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Endothelial NO Synthase (eNOS) phosphorylation regulates coronary diameter during ischemia-reperfusion in association with oxidative stress

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Abstract

The link between endothelial nitric oxide synthase (eNOS) activation and vascular diameter during ischemia-reperfusion was investigated in the rat heart. After short (<30 min) and long (>45 min) time of ischemia conferred by coronary artery occlusion of the rats, reperfusion caused dilatation and constriction of arterioles, respectively. Partial oxygen pressure (pO_2) measurement of the heart by the electrode confirmed the hyper-perfusion and no-reflow phenomena during reperfusion, as well as myocardial ischemia. The vascular diameter was correlated with phosphorylation of Akt and serine 1177 residue of eNOS, and formation of NO-bound guanylate cyclase (GC) by immuoflorescence study. Western blotting confirmed the phosphorylation of eNOS-Ser1177 depending on ischemia time. The constriction during reperfusion after 45 min of ischemia is supposedly caused by the inhibition of Akt-mediated eNOS-Ser1177 phosphorylation, which was suppressed by a PKC inhibitor chelerythrine, or ROS scavengers N-2-mercaptopropionyl glycine (MPG) and 4,5-Dihydroxy-1, 3-benzenedisulfonic acid disodium salt (Tiron). However, an endothelin receptor antagonist BQ123 alleviated the vasoconstriction by increasing NO availability but not eNOS-Ser1177 phosphorylation. Thus, vascular patency is correlated with eNOS-Ser1177 phosphorylation in association with ROS, and PKC during reperfusion. Endothelin inhibits vasodilatation by reducing NO availability during reperfusion.

Keywords: *Ischemia-reperfusion, nitric oxide, reactive oxygen, vasoconstriction/dilation*

Introduction

Nitric oxide (NO), a key vasodilator, is generated by vascular endothelial NO synthase (eNOS) [1,2]. Reperfusion following brief and sustained ischemia induces hyper- and hypo-perfusion, respectively [6]. During early reperfusion, eNOS causes reactive hyperemia [7], but it is unknown as to whether eNOS persistently regulates flow during post-ischemic reperfusion.

The eNOS is primarily activated by phosphorylation at serine-1177 residue (Ser1177) by Akt that is activated by PI3 kinase-dependent phosphorylation in response to shear stress, insulin and H_2O_2 etc. [14–17]. By contrast, Protein Kinase C (PKC) down-regulates eNOS by phosphorylation at threonine-495 [2,18] or serine-116 [1]. The link between eNOS phosphorylation and vascular diameter during ischemia-reperfusion is the first goal of this study.

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Although reactive oxygen species (ROS) reduces perfusion through endothelial injury [8–10], it is unknown whether ROS acts on NOS. ROS is generated by NAD(P)H oxidase, mitochondrial respiratory chain [11,12], or even eNOS upon uncoupling under pathological settings [5]. Protein Kinase C (PKC) up-regulates NADPH oxidase [29]. On the other hand, superoxide anion ($O_2^{\cdot-}$) reacts with NO to form peroxynitrite ($ONOO^-$). $ONOO^-$ promotes reperfusion injury through nitrotyrosin modification of proteins [3–4], while it inactivates eNOS through zinc-thiosulfate modification [5].

Endothelin (ET)-1 promotes endothelial injury in ischemia-reperfusion, which is alleviated by ET-A receptor blockade [21,22], and is enhanced by PKC-dependent ROS production [9]. Thus, the second goal of this study is to investigate as to whether ROS, PKC, or ET-1 modulates eNOS phosphorylation during ischemia-reperfusion.

Materials and methods

N^G-Nitro-L-arginine methyl ester hydrochloride (L-NAME) was purchased from Wako Pure Chemical (Osaka, Japan), N-2-mercaptopropionyl glycine (MPG), chelerythrine-chloride and 4,5-Dihydroxy-1, 3-benzenedisulfonic acid disodium salt (Tiron) from Sigma (St. Louis, MO, USA) and endothelin (ET)-A receptor antagonist (BQ123) from Calbiochem® (Darmastadt, Germany). Anti-eNOS monoclonal antibody (mAb) and anti-phospho- Ser472/473/474-Akt polyclonal antibody (pAb) were obtained from BD Biosciences (San Diego, CA, USA), anti-phospho-Ser1177-eNOS pAb from Cell Signaling technology (Beverly, MA, USA), anti-phospho-Thr 495-eNOS-pAb and Ser116-eNOS pAb from Upstate Biotechnology (Lake Placid, NY, USA). The anti-NO-GC monoclonal antibody (mAb), specific for the GC underwent conformational change upon activation by NO, was produced as we reported [23]. FITC-labeled anti-mouse IgG (H + L) was obtained from Vector Laboratories (Burlingame, CA, USA), phycoerythrin (PE)-labeled anti-rabbit IgG (whole molecule) from Sigma, 2% normal horse serum and Vectashield mounting medium from Vector Laboratories (Burlingame, CA, USA).

The protocol for surgical procedure was approved by the committee of the animal experiments of Graduate School of Medicine, University of Tokyo. Male male Sprague-Dawley rats, aged 8 weeks, were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and ventilated by an artificial ventilator with room air through a bronchial tube, while the body temperature was maintained at 37–38°C, as previously reported [13]. After left thoractomy, ischemia was induced by occlusion of the left anterior descending coronary artery (LAD) with a 6–0 silk suture, and the reperfusion by its release.

For histological study, rats ($n = 10$ for each group) were randomly assigned to either of the sham, ischemia for 30 or 60 min followed by 60 min of reperfusion. After ischemia-reperfusion, the hearts were washed with physiological saline, fixed with 10% paraformaldehyde in phosphate buffer, embedded in paraffin, and sectioned at 3 μm. The sections were stained with Elastica-Masson.

