Evidence for enhanced eNOS function in coronary microvessels during the second window of protection

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Kim SJ, Zhang X, Xu X, Chen A, Gonzalez JB, Koul S, Vijayan K, Crystal GJ, Vatner SF, Hintze TH. Evidence for enhanced eNOS function in coronary microvessels during the second window of protection. Am J Physiol Heart Circ Physiol 292: H2152–H2158, 2007. First published January 19, 2007; doi:10.1152/ajpheart.00326.2006.—Nitric oxide (NO) derived from endothelial NO synthase (eNOS) has been identified as a trigger for the second window of protection (SWOP), but its role as a mediator during the SWOP is a matter of debate. Eighteen mongrel dogs were chronically instrumented to measure left ventricular function, coronary blood flow, and wall thickening. Myocardial preconditioning was induced by 10 min coronary artery occlusion. After 24 h of reperfusion (during the SWOP), the hearts were excised. Coronary microvessels were isolated and incubated in presence of J) the endothelium-dependent agonists carbachol and bradykinin, 2) the calcium ionophore A23187, and 3) the angiotensin-converting enzyme (ACE) inhibitors enalaprilat and ramiprilat. Nitrite, a metabolite of NO, was measured. Under baseline conditions, nitrite production in microvessels from SWOP was 30% higher than that from normal (P < 0.01, respectively). Nitrite production in response to carbachol, bradykinin, and A23187 was also enhanced in microvessels from SWOP (P < 0.05). These enhanced responses were abolished by N3-nitro-l-arginine methyl ester (l-NNAME) or the endothelial receptor-specific antagonists atropine and HOE-140. The level of eNOS protein in the SWOP myocardium was twofold higher than that in the non-SWOP myocardium. Nitrite production in response to the ACE inhibitors was greater in microvessels from SWOP. These effects were blocked by l-NNAME, HOE-140, or dichloroisoucomarin (which inhibits kinin formation). We found that a brief ischemic episode induced delayed, enhanced NO production in coronary microvessels and an upregulation of eNOS protein. These findings suggest that eNOS is a mediator during the SWOP. The ability of ACE inhibitors to enhance NO release during the SWOP points to an additional clinical application for these drugs.

ischemic preconditioning; coronary circulation; endothelium; nitric oxide; angiotensin-converting enzyme inhibitors

ISCHEMIC PRECONDITIONING is the phenomenon by which a single brief period of ischemia producing reversible myocyte injury increases resistance to lethal myocyte injury following a subsequent, more sustained, period of ischemia (31). The early ischemic preconditioning (classic preconditioning) occurs early and is relatively short acting, i.e., wanes after 1–2 h (20, 29), whereas late ischemic preconditioning (a second window of protection, SWOP) occurs within 6 h after the initial ischemic stimulus and persists for a few days (27, 30). This delayed protection has been primarily attributed to upregulation or de novo synthesis of proteins (4). One protein that has been implicated in SWOP is nitric oxide (NO) synthase (NOS) via its ability to regulate NO production. NO derived from inducible NOS (iNOS), and mostly contained within the myocytes, is an important mediator of SWOP (9, 17, 40, 42). NO derived from endothelial NOS (eNOS) has been identified as a trigger for the SWOP (7, 14, 42); however, its role as a mediator during SWOP is a matter of debate (3, 7, 25, 28, 41).

Our previous study demonstrated enhanced coronary blood flow responses to the endothelium-dependent vasodilators acetylcholine and bradykinin during the SWOP in the intact conscious dog (25). These findings are suggestive of an upregulation of NO and an enhancement of NO release in the coronary microcirculation, which would support a role for eNOS as a mediator in the coronary vessels during the SWOP. However, they could also be due, at least in part, to an enhanced activation of non-NOS-dependent coronary vasodilating pathways, such as prostaglandins and hyperpolarizing factor, or to augmented dilation of large coronary conducting vessels.

The current study was performed to directly test the hypothesis that NO production is enhanced in coronary microvessels (resistance vessels) during the SWOP. This was accomplished by subjecting a region of the canine heart to a single brief period of ischemia followed by 24 h of reperfusion, removing the heart, isolating the coronary microvessels, and comparing baseline and drug-induced NO production in these microvessels to normal coronary microvessels. The drugs investigated were the endothelium-dependent agonists carbachol and bradykinin and the receptor-independent agonist A23187 (calcium ionophore). In addition, the change in eNOS protein expression during the SWOP was determined. Studies were also performed using angiotensin-converting enzyme (ACE) inhibitors, which are widely used clinically (1, 11a, 39a) and known to stimulate NO production in the vascular endothelium (2, 45).

