Hydrogen Peroxide Generated From Cardiac Myocytes Impacts Metabolic Dilation in Coronary Arterioles

Atsushi Otake,† MD, Shu-ichi Saitoh,‡ MD, and Yasuchika Takeishi,‡ MD

**Summary**

During oxidative cardiac metabolism, the myocardium produces reactive oxygen species, such as superoxide and hydrogen peroxide (H₂O₂). We hypothesized H₂O₂ is a coronary metabolic dilator linking regulation of coronary tone with myocardium metabolism. Dilation of isolated, pressurized coronary arterioles (76 ± 10 μm, diameter) in reaction to supernatant collected from enzymatically isolated cardiac myocytes was measured. Isolated rat myocytes were stimulated electrically [unpaced or stimulated at 200, 400 beats/min (bpm)]. H₂O₂ was significantly generated by pacing (400 bpm n = 11, 9.3 ± 0.4 μM P < 0.01, versus unpaced) and the addition of this supernatant caused vasodilation (500 μL to 2 mL bath, 14.6 ± 0.7%, P < 0.01 versus unpaced). Supernatant from unpaced myocytes was not vasoactive. To clarify the source of H₂O₂, myocytes were also stimulated at 400 bpm following treatment with Mn-TBAP (25 μM), which mimics the action of Mn-SOD, and apocynin (3 mM), an NADPH oxidase inhibitor (n = 11, each). Mn-TBAP increased H₂O₂ generation in myocyte supernatant stimulated at 400 bpm (12.2 ± 0.8 μM, P < 0.01 versus 400 bpm stimulation only). Treatment of the myocytes with Mn-TBAP augmented vasodilation by the stimulated myocyte supernatant (19.6 ± 1.1%, P < 0.01 versus untreated myocyte supernatant). Apocynin did not alter vasodilation to myocyte supernatant. These results suggest that the main source of superoxide by metabolic stimuli is cardiac myocytes and Mn-SOD is a scavenger from superoxide to H₂O₂. We conclude that H₂O₂ is a key metabolic vasodilator produced by myocardium. (Int Heart J 2010; 51: 125-128)

**Key words:** Cardiac myocyte, Coronary arterioles, Hydrogen peroxide

Although the principle driving force for the control of coronary blood flow is myocardial metabolism, this intimate relationship between cardiac metabolism and coronary blood flow may be modified by a variety of conditions and stimuli. However, the link between coronary blood flow and myocardial metabolism has not yet been unequivocally established. Recent investigations show that reactive oxygen species can play an important supportive role in regulatory vascular function. For example, hydrogen peroxide (H₂O₂) hyperpolarizes and dilates human coronary arterioles through opening of Ca²⁺-activated K⁺ channels. However, there is a paucity of information concerning the source of H₂O₂. During oxidative cardiac metabolism, cardiac myocytes produce superoxide. Superoxide is converted to H₂O₂ by superoxide scavenger (SOD). Thus, we hypothesized that the production of H₂O₂ by cardiac myocytes links cardiac metabolism to coronary vascular tone.

**METHODS**

All experimental procedures and protocols used in this study were reviewed and approved by the Animal Care and Use Committees of our institutions and conformed to the "Guiding Principles in the Care and Use of Animals" of the American Physiological Society.

**General procedures:** Male Wister rats (125-300g; Harlan Sprague Dawley) were anesthetized with pentobarbital sodium (65 mg/kg ip) and decapitated. The thorax was opened and the heart was removed and placed in ice-cold physiological saline solution (PSS). The hearts were then used for either dissection of coronary arterioles or isolation of cardiac myocytes.

**Isolation of cardiac myocytes:** Myocytes were isolated using a modified Langendorff set up. Briefly, the heart was perfused with buffer containing (in mM) 123 NaCl, 2.6 KCl, 1.2 KH₂PO₄, 7 MgSO₄, 1.2 H₂O, 25 HEPES, 11 glucose, 20 taurine, 20 creatine, and 1 CaCl₂ (pH 7.4) for 3 minutes, after which buffer was replaced by one with the same composition but without calcium for 6 minutes. Collagenase type II (0.6 mg/mL; Worthington) and CaCl₂ (30 μM) were then added, and the heart was perfused for another 15-18 minutes. The heart was then cut into small pieces and resuspended in perfusion buffer to which BSA (1%) was added. After 5 minutes, during which the suspension was gently titrated, the buffer containing the myocytes was filtered through surgical gauze to remove big clumps of myocytes and gently spun down. Calcium was reintroduced to the myocytes in a stepwise manner (200 μM, 500 μM,
and 1mM CaCl₂, respectively). The myocytes were allowed to settle under gravity between the CaCl₂ steps. The supernatant was removed and the pellet resuspended in buffer containing more calcium. Because rod-shaped living cells settle faster than dead cells, this increased the percentage of living cells in the suspension. Only preparations containing at least 80% rod-shaped cells were used.