For myocardial partial oxygen pressure (pO_2) monitoring, rats ($n = 10$ for each group) were assigned to either of ischemia for 30 or 60 min followed by 60 min of reperfusion. To measure pO_2, the rats were pre-treated with atropine (0.05 mg/kg, s.c.) and anesthetized with sodium pentobarbital (50 mg/kg, i.p.) plus ketamine (100 mg/ml, i.p.). The other procedures were the same as described above. The pO_2 was recorded by use of the Dual Channel Oxygen Monitor (AD Instruments, NSW, Australia), equipped with a data analyzer (PowerLab/8sp, AD Instruments, NSW, Australia). The pO_2 sensor and reference electrodes (AD Instruments, NSW, Australia) were inserted in the sub-epicardial portion in the risk area, and a cervical muscle, respectively. The pO_2 sensor electrode was inserted to the expected center of the ischemic-risk area at the left side of anterior left ventricular wall at about 1/3 of the height from the apex. After stabilization of pO_2 at 30 ~ 40 mmHg and body temperature at 37.0 ~ 38.0°C, kept by a hot plate on the back equipped with a thermostat (AD Instruments, NSW, Australia), for at least 10 min, LAD was occluded. As the ischemic area became pale, we could confirm that the electrode had been inserted around the center of the ischemic area.

For immunofluorescence study, rats ($n = 5$ for each group) were randomly assigned to either of the following groups: (1) sham, (2) ischemia for 15 min (I15), (3) ischemia for 30 min (I30), (4) I30 + NOS inhibitor L-NAME (30 mg/kg), (5) ischemia for 45 min (I45), (6) I45 + endothelin (ET)-A receptor antagonist BQ123 (0.5 mg/kg), (7) I45 + PKC inhibitor chelerythrine (5 mg/kg), (8) I45 + MPG (scavenger for $^{\cdot}OH$) (10 mg/kg), (10) I45 + Tiron (scavenger for $O_2^{\cdot-}$) (1 mg/kg). (11) ischemia for 60 min and (I60), with each of ischemic heart underwent reperfusion for 60 min, unless otherwise specified. The reagents were injected (i.v.) shortly before coronary occlusion, unless otherwise specified. To the groups (8), (9) and (10), the same doses of the reagents were re-injected before reperfusion and at 10 min of reperfusion. In the sham group, we performed the same procedure except for the LAD occlusion.

To confirm the ischemia area (particularly necessary for hyper-perfusion), the LAD was re-occluded and 2.0 ml of 0.3% Evans Blue in physiological saline was injected in the left atrium. We could confirm the ischemic area by dye-exclusion and separate ischemic from non-ischemia area. After the hearts were quickly removed, excised, rinsed in physiological saline, cut

horizontally into 3 portions, immersed in OCT compound (Sakura Finetek Inc, Tokyo, Japan), or the ischemic area was cut out for western blotting. The samples were snap-frozen in liquid nitrogen in both the cases.

The 8-μm-thick cryo-sections were placed on silan-coated slides (Matsunami, Osaka, Japan), fixed in acetone (for 10 min at room temperature), washed with Phosphate-Buffered Saline (PBS, pH 7.4) (3 times, 5 min each), blocked with 2% normal horse serum in PBS (for 60 min at room temperature), and then, incubated with anti-NO-GC monoclonal antibody (mAb, 1:1000) and either of diluted (1:1000) polyclonal phosphor specific antibodies (pAb)s to Ser-1177, Ser116, or Thr495 of-eNOS (1:1000), or phospho-Akt-Ser472/473/474 (1:1000) in 1% Bovine Serum Albumin (BSA)/PBS overnight at 4°C. The sections were washed with PBS and incubated with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG and phycoerythrin (PE)-labeled anti-rabbit IgG (1:10,000) in 1%BSA/PBS, washed with PBS, and mounted on glass slides with Vectashield mounting medium. The fluorescence images were acquired with the immunofluoresence microscopy (Axioskop 2 routine microscope, Zeiss, Jena, Germany), equipped with an image analyzer. As negative controls, sections were incubated either with secondary antibodies or non-immune IgG.

We measured the minimum diameters of the sub-epicardial arterioles (5 $\sim$ 6 for each of three sections for each heart) in the ischemic area, and measured the immunofluorescence intensity for each vessel under the microscope by use of the software equipped with the image analyzer (Image-Pro Plus Ver.4.0, Media Cybernetics, Maryland, USA). All arterioles in the ischemic area, except for few vessels sectioned obliquely, were observed. Each minimum diameter was measured, on each image, by use of an objective micrometer as a scale on each slide. The minimum diameter (mean $\pm$ SE) for each condition ($n = 5$) was calculated from the mean values for each heart (3 sections each). To semi-quantify the immunofluorescence intensity, we used In Speck™ Microscope Image Intensity Calibration Kits (InSpeck Red, InSpeck Green, Molecular Probes, Eugene, OR, USA).

Subcellular fractionation of the frozen hearts was performed, as we previously reported [24]. The 100,000 × g pellet is the membrane fraction [24], containing most of eNOS immunoreactivity. The protein concentrations were determined using protein