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MATERIALS AND METHODS

*In vivo studies.* The study was conducted after approval from the Institutional Animal Care Committee. The animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, Revised 1996).

Eighteen dogs of either sex, weighing 25–30 kg, were sedated with acepromazine (0.3 mg/kg im) and anesthetized with pentobarbital sodium (25 mg/kg iv). With the use of sterile surgical techniques, the heart was exposed through a left thoracotomy at the fifth intercostal space and instrumented as described previously (25, 26). Briefly, Tygon catheters were implanted in the descending aorta and in the left atrium for the measurement of respective pressure. Left ventricular (LV) pressure was measured by implanting a solid-state miniature pressure gauge (model P7, Konigsberg Instruments, Pasadena, CA) in the left ventricle through an apical incision. A hydraulic occluder was placed around the left circumflex coronary artery to induce regional myocardial ischemia as described previously (25, 26). A pair of 5-MHz ultrasonic crystals was implanted transmurally in the anterior (control) and posterior (ischemic) regions of the LV free wall for measurement of regional myocardial wall thickening (WT).

At least 10–14 days of recovery from surgery were allowed before initiating the SWOP protocol in seven animals. Just before coronary artery occlusion (CAO) was induced, the animals received an injection of morphine sulfate (0.2 mg/kg im). After control measurements were recorded, myocardial ischemia was produced by infusing the hydraulic occluder. The coronary occlusion was maintained for 10 min, after which time the occluder was deflated permitting reperfusion. Bolus injections of 2% lidocaine were made to prevent premature ventricular contractions during ischemia-reperfusion. Measurements of hemodynamic variables [aortic pressure, LV pressure, left atrial pressure, rate of rise of LV pressure (dP/dt max), LV anterior and posterior wall thicknesses, heart rate, and lead II ECG] were obtained at baseline, during the CAO, and 1 h after restoration of coronary blood flow. In seven animals (Normal group), a sham occlusion of the coronary artery was performed; otherwise, the animals were treated identically to those in the SWOP group. To confirm the effectiveness of our protocol to produce SWOP, four animals underwent 10 min CAOs 24 h apart, and the recovery of contractile function was evaluated. To confirm the effectiveness of our protocol to produce SWOP, four animals underwent 10 min CAOs 24 h apart, and the recovery of contractile function was evaluated. To confirm the effectiveness of our protocol to produce SWOP, four animals underwent 10 min CAOs 24 h apart, and the recovery of contractile function was evaluated.

**Baseline CAO CAR (1 h)**

<table>
<thead>
<tr>
<th></th>
<th>MAP, mmHg</th>
<th>LVSP, mmHg</th>
<th>LVEDP, mmHg</th>
<th>+dP/dt max, mmHg/s</th>
<th>HR, beats/min</th>
<th>CBF, ml/min</th>
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<tbody>
<tr>
<td>Baseline</td>
<td>102±5</td>
<td>126±6</td>
<td>7±1</td>
<td>2,923±124</td>
<td>92±6</td>
<td>30±6</td>
</tr>
<tr>
<td>CAO</td>
<td>122±6</td>
<td>143±6</td>
<td>16±1*</td>
<td>2,389±216</td>
<td>139±13*</td>
<td>0*</td>
</tr>
<tr>
<td>CAR (1 h)</td>
<td>104±5</td>
<td>128±6</td>
<td>7±1</td>
<td>2,844±303</td>
<td>99±9*</td>
<td>30±2</td>
</tr>
</tbody>
</table>

*WT% change from baseline*

<table>
<thead>
<tr>
<th></th>
<th>AWT</th>
<th>PWT</th>
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<tbody>
<tr>
<td>Baseline</td>
<td>–3±12</td>
<td>–112±14*</td>
</tr>
<tr>
<td>CAO</td>
<td>2±12</td>
<td>–62±15*</td>
</tr>
</tbody>
</table>

Data are means ± SE, n = 7. CAO, coronary artery occlusion; CAR, coronary artery reperfusion; MAP, mean aortic pressure; LVSP, left ventricular peak systolic pressure; LVEDP, end-diastolic pressure; +dP/dt max, maximal rate of LV pressure change; HR, heart rate; CBF, coronary blood flow; WT, wall thickening; AWT, anterior wall thickening; PWT, posterior wall thickening. *P < 0.05 vs. baseline.
exposures were in the linear range of detection, and the intensities of the resulting bands were quantified by densitometry (ImageQuant 5.2).