Myocytes (500,000 cells in 4 mL) were stimulated for 20 minutes at different rates [0, 200, 400 beats/min (bpm)] in a custom-designed chamber. To determine the role of SOD and NADPH-oxidase, we prepared the 400 bpm stimulated myocyte supernatant pretreatment with Mn-TBAP (25 μM), mimic Mn-SOD, and apocynin (3mM), an inhibitor of NADPH oxidase. Myocytes were treated for 30 minutes in buffer containing agents as mentioned above, then supernatant was removed and the pellet resuspended in buffer without containing these agents to perform electric stimulation. Their production of hydrogen peroxide (H₂O₂) was measured by the withdrawal of the myocyte supernatant using an electrode system (Apollo 4000, WPI Inc., Florida). Apollo 4000 calculates the H₂O₂ concentration by measuring the small amount of current which is produced when H₂O₂ oxidation occurs.5,6

Dissecting of coronary arterioles: Single arterioles (42-112 μm passive diameter) were dissected from the left ventricle of the septum of the rat heart, as previously described,7 and placed in ice-cold PSS containing 1% BSA (USB-Amer sham). The PSS was composed of (in mM) 145.0 NaCl, 4.7 KCl, 2.9 CaCl₂, 1.17 MgSO₄, 1.2 NaH₂PO₄, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, and 3.0 3-(N-morpholino) propanesulfonic acid, buffered to pH 7.4 at 4°C and filtered (dissection buffer). Unless otherwise mentioned, all drugs were obtained from Sigma.

The vessels were cannulated on both ends with micropipettes (~20-60 μm outer diameter, depending on the size of the vessel) connected to pressurized reservoirs filled with PSS buffered at pH 7.4 at 37°C. The height of these reservoirs was set to obtain the desired intraluminal pressure (60 mmHg). Vessels that failed to maintain pressure were excluded from analysis. The internal diameter of coronary microvessels was measured with a videomicroscope (Zeiss Inverted Scope and Sony CCD-IRIS camera and videocaliper). The vessel was slowly warmed to 37°C and allowed to develop spontaneous tone.

**Protocol:** Aliquots of myocyte supernatant (100, 200, and 500 μL) were added to the vessel bath (2 mL vol). Vascular diameter was measured 5 minutes after the addition of the supernatant and after a 10 minute washout.

**Data analysis and statistics:** All statistical analyses were performed on StatView software (Abacus Concepts; Berkeley, CA). Data from the experiments were compared using ANOVA with repeated measures with Scheffe’s test as a post hoc multiple-comparison test. Vascular diameters were normalized to the diameter with tone before administration of the supernatant. Data are expressed as the mean ± SEM. Significance was accepted at P < 0.05 in all experiments.

**Results**

**H₂O₂ generation:** The H₂O₂ concentration of nonstimulated myocyte supernatant was 0.09 ± 0.01 μM (n = 11). H₂O₂ increased dramatically when the myocytes were electrically stimulated [200 bpm 6.9 ± 0.4 μM (n = 11), P < 0.01 versus nonstimulated myocyte supernatant, 400 bpm 9.3 ± 0.4 μM (n = 11), P < 0.01 versus nonstimulated or 200 bpm stimulated myocyte supernatant] (Figure 1). Pretreatment with Mn-TBAP increased the H₂O₂ concentration in 400 bpm stimulated myocyte supernatant (n = 11, 12.2 ± 0.8 μM, P < 0.01 versus 400 bpm stimulated myocyte supernatant). Pretreatment with apocynin did not change the H₂O₂ concentration of 400 bpm stimulated myocyte supernatant (8.5 ± 0.5 μM, n = 11) (Figure 2).

**Vasoactive properties:** Supernatant of cardiac myocytes was added to isolated coronary arteries, and their responses measured. Nonstimulated myocyte supernatant did not change the vessel diameter. Supernatant from myocytes stimulated at 200 bpm caused modest dilation (n = 11, 100 μL 2.0 ± 0.3%, 200 μL 4.3 ± 0.5%, 500 μL 6.7 ± 0.4%) compared with nonstimulated myocytes (P < 0.01, n = 11) and that from myocytes stimulated at 400 bpm resulted in a further increase in vasodilator properties of the supernatant (n = 11, 100 μL 5.1 ± 0.9%, 200 μL 8.9 ± 1.2%, 500 μL 11%)

**Figure 1.** H₂O₂ concentration in myocyte supernatant with electrical stimulation. H₂O₂ concentration in nonstimulated myocyte supernatant was negligible. Stimulation of myocytes at 200 and 400 bpm resulted in rate-dependent increases in H₂O₂ concentration (n = 11, each). P < 0.01 versus nonstimulated myocyte supernatant. # P < 0.01 versus 200 bpm stimulated myocyte supernatant.

