Restoration of impaired endothelium-dependent coronary vasodilation in failing heart: role of eNOS phosphorylation and CGMP/cGK-I signaling

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Gill RM, Braz JC, Jin N, Etgen GJ, Shen W. Restoration of impaired endothelium-dependent coronary vasodilation in failing heart: role of eNOS phosphorylation and CGMP/cGK-I signaling. Am J Physiol Heart Circ Physiol 292: H2782–H2790, 2007. First published February 23, 2007; doi:10.1152/ajpheart.00831.2006.—In congestive heart failure (CHF), coronary vascular relaxation is associated with endothelial dysfunction and nitric oxide (NO) deficiency. This study explored the reversibility of this process in hearts recovering from CHF and its related mechanisms. Dogs were chronically instrumented to measure cardiac function and coronary blood flow (CBF). Heart failure was induced by right ventricular pacing at 240 beats/min for 3–4 wk, and cardiac recovery (CR) was allowed by the termination of cardiac pacing for 3–4 wk after the development of CHF, in which left ventricular contractile function was restored by 80–90%. The endothelium-dependent CBF response to bradykinin and acetylcholine was depressed in CHF and fully restored in CR. Myocardial NOx (nitrate/nitrite), endothelial NO synthase (eNOS) mRNA expression, total protein, and phosphorylated eNOS decreased significantly in failing hearts. However, myocardial NOx recovered to 78% of control and phosphorylated eNOS was fully restored in CR, despite the fact that eNOS mRNA expression and protein levels remained lower than control. Furthermore, the endothelium-independent CBF response to nitroglycerin did not change in CHF; however, it increased by 75% in CR, in conjunction with a near threefold increase in the phosphorylation of vasodilation-stimulated phosphoprotein (VASP) at Ser239 in recovering hearts. Thus the complete restoration of endothelium-dependent coronary vascular relaxation during cardiac recovery from CHF was mediated by 1) a restoration of phosphorylated eNOS for partial recovery of the NO production and 2) an increase in cGMP/cGMP-dependent protein kinase-I pathway signaling activity for the enhancement of coronary vascular smooth muscle relaxation in response to NO.

congestive heart failure; endothelial nitric oxide synthase; cGMP-dependent protein kinase-I; vasodilation-stimulated phosphoprotein; coronary blood flow; cardiac recovery

ENDOTHELIUM-MEDIATED vascular relaxation plays an important role in the regulation of myocardial blood flow. In congestive heart failure (CHF), coronary vascular endothelial dysfunction and nitric oxide (NO) deficiency, resulting in the depression of coronary vasodilation response to endothelium-dependent challenge, have been well documented in experimental animal studies (4, 25, 34, 36) and clinical observations in patients with CHF (15, 21, 24, 31). A decrease in bioavailability of endothelium-derived NO, primarily due to direct reduced expression of endothelial NO synthase (eNOS) (5, 29), has been demonstrated as a major contributor to endothelial dysfunction in CHF. Thus it becomes clinically important to investigate the reversibility of coronary vascular NO deficiency in the failing heart and to identify potential compensatory mechanisms for improving coronary vascular endothelium-dependent relaxation in CHF by enhancing either NO production or vascular response to NO.

eNOS is mainly located in the vascular endothelium, and its expression is under translational control by mRNA levels of eNOS. However, it was not clear whether reduced expression of eNOS in CHF is associated with a decrease in its messenger level and whether this is reversible during cardiac recovery (CR). Moreover, eNOS has been traditionally characterized as a calcium/