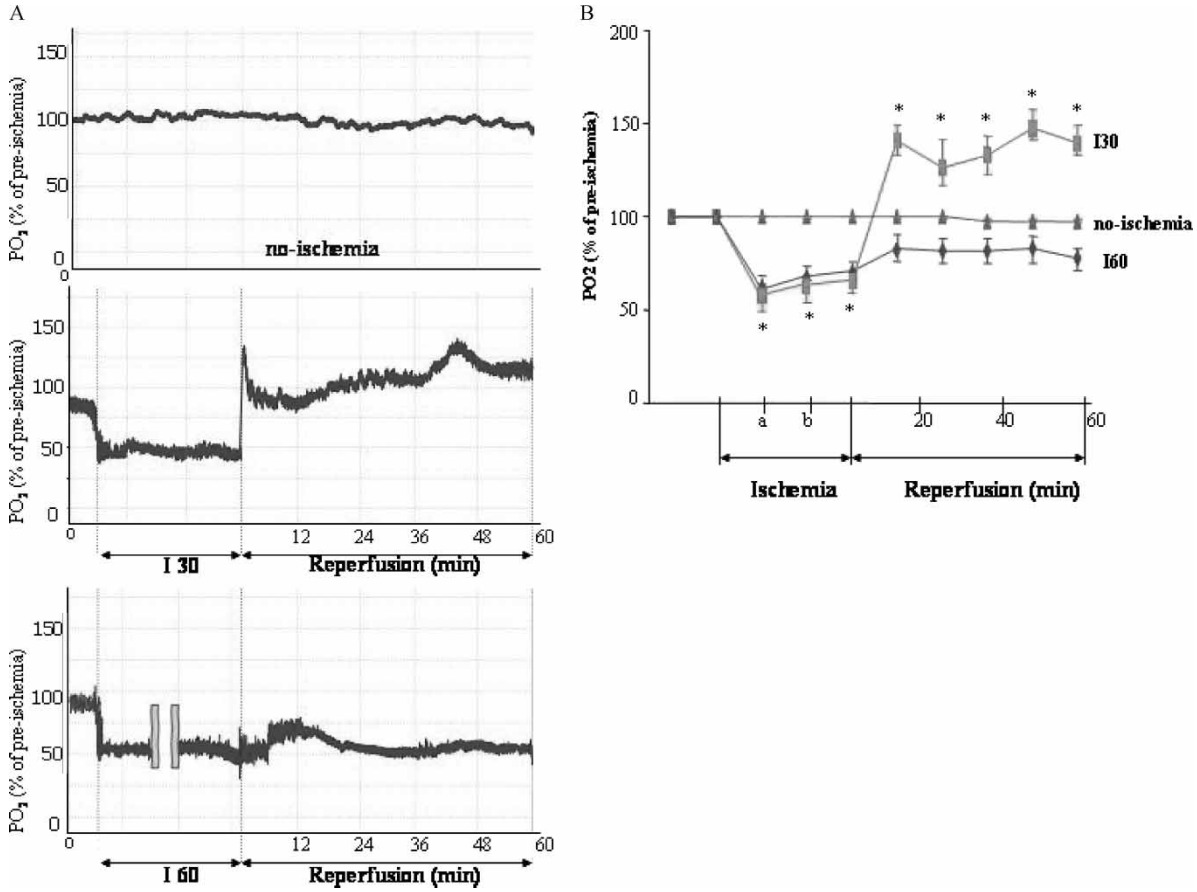

Figure 1. A: Representative patterns of reduced-oxygenation during ischemia, enhanced- and hypo-oxygenation of myocardium during reperfusion in sub-epicardium, following 30 and 60 min of ischemia (I30, I60), respectively, as detected by pO_2 electrode. B: Quantification ($n = 10$) of pO_2. (pre-ischemic value = 100%; a: Shortly after ischemia, b: At the middle of ischemia) (⋆: $P < 0.05$ vs sham).

assay reagent kit (PIERCE, Pockford, IL). SDS-polyacrylamide gel (6.5%) electrophoresis [25] and immunoblotting [26] were performed as previously described [13,24], with equal loading of protein (30 μg/lane), by use of the antibodies to various eNOS antibodies, listed as above.

Data are presented as means ± SDs or SEs of the ratios to the mean value of the sham. Statistical significance ($P \leq 0.05$) was determined by the analysis of variance (ANOVA) followed by the post-hoc analysis of Fisher.

Results

We monitored partial oxygen pressure (pO_2) by the electrode inserted into sub-epicardial myocardium. The reduction in pO_2 (oxygenation) directly indicates myocardial ischemia. The increased oxygenation in early phase of reperfusion after 30 min of ischemia reflects the "hyper-perfusion" (Figure 1A, B) [6,7]. Notably, the increase in pO_2 was enhanced toward the end of 60 min reperfusion (Figure 1A, B). By contrast, myocardial oxygenation was not increased during reperfusion following 60 min of ischemia (Figure 1A, B), representing "no-reflow" phenomenon [8]. This is the first successful demonstration of the temporal change in myocardial oxygenation state during ischemia-reperfusion *in situ*, caused by the change in blood flow.

Consistent with the pO_2 data, the arterioles visualized by Elastica-Masson staining of the formalin-fixed tissue showed vascular dilation after 30 min, and constriction after 60 min of ischemia (Data not shown). The long axis of the arterioles oriented variously, suggesting that the ovoid-shape is not due to the experimental artifacts. The coronary perfusion of formalin just before the end of reperfusion artificially dilated the vessels after ischemia for 60 min (data not shown). Accordingly, we performed immuno-fluorescence study on cryo-sections of non-fixed samples.

Effects of ischemia duration on eNOS phosphorylation and vaso-diameter after reperfusion were examined. Representative views of the arterioles in the ischemic area, as demonstrated by double immunofluoresence method using anti-phospho-Ser1177-eNOS and anti-NO-bound NO-GC antibodies, are shown in Figure 2A, while their quantifications and vascular diameters are shown in Figure 2B. The diameter of the arterioles ranged from 10 to 150 μm, in consistent with those of resistance vessels [27]. The NO-GC antibody specifically recognizes the GC that changed in conformation after activation by NO [23]. After 15 and 30 min of ischemia followed by 60 min of reperfusion, the vascular diameter was increased with a maximum vasodilatation at 30 min to about 180% of that of the sham. Reperfusion for 60 min following 45 and 60 min (to about 15% vs sham) of ischemia induced vasoconstriction. The vascular diameter correlated closely with the fluorescence intensities of eNOS phosphorylated at Ser1177 and NO-GC on vessels, at each ischemia time point (Figure 2). After ischemia for 30 min, reperfusion for 60 min induced greater vasodilatation than reperfusion for 30 and 180 min, whereas reperfusion for 30 and 60 min showed no difference in vasoconstriction after 45 min of ischemia (data not shown). Therefore, the hearts underwent reperfusion for 60 min following 15 and 60 min of ischemia in this study, unless otherwise specified.