**Figure 2.** H₂O₂ concentration in 400 bpm stimulated myocyte supernatant. Pretreatment with Mn-TBAP (25 μM) increased H₂O₂ concentration. Apocynin did not alter the H₂O₂ concentration. n = 11, each, P < 0.01 versus 400 bpm stimulated myocyte supernatant.
Coronary arteriole vasodilation by electrically stimulated myocyte supernatant. Dilation of coronary arterioles by myocytes was dose-dependent and increased significantly with increasing metabolic activity of the myocytes. Resting diameters of the vessels were $72 \pm 9.8$ (range: 42-108) $\mu m$ (nonstimulated), $78 \pm 9.2$ (50-118) $\mu m$ (stimulated 200 bpm), and $84 \pm 8.8$ (42-140) $\mu m$ (stimulated 400 bpm). $P < 0.01$ versus nonstimulated myocytes, $\# P < 0.01$ versus 200 bpm stimulated.

**Discusssion**

In our study, we have shown that adult rat cardiac myocytes produce H2O2 during cardiac metabolism. The production of H2O2 is proportionate to increases in cardiac metabolism. Supernatant of stimulated myocytes diluted the coronary arterioles dose-dependently. Mn-TBAP, which mimics the action of Mn-SOD, augmented not only H2O2 production in myocytes but also vasodilation induced by myocyte supernatant. Based on these results, we speculate that H2O2 released from cardiac myocytes is a coronary metabolic dilator linking regulation of coronary tone with myocardial metabolism.

Several recent studies have indicated that H2O2 is a potent dilator in coronary artery and there are multiple sources for production of H2O2. Although the myocardium is one important source of H2O2 in metabolism, coronary blood flow and myocardial metabolite H2O2 have not been unequivocally established as sources. In many organs and cells, including cardiac myocytes, superoxide is a product of oxidative metabolism by the 1 e- reduction of $O_2^-$. When $O_2$ is reduced and charged, it is no longer freely diffusible, and its diffusion is confined through large pores and anionic channels of the inner mitochondrial membrane to the space between the inner and outer mitochondrial membranes. There is a tremendous amount of Mn-SOD between the inner and outer membranes, and the activity is so high that all superoxide produced by the mitochondria will enter the cytosol as $H_2O_2$. H2O2 is an oxidant that has a short half-life because of the enzyme catalase, which metabolizes two molecules into 2H2O and O2. However, H2O2 is vasoactive, and can induce hyperpolarization of smooth muscle, and thus vasodilation, and we believe that during oxidative metabolism, the production of H2O2 links cardiac metabolism to coronary vascular tone. Our data support this hypothesis by showing that during increases in cardiac metabolism, cardiac myocytes produce increased amounts of H2O2, and that the vasodilatory properties of the fluid bathing working cardiac myocytes is abolished by catalase (data not shown).

NADPH oxidase, another putative source for generating oxygen free radicals, is a membrane-bound flavocytochrome present in many types of cells, including vascular endothelial cells. It uses both flavin and heme groups to shuttle electrons from NADPH to oxygen, yielding oxygen free radicals. To examine the involvement of NADPH oxidase in myocytes induced to vasodilation, we used apocynin, an inhibitor selective for the NADPH oxidase that acts by inhibiting incorporation of the p47phox subunit of NADPH oxidase into the membrane unit, thereby inhibiting enzyme function. We found that apocynin did not change the generation of H2O2 or myocyte induced vasodilation. These results indicate a lack of involvement of NADPH oxidase in myocyte induced vasodilation and H2O2 generation in the rat coronary microcirculation. Mitochondria serve as an important source for reactive oxygen species in many pathological conditions. The vast majority of ROS produced by cells originates from mitochondrial metabolism. The significant contribution of free radicals to metabolic dilation in coronary arterioles suggests a mechanism for preserving myocardial perfusion in disease states such as ischemia/reperfusion, diabetes, coronary artery disease, and hypertension where ROS generation is increased and dilator mechanisms involving NO may be inhibited.

**Conclusion:** H2O2 is a key metabolic vasodilator produced by working cardiac myocytes.
REFERENCES