0.2 kg) were used and prepared as described in our previous studies (2-4). Briefly, the dogs were anesthetized with α-chloralose (60 mg/kg, i.v. Wako Pure Chemicals, Osaka, Japan) and artificially ventilated. The right jugular vein and the carotid artery were cannulated for the administration of anesthetics and for the measurement of the aortic pressure, respectively. Thoracotomy and pericardiotomy were performed to expose the heart surface. The heart was paced at 120 beats/min by left atrial pacing after the suppression of the sinoatrial node with formaldehyde injection. Two 22-gauge, stainless-steel needles were inserted horizontally (5 to 7 mm apart) into the mid-myocardium of the left ventricle. Both ends of each needle were fixed to a needle holder. This apparatus limits excessive movement of the heart, thereby keeping the region of interest within the observation field.

The incubated DV was gently placed on the beating left ventricle of the dog and pressurized to 80 cmH_2O to produce intrinsic tone. Only one DV was used for each experiment. DV and the heart surface were kept moist throughout the experiment by continuous suffusion of Krebs solution (38°C) at a rate of 1-1.5 mL/min with an infusion pump (model STC-521, Terumo, Tokyo, Japan).

For visualization of DV placed on the beating left ventricle, an intravital microscope system equipped with a floating objective and a charge-coupled device (CCD) camera (HV-D28S, Hitachi, Tokyo) was used. This optical system was originally developed in our laboratory (5) for the visualization of epimyocardial coronary microvessels in vivo. The microscope objective was a Leitz model PL-fl (×10, numerical aperture: 0.30). An objective lifter was used to carefully adjust the distance between the objective and DV, so as not to compress DV. The distance between the floating objective and the heart surface was ~0.5 mm, and that space was filled with Krebs solution, which was suffused as described above. The spatial resolution of this optical system was 2 μm.

Epi-illumination with a mercury lamp was applied to obtain the DV images. Obtained images were recorded with a digital video cassette recorder (DSR-20, Sony, Tokyo) and were captured on a PC monitor, and the inner diameters were measured with Scion Image (version β4.0.2, Scion, Frederick, MD) at least three times during the end-diastolic phase.

H_2O_2 detection

In 5 anesthetized open-chest beagle dogs, epimyocardial biopsy of the left ventricle was performed at the heart rates of 120, 160, 200 and 240 bpm every ten

minutes after all hemodynamic variables and blood gas parameters had become stable using a biotome (Biopsy Forceps 504-300, Cordis). The obtained specimens were loaded with 10 μmol/L 2',7'-dichlorodihydrofluorescein diacetate (Sigma) for 60 minutes at 37°C in the dark. This dye is taken up by cells and metabolized by intracellular esterases to 2',7'-dichlorodihydrofluorescein and sequentially oxidized to fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of H_2O_2 (6). The specimens were frozen in the liquid nitrogen, subjected to cryosection (thickness: 20 μm) and placed on the glass slides. The fluorescent images were acquired using a laser scanning confocal microscope (C1, Nikon) with a ×40 objective at an excitation wavelength of 488 nm and emission wavelength of 522 nm. The fluorescence intensity was quantitated with an image analyzer (AxioVision v4.5.0.0, Carl Zeiss, Jena, Germany). In another set of experiments (n=2), the myocardial specimens were preincubated with PEG-catalase (1000 U/mL, Sigma), which is cell-permeable and easy to metabolize H_2O_2 to water in the cardiomyocyte, for 2 hours before the above-mentioned procedures to confirm the specificity of DCF signal for the H_2O_2 detection.

Immunohistochemistry

We performed immunohistochemistry for sarcomeric α–actinin to stain the

myocardium to examine the coexistence of DCF signal with the myocardium. After nonspecific binding was blocked with 10% goat serum for ten minutes, the frozen myocardial sections (thickness: 5 μm) were incubated with anti-sarcomeric α-actinin antibody (1:250, mouse monoclonal IgG1, Sigma) for 1 hour at room temperature. After rinsing three times with PBS, the sections were incubated with Alexa 546-conjugated goat anti-mouse IgG1 secondary antibody (1:250, Molecular Probes, Eugene, OR) for 1 hour at room temperature. The sections were washed three times in PBS and mounted with glass coverslips using ProLong®Gold mounting reagent (Molecular Probes). Anti-sarcomeric α-actinin is specific for α-skeletal muscle actinin and α-cardiac muscle actinin, and does not bind to non-sarcomeric muscle elements such as the connective tissue, smooth muscle, the epithelium and nerves (7). The fluorescence images were acquired using a laser scanning confocal microscope (LSM5 PASCAL, Carl Zeiss) with a ×40 objective at an excitation wavelength of 543 nm and emission wavelength of 573 nm.

In vitro studies

To confirm the vasoactive effect of hydrogen peroxide and the specificity of catalase, we performed *in vitro* study.

Coronary arterial microvessels (n=16, 198 ± 8 μm, at maximal diameter) were

isolated from rabbit hearts (n=9; body weight, 2.1 ± 0.1 kg) and cannulated with dual glass micropipettes in a vessel chamber (CH/2/M, Living Systems Instrumentation, Burlington, VT) containing Krebs solution. They were pressurized to 60 cm H_2O without flow throughout the experiment and bathed at 38°C by the continuous circulation of warmed Krebs solution through the vessel chamber. Vessels with any leaks were not used for further experiments. The microvessel chamber was transferred to the stage of an inverted microscope (DIAPHOT TM-O, Nikon) equipped with a CCD camera (HV-D28S, Hitachi, Tokyo, Japan). The images of the vessels were captured on a PC monitor, and the inner diameters were measured as described above.

After the inner diameters were reduced by 20-50% by the development of the spontaneous tone, the responses of coronary arterial microvessels to extraluminal administration of H_2O_2 (10 nmol/L to 300 μmol/L, Wako Pure Chemicals) and sodium nitroprusside (1 nmol/L to 100 μmol/L) was examined in the absence and presence of extraluminal cell-impermeable catalase (500 U/mL). In some cases, the endothelium of the isolated vessels was removed by the air injection as mentioned above and the endothelial denudation was verified by the lack of vasodilation in response to 1 μmol/L acetylcholine.

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