**Data analyses.** Data are reported as means ± SE. Differences between means were considered statistically significant if the probability of their occurring by chance was <5% (P < 0.05). Hemodynamic changes within an experimental group were evaluated using a one-way ANOVA for repeated measures combined with the Student-Newman-Kuels test for post hoc analysis. Comparisons of the data for microvessels from normal and SWOP groups and for wall thickness after the first and the second CAO were performed using the Student's t-test.

**RESULTS**

Table 1 presents values for regional myocardial function and systemic hemodynamic variables for the SWOP group before CAO, during occlusion, and after 1 h of reperfusion. The coronary occlusion reduced blood flow to zero; flow had returned to the baseline value after 1 h of reperfusion. The occlusion converted normal myocardial WT into paradoxical thinning in the posterior (ischemic region). Posterior WT remained depressed even after 1 h of reperfusion, indicating myocardial stunning. WT in the anterior (control) region was unaffected by the coronary occlusion. Coronary occlusion was associated with increases in mean aortic pressure, LV systolic and end-diastolic pressures, and heart rate. LV dP/dt max tended to decrease, but this effect did not achieve statistical significance. After 1 h of reperfusion, all systemic hemodynamic values had returned to baseline levels.

Figure 1 compares the recovery of WT for the two CAOs 24 h apart. The extent of paradoxical WT during the first and second CAO was identical, indicating comparable degrees of myocardial ischemia. Noteworthy was that WT recovered more rapidly following the second CAO. At 30 min of reperfusion following the second CAO, WT was 22 ± 7% below baseline versus 60 ± 11% below baseline at the same time point following the first CAO (P < 0.05). At 2 h of reperfusion following the second CAO, WT had returned to baseline, whereas it remained markedly reduced (−34 ± 8%) at the same time point following the first CAO.

Basal nitrite production in coronary microvessels in the SWOP group (96 ± 4 pmol/mg) was 30% greater than that from coronary microvessels in the normal group (74 ± 3 pmol/mg, P < 0.01). Figure 2A shows the effect of an increasing concentration of carbachol on nitrite production of coronary microvessels. At each concentration, carbachol caused greater nitrite production from microvessels in the SWOP group compared with that in the normal group. At the highest concentration of carbachol (10⁻⁵ M), nitrite production was 47% higher in microvessels from the SWOP group. The increased production of nitrite caused by carbachol was abolished by L-NAME or atropine (Fig. 2B). In similarity to the findings with carbachol, both bradykinin and the calcium ionophore A23197 (Figs. 3 and 4) caused greater production of nitrite from microvessels in the SWOP group compared with that from microvessels from the normal group. The increased release of nitrite by bradykinin and A23197 was abolished by L-NAME or HOE-140.

**Response to Carbachol (CCh)**

![Fig. 2. A: increase in nitrite production in response to carbachol (CCh) for coronary microvessels from previously ischemic myocardium (second window of protection, SWOP, n = 7 animals) compared with that from normal myocardium (Normal; n = 6 animals), showing an upward shift of the dose-response curve. B: enhanced production of nitrite caused by the highest concentration of carbachol (10⁻⁵ M) in microvessels from SWOP group was abolished by N⁶-nitro-L-arginine methyl ester (L-NAME, 10⁻⁴ M) or atropine (10⁻⁵ M). *P < 0.05 vs. respective baseline. †P < 0.05 vs. Normal. Values are means ± SE.](http://ajpheart.physiology.org/)

![Fig. 1. A comparison of changes in wall thickness (WT) following release of two coronary artery occlusions (CAO) 24 h apart. The results indicate a more rapid recovery of WT during reperfusion following the second CAO. CAR, coronary artery reperfusion; B, baseline. *P < 0.05 vs. respective value of the first CAO. Values are means ± SE; n = 4 animals.](http://ajpheart.physiology.org/)
Figure 5 presents representative Western blots and a summary of the eNOS protein results. It shows that the level of eNOS protein was increased twofold in myocardium obtained from the previously ischemic region compared with the nonischemic region in the same hearts.