To confirm eNOS phosphorylation, we performed western blotting of the membrane fraction (Figure 3A). Phosphorylation of eNOS at Ser1177 was enhanced after 15 and 30 min, but reduced after 45 and 60 min of ischemia (Figure 3B), in consistent with the immunofluorescence data (Figure 2). However, there was neither significant change in weak phosphorylation of eNOS at Thr495, nor any band of eNOS-Ser116 detected.

We examined the effect of NOS inhibitor on vasodilatation. A NOS inhibitor L-NAME inhibited the vasodilatation induced by 30 min of ischemia to the sham level (Figure 4). The vascular diameter correlated closely with the fluorescence intensity of

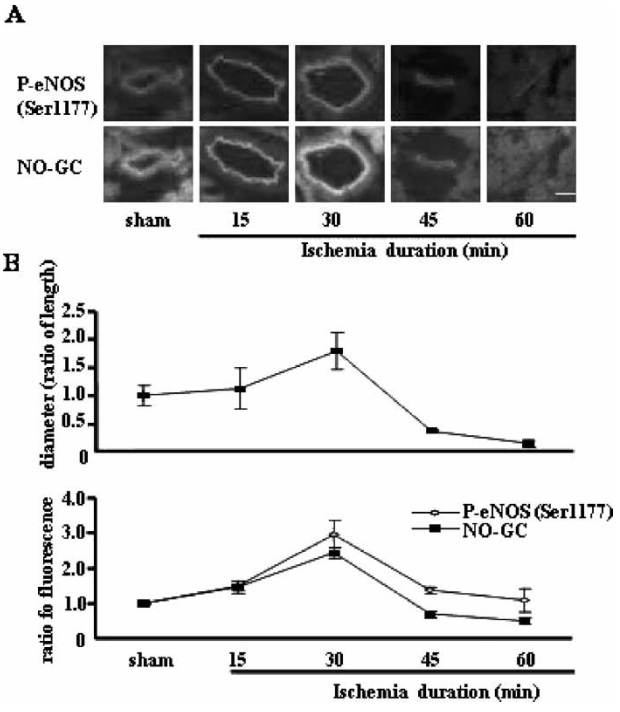

Figure 2. A: Representative immunofluorescence patterns of phospho-eNOS (Ser1177) and NO-bound guanylate cyclase (NO-GC) on the vessels in the sham, ischemia (I) for 15, 30, 45 and 60 min, each followed by 60 min of reperfusion. B: Quantifications of fluorescence intensity of phospho-eNOS-Ser1177, NO-GC and vascular diameters are shown as the ratio to the mean sham value ($\star P < 0.05$).

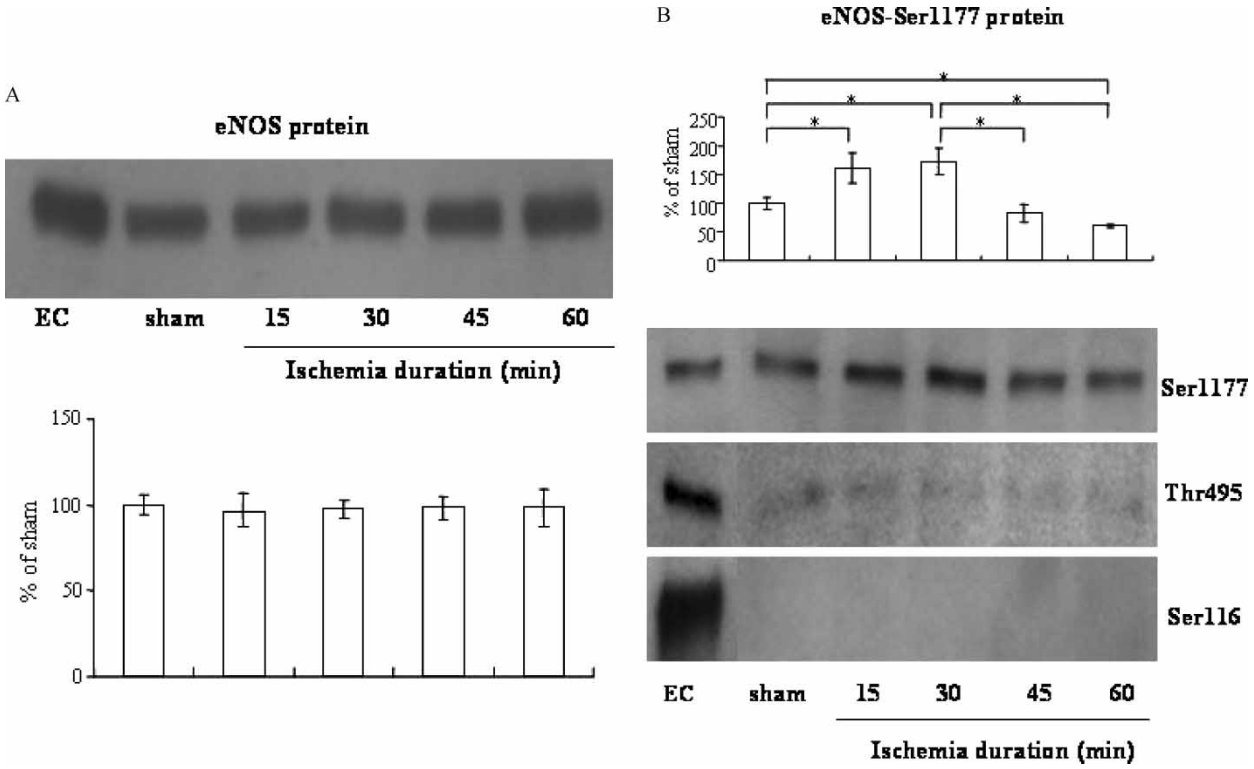

Figure 3. Representative western blots on eNOS and vascular diameters during ischemia (I) of various durations, and the quantification. A: Total eNOS. B: Phosphorylated form of eNOS at Ser1177, Thr495 and Ser116. The phosphorylation of eNOS at Ser1177 was enhanced at 15 and 30 min, but reduced at 45 and 60 min of ischemia, followed by reperfusion. The phosphorylation of eNOS at Thr495 was weak and not changed, while that at Ser116 was not detected (⋆: $P < 0.05$ vs sham). EC (human umbilical endothelial cell) is a positive control for eNOS.

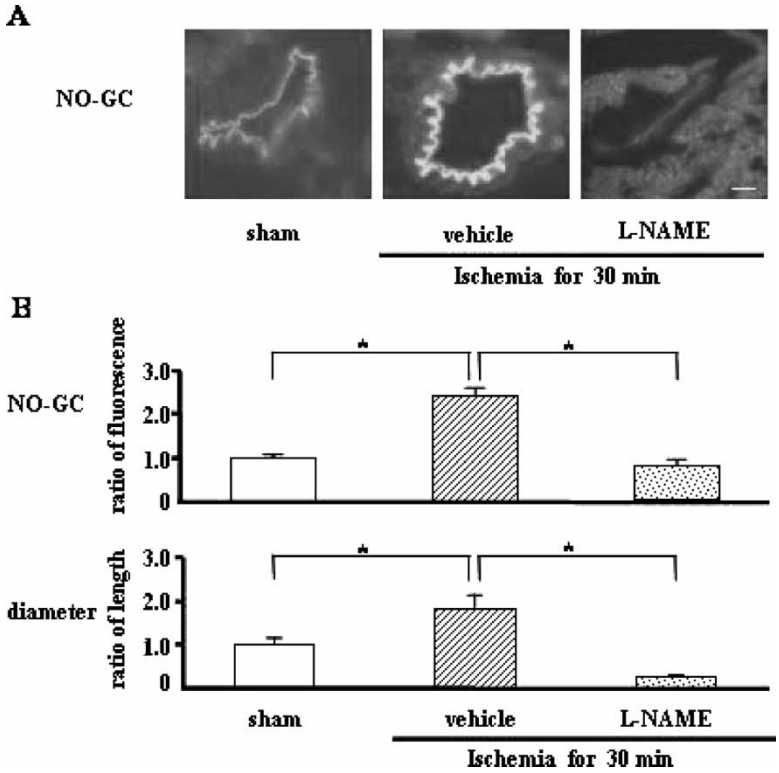

Figure 4. NOS inhibitor (L-NAME) inhibits dilation of arterioles after 30 min of ischemia (I) followed by 60 min of reperfusion and fluorescence of NO-bound NO-GC on the vessels. A: Representative arterioles. B: Quantifications of fluorescence intensity of NO-GC and vascular diameters shown as the ratio to the mean of sham (⋆$P < 0.05$ vs vehicle).

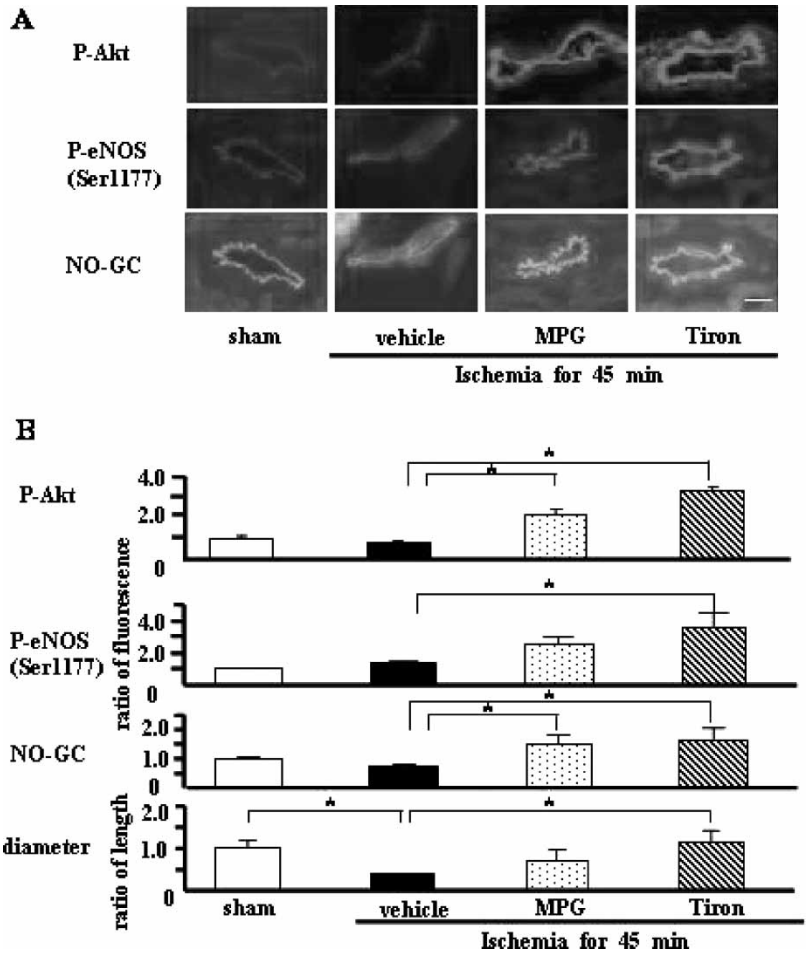

Figure 5. Effects of MPG or Tiron on vascular diameters and immunofluorescence of phosphor-Akt, phospho-eNOS-Ser1177 and NO-GC in the heart after ischemia for 45 min and reperfusion. A: Representative arterioles. B: Quantifications of fluorescence intensity on the arterioles and the diameters shown as the ratio to the mean of sham ($\star P < 0.05$ vs vehicle).