The effects of the ACE inhibitors ramiprilat and enalaprilat on nitrite release from the coronary microvessels were similar; thus the data were combined (Fig. 6). The ACE inhibitors caused more pronounced increases in nitrite production from microvessels in the SWOP group compared with those in the normal group (Fig. 6A). This enhanced nitrite production in response to the ACE inhibitors was abolished by L-NAME, HOE-140, or DCIC (Fig. 6B).

**DISCUSSION**

The main findings of this study were that a brief ischemic episode (preconditioning stimulus) induced 1) a delayed enhanced release of NO from coronary microvessels, which occurred under baseline conditions, as well as in response to receptor-dependent agonists (carbachol and bradykinin), a receptor-independent agonist (calcium ionophore), and ACE inhibitors; and 2) an upregulation of eNOS protein in previously ischemic myocardium. Most prior studies on the SWOP used multiple ischemic preconditioning stimuli. In the current study, we used a single 10-min period of ischemia. A more rapid recovery of contractile function, i.e., attenuated myocardial stunning, following the second 10-min CAO (Fig. 1) provided evidence that our protocol was sufficient in the conscious state to produce the SWOP.

Enhanced biosynthesis of NO has been shown to play a role in late ischemic preconditioning. Considerable effort has been undertaken to determine which NOS is upregulated during the SWOP (3, 8, 19, 21, 25, 26). Studies in a variety of animal models in our laboratory and others (7, 14, 17, 26) have provided convincing evidence for involvement of iNOS from myocytes. This includes 1) an increased iNOS activity in samples of myocardium and in isolated myocytes (42), 2) the ability of treatment with selective iNOS inhibitors, e.g., amino- guanidine and S-methylisothiourea sulfate, to attenuate the SWOP (as well as the increase in iNOS activity) (9, 19, 40), and 3) an abrogation of SWOP in iNOS knockout mice (17).

A role for eNOS in the mediation of cardioprotective effects during the SWOP is more controversial. Arguing against a role for eNOS are studies in rats and rabbits showing increased cardiac iNOS and nNOS expression, but not eNOS expression, during the SWOP (9, 17, 19). On the other hand, an increased eNOS expression was demonstrated during the SWOP in pig hearts (3). An upregulation of eNOS was observed in mouse...
hearts preconditioned with the mitochondrial ATP-sensitive K⁺ channel opener diazoxide (41). Delayed preconditioning-induced endothelial protection was shown in rat hearts that could be abolished with L-NAME but not with selective nNOS or iNOS inhibitors, implying involvement of NO produced by eNOS (28). Finally, in a previous in vivo study in dogs (25), we observed augmented baseline release of NO metabolites (nitrate and nitrite) from the myocardium and enhanced coronary blood flow responses to the endothelium-dependent vasodilators acetylcholine and bradykinin during the SWOP. Discrepancies in the relative roles of iNOS and eNOS during the SWOP in previous studies are likely due, at least in part, to the use of different animal models and protocols in assessing the contribution of each NOS isoform. The advantage of our approach was that it provided the unique opportunity to assess NOS-induced responses from myocytes and coronary microvessels isolated from the hearts exposed to the same preconditioning protocol. In our previous report, we presented evidence for a role for iNOS contained within myocytes in the SWOP (26). In the present study, we showed that eNOS contained within the coronary microvessels is also involved.

Previous studies that used NO levels in the coronary venous effluent as an index of eNOS activity were limited by uncertainties relating to the cellular source of NO (25). The use of isolated coronary microvessels simplified interpretation of our findings since other cell types, e.g., myocytes and fibrocytes, were excluded (41, 42). Although our measurements of NO release did not distinguish among the various NOS isoforms, the upregulation of eNOS protein suggests that eNOS played a role. This is supported by our additional findings indicating that two different receptor-mediated agonists, carbachol and bradykinin, and a calcium ionophore augmented NO release from the microvessels. These latter results would seem to rule out iNOS, since its activity is not receptor or calcium mediated. Furthermore, iNOS is known to evoke substantial baseline arteriolar vasodilation and to show sustained activity resulting in tissue damage (18, 32). Neither of these responses was evident in vivo during the SWOP in our model (25). Neuronal NOS is expressed at very low levels in the heart (10).