NO-GC on the vessels in the presence or absence of L-NAME, indicating that the vasodilation is caused chiefly by NOS activation through phosphorylation at eNOS-Ser1177.

The effect of ROS scavengers on vasoconstriction was examined. After 45 min of ischemia followed by 60 min of reperfusion, the vascular diameter was decreased to about 35% of that of the sham, which was alleviated by ROS scavenger MPG or Tiron (Figure 5). Tiron increased the vascular diameter and the fluorescence intensity of phosphorylated Akt, eNOS phosphorylated at Ser1177 and NO-GC on the vessels more effectively than MPG did (Figure 5A). These data suggest that ROS mediate vasoconstriction through inhibition of Akt-mediated phosphorylation of eNOS-Ser1177. On the other hand, the extent of vasoconstriction after 45 min of ischemia was greater than that of reduction in NO availability as shown by NO-GC fluorescence (Figure 5B), suggesting the contribution of a vasoconstrictor such as endothelin.

A selective PKC inhibitor (chelerythrine), or an ET-A receptor antagonist (BQ123) alleviated vasoconstriction after 45 min of ischemia. The increase in the vaso-diameter by each inhibitor correlated closely with the increase in fluorescence intensities of phospho-Akt, eNOS-Ser1177 and NO-GC on each vessel under each condition except for BQ123. BQ123 improved NO availability (NO-GC fluorescence) and vasoconstriction, but not phosphorylation of Akt and eNOS-Ser 1177, indicating that endothelin reduces NO availability downstream from Akt-mediated phosphorylation of eNOS at Ser1177 (Figure 6).

Phosphorylation of eNOS at Thr495, a PKC phosphorylation site, was unaltered during ischemia-reperfusion, and not affected by any reagent (Figure 7), showing that PKC does not directly induce vasoconstriction through modulation of eNOS phosphorylation.

Discussion

This study demonstrated the functional-morphological correlation between eNOS phosphorylation at Ser1177 and vascular diameter in the coronary arterioles in the rat heart after ischemia-reperfusion for the first time to our knowledge. The vasodilatation

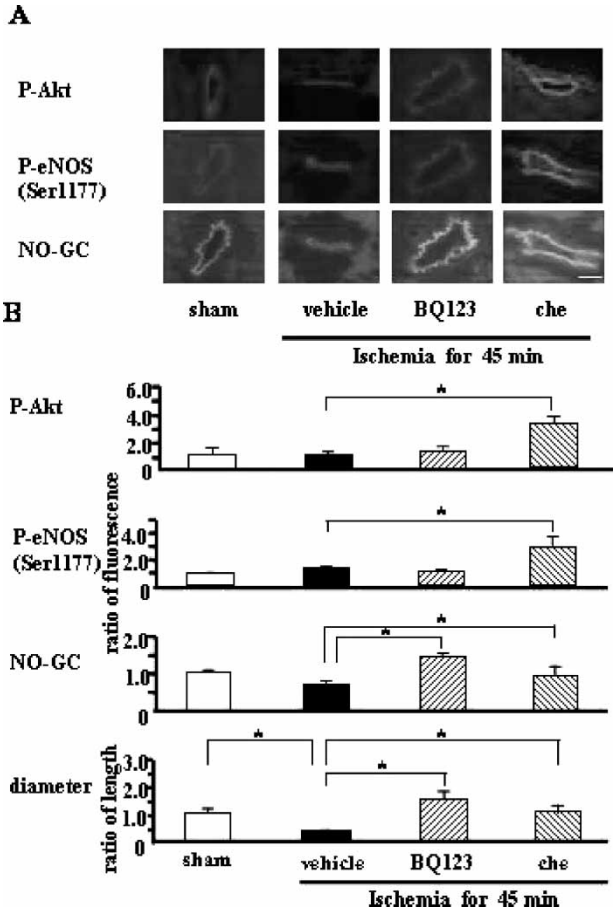

Figure 6. Effect of BQ123, or chelerythrine, on vascular diameters and immunofluorescence of phospho-Akt, phospho-eNOS-Ser1177 and NO-GC in the heart after ischemia for 45 min and reperfusion. A: Representative arterioles. B: Quantifications of fluorescence intensity on arterioles and diameters shown as the ratio to the mean of sham ($*P < 0.05$ vs vehicle).

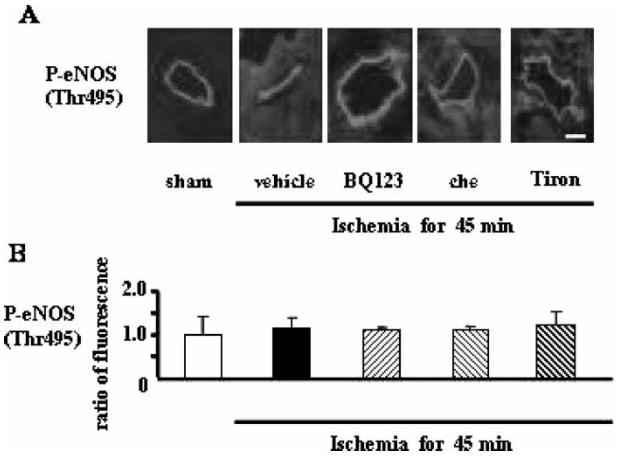

Figure 7. Effect of BQ123, chelerythrine or Tiron on immunofluorescence of phospho- Thr495 and in the heart after ischemia for 45 min and reperfusion. A: Representative arterioles. B: Quantifications of fluorescence intensity on the arterioles shown as the ratio to the mean of sham ($*P < 0.05$ vs vehicle).

after 15 and 30 min of ischemia followed by reperfusion is closely correlated with the phosphorylation of eNOS-Ser1177 (immunofluorescence and western blotting), as well as NO-mediated activation of GC, as detected by the unique antibody that specifically recognizes NO-bound-GC (NO-GC) (Figure 2, 3) [23]. The NOS inhibitor L-NAME inhibited eNOS-GC activation and vasodilatation (Figure 4).

The causes of vasoconstriction after 45 min of ischemia followed by reperfusion were: (1) inhibition of Akt-mediated eNOS-Ser1177 phosphorylation by PKC, and ROS generation, or by (2) reduction of NO availability by endothelin (Figure 5, 6). The no-reflow phenomenon persisted long-term during reperfusion after prolonged ischemia predicts infarct expansion [19].

The validity of our strategy was confirmed by myocardial pO_2 monitoring that demonstrated the "hyper-perfusion" and "no-reflow" during early reperfusion following 30 and 60 min, respectively, of ischemia (Figure 1). We were the first to demonstrate the temporal change in ischemic/normoxic state of the myocardium during ischemia-reperfusion by pO_2 monitoring. The initial hyper-reperfusion following 30 min of ischemia would reflect "reactive hyperemia" [7]. Notably, the myocardial perfusion was greatly increased toward the end stage of 60 min reperfusion. Although eNOS-Ser1177 phosphorylation was persisted at the end of reperfusion (Figure 3), the underlying mechanisms of the initial and later hyperperfusion remain to be elucidated.

This study also demonstrated the inhibition of the Akt-eNOS-GC pathway and vosodilation by ROS in ischemia-reperfusion for the first time to our knowledge (Figure 5). It was reported that insulin promotes ROS-mediated phosphatase activation and eNOS dephosphorylation [20]. However, our findings that the two anti-oxidative reagent Tiron and MPG inhibited the phosphorylation of Akt and eNOS-Ser1177 (Figure 5, 6) suggest that ROS inhibits Akt-dependent eNOS phosphorylation in their upstream. The target of ROS remains to be identified.

A PKC inhibitor chelerythrine also enhanced phosphorylation of Akt and eNOS-Ser1177 (Figure 6). In diabetic state, ROS generated from mitochondria respiratory chain activates PKC [33], whereas insulin reverses the PKC-mediated inhibition on PI3 kinase-Akt-eNOS activation during myocardial ischemia-reperfusion of the heart [15], probably through its PI3 kinase activation. However, we could neither find direct evidence for the involvement of PKC-mediated phosphorylation of eNOS at Thr495 (Figure 3B, 7), nor at Ser116 (Figure 3B).

It was shown that PKC activates endothelial NADPH oxidase after stimulation with TNF-α or angiotensin II [28,29], whereas TNF-α is upregulated in reperfusion [30]. Additionally, it was shown that endothelial and leukocyte NADPH

oxidases are involved in the contractile dysfunction in ischemia-reperfusion [33]. Angiotensin II induces NADPH oxidase over-expression and O_2^- production, thereby scavenging NO and constricting resistant vessels in the spontaneously hypertensive rat [32]. However, angiotensin blocker or α_1-adrenoceptor blocker was not effective under the same protocol [9]. These findings suggest that PKC, and ROS inhibit eNOS-phosphorylation at Ser1177 and vasodilatation during reperfusion following ischemia for 45 min or more. The causality of each remains undetermined.

Endotelin (ET)-1 apparently enhances vasoconstriction independently of eNOS phosphorylation. An ET blocker BQ123 improved NO availability and vasoconstriction, but did not affect phosphorylation of Akt and eNOS-Ser 1177 (Figure 6). Besides the vaso-constrictive role, ET-1 can enhance generation of ROS that scavenges NO during ischemia-reperfusion, as supported by the report on the protective effect of an endothelin blocker bonsentan on ROS generation and endothelial dysfunction after ischemia-reperfusion [9]. However, since the effects of ROS scavengers and ET were different (Figure 5, 6), ET-1 may exert its effect independent of ROS. In support of the latter explanation, Endothelium-derived ROS and ET-1 attenuate NO-dependent pulmonary vasodilation following chronic hypoxia in an additive fashion [33]. The mechanism of the effect of ET-1 on NO-dependent vasodilation remains to be addressed.

In conclusion, we showed the correlation of Akt-mediated eNOS-Ser1177 phosphorylation and vascular diameter after ischemia-reperfusion in the *in vivo* myocardial ischemia-reperfusion, in association with PKC, and ROS. Additionally, endothelin may be involved in the vasoconstriction through reducing NO availability.

Acknowledgements

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References

[1] Kou R, Greif D, Michel T. Dephosphorylation of endothelial nitric-oxide synthesis by vascular endothelial growth factor. J Biol Chem 2002;277:29669–29673.

[2] Michell BJ, Chen Zp, Tiganis T, et al. Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. J Biol Chem 2001;276:17625–17628.

[3] Liu PT, Hock CE, Nagele R, et al. Formation of nitric oxide, superoxide, and peroxynitrite in myocardial ischemia-reperfusion injury in rats. Am J Physiol 1997;272: H2327–H2336.

[4] Nakazawa H, Fukuyama N, Takizawa S, Tsuji C, Yoshitake M, Ishida H. Nitrotyrosine formation and its role in various pathological conditions. Free Radic Res 2000;33:771–784.

[5] Zou MH, Shi C, Cohen RA. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. J Clin Invest 2002;109:817–826.

[6] Muramatsu K, Numaguchi K, Egashira K, et al. Effects of N-nitro-L-arginine on coronary artery tone and hyper-perfusion after brief coronary occlusion in conscious dogs. Coron Art Dis 1994;5:815–820.

[7] Tsunoda R, Okumura K, Ishizaka H, et al. Enhancement of myocardial hyper-perfusion with manganese-superoxide dismutase: Role of endothelium-derived nitric oxide. Cardiovasc Res 1996;31:537–545.