The mechanism responsible for the apparent increase in eNOS function during the SWOP was beyond the scope of the present study. However, previous studies have demonstrated that eNOS is a novel substrate for AKT and thus that enhanced eNOS function may be associated with the PI3-AKT-eNOS signaling cascade (13, 15).

Augmented NO release from the coronary vascular endothelium could have important consequences relating to cardiac protection during ischemia-reperfusion. Among them are a preservation of endothelial cell integrity and an inhibition of platelet aggregation (no-reflow phenomenon), neutrophil infiltration and adherence, and release of cytokines (12, 34, 43). Furthermore, because NO relaxes coronary vascular smooth muscle, increased endothelial NO release may promote effective matching of myocardial blood flow with its oxygen demands and reduce the risk of coronary spasm (36, 37, 39).

Fig. 5. Representative Western blots (A) and summarized data (B) showing increased level of endothelial nitric oxide synthase (eNOS) protein in myocardium during SWOP. 1 day after CAO, the levels of eNOS protein were significantly increased in the previously ischemic myocardium (I) compared with the nonischemic myocardium (NI). *P < 0.05 vs. NI. n = 3 animals.

Fig. 6. A: increase in nitrite production in response to the ACE inhibitors (ACEI) ramiprilat and enalaprilat (both 10⁻⁷ M) for coronary microvessels from previously ischemic myocardium (SWOP, n = 7 animals) compared with that from normal myocardium (Normal; n = 7 animals). Findings for ramiprilat and enalaprilat were qualitatively similar and thus were combined. B: enhanced production of nitrate caused by the ACE inhibitors in microvessels from the SWOP group was abolished by L-NAME (10⁻⁴ M), HOE-140 (10⁻⁸ M), or dichloroisourocumarin (DCIC, 10⁻⁵ M). *P < 0.05 vs. respective baseline. †P < 0.05 vs. Normal. Values are means ± SE.
ACE inhibitors are widely used in the treatment of various cardiac diseases, including hypertension, congestive heart failure, and myocardial ischemia (1, 11a, 39a). The primary beneficial effect of ACE inhibitors is via inhibition of angiotensin II formation. However, ACE inhibitors have additional beneficial effects via actions on the kallikrein-kinin pathway. ACE inhibitors block kininase II, which increases local kinin concentration in many tissues, including the heart and blood vessels. This leads to the formation of NO and PGL2 via the endothelial B2 kinin receptors. Various lines of evidence link activation of the kallikrein-kinin pathway with a cardioprotective effect. For example, coronary sinus kinin concentration rapidly increased during ischemic preconditioning in dogs (33) [as well as in patients undergoing angioplasty (35)], whereas pharmacological blockade of the kinin receptors abolished the cardioprotective effect of preconditioning (11). Furthermore, the cardioprotective effect of preconditioning was abolished in B2 kinin receptor knockout mice and in transgenic rats with low levels of high-molecular-weight kininogen, which is converted to kinin by plasma kallikrein (44). In the present study, the ACE inhibitors (enalaprilat and ramiprilat) markedly enhanced nitrite production in coronary microvessels from myocardium subjected to ischemic preconditioning (Fig. 6) when compared with those obtained from normal myocardium. These effects were abolished by l-NAME, HOE-140, or DCIC, suggesting that ACE inhibitors stimulate NO production via the kallikrein-kinin system. The present findings imply that treatment with ACE inhibitors may function to enhance endothelial and cardiac protection during the SWOP via an eNOS-mediated, NO-dependent mechanism.

In summary, a brief ischemic episode induced delayed, enhanced NO production in coronary microvessels within the endothelial cells via upregulation of eNOS protein. This NO would be expected to provide protection to the endothelium itself, as well as to the surrounding myocytes, during ischemia-reperfusion. The ability of ACE inhibitors to enhance NO release from the vascular endothelium during the SWOP points to an additional clinical application for these drugs.

REFERENCES


GRANTS

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BRIEF ISCHEMIA INCREASES NO PRODUCTION IN CORONARY MICROVESSELS 24 HOURS LATER

H2157