[8] Reffelmann T, Hale SL, Dow JS, Kloner RA. No-reflow phenomenon persists long-term after ischemia/reperfusion in the rat and predicts infarct expansion. Circulation 2003;108:2911–2917.

[9] Maczewski M, Beresewicz A. The role of endothelin, protein kinase C and free radicals in the mechanism of the post-ischemic endothelial dysfunction in guinea-pig hearts. J Mol Cell Cardiol 2000;32:297–310.

[10] Hink U, Li H, Mollnau H, et al. Mechanisms underlying endothelial dysfunction in diabetes mellitus. Circ Res 2001;88:E14–E22.

[11] Zulueta JJ, Yu FS, Hertig IA, Thannickal VJ, et al. Release of hydrogen peroxide in response to hypoxia-reoxygenation: Role of an NAD(P)H oxidase-like enzyme in endothelial cell plasma membrane. Am J Respir Cell Mol Biol 1995;12:41–49.

[12] Waypa GB, Marks JD, Mack MM, et al. Mitochondrial reactive oxygen species trigger calcium increases during hypoxia in pulmonary arterial myocytes. Circ Res 2002;91:719–726.

[13] Kawata H, Yoshida K, Kawamoto A, et al. Preconditioning upregulates vascular endothelial growth factor mRNA expression and neovascularization via nuclear translocation of protein kinase C epsilon in the rat ischemic myocardium. Circ Res 2001;88:696–704.

[14] Dimmler S, Fleming I, Fisslthaler B, et al. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 1999;399:601–605.

[15] Gao F, Gao E, Yue TL, Ohlstein EH, Lopez BL, Christopher TA, Ma XL. Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: The roles of PI3-kinase, Akt, and endothelial nitric oxide synthase phosphorylation. Circulation 2002;105:1497–1502.

[16] Isenovic ER, Divald A, Milivojevic N, Grgurevic T, Fisher SE, Sowers JR. Interactive effects of insulin-like growth factor-1 and beta-estradiol on endothelial nitric oxide synthase activity in rat aortic endothelial cells. Metabolism 2003; 52:482–487.

[17] Thomas SR, Chen K, Keaney Jr, JF. Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. J Biol Chem Feb 22 2002;277:6017–6024.

[18] Fleming I, Fisslthaler B, Dimmeler S, et al. Phosphorylation of Thr (495) regulates $Ca(2^+)$/calmodulin-dependent endothelial nitric oxide synthase activity. Circ Res 2001;88:E68–E75.

[19] Reffelmann T, Hale SL, Dow JS, Kloner RA. No-reflow phenomenon persists long-term after ischemia/reperfusion in the rat and predicts infarct expansion. Circulation 2003;108:2911–2917.

[20] Seo JH, Ahn Y, Lee SR, Yeo CY, Hur KC. The major target of the endogenously generated reactive oxygen species in response to insulin stimulation phosphatase and tensin homolog and not Phosphoinositide-3 Kinase (PI-3 Kinase) in the PI-3 Kinase/Akt Pathway. Mol Biol Cell 2005; 16(1):348–357.

[21] Kurzelewski M, Czarnowska E, Beresewicz A. Endothelin in the mechanism of endothelial injury and neutrophil adhesion in the post-ischemic guinea-pig heart. Eur J Pharmacol 2002;434:95–107.

[22] Wang QD, Uriuda Y, Pernow J, et al. Myocardial release of endothelin (ET) and enhanced ET (A) receptor-mediated coronary vasoconstriction after coronary thrombosis and thrombolysis in pigs. J Cardiovasc Pharmacol 1995;26: 770–776.

[23] Tsuyama S, Yamazaki E, Tomita T, et al. Characterization of a novel monoclonal antibody that senses nitric oxide-dependent activation of soluble guanylate cyclase. FEBS Lett 1999;455:291–294.

[24] Yoshida K, Hirata T, Akita Y, et al. Translocation of protein kinase C-alpha, delta and epsilon isoforms in ischemic rat heart. Biochim Biophys Acta 1996;1317:36–44.

[25] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–685.

[26] Towbin HT, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350–4354.

[27] Chilian WM. Coronary microcirculation in health and disease. Summary of an NHLBI workshop. Circulation 1997;95:522–528.

[28] Frey RS, Rahman A, Kefer JC, et al. PKC ζ regulates TNF-α-induced activation of NADPH oxidase in endothelial cells. Circ Res 2002;90:1012–1019.

[29] Mollnau H, Wendt M, Szocs K, et al. Effects of Angiotensin II infusion on the expression and function of NAD(P)H oxidase and uncomponents of nitric oxide/cGMP signaling. Circ Res 2002;90:e58–e65.

[30] Frangogiannis NG, Lindsey ML, Michael LH, Youker KA, Bressler RB, Mendoza LH, Spengler RN, Smith CW, Entman ML. Resident cardiac mast cells degranulate and release preformed TNF-alpha, initiating the cytokine cascade in experimental canine myocardial ischemia/reperfusion. Circulation 1998;98(7):699–710.

[31] Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 2000;404(6779):787–790.

[32] Dantas AP, Franco Mdo C, Silva-Antonialli MM, Tostes RC, Fortes ZB, Nigro D, Carvalho MH. Gender differences in superoxide generation in microvessels of hypertensive rats: Role of NAD(P)H-oxidase. Cardiovasc Res 2004;61:22–29.

[33] Jernigan NL, Walker BR, Resta TC. Endothelium-derived reactive oxygen species and endothelin-1 attenuate NO-dependent pulmonary vasodilation following chronic hypoxia. Am J Physiol Lung Cell Mol Physiol 2004;287: L801–L808.

[34] Ikeda Y, Young LH, Scalia R, Ross CR, Lefer AM. PR-39, a proline/arginine-rich antimicrobial peptide, exerts cardioprotective effects in myocardial ischemia-reperfusion. Cardiovasc Res 2001;49:69–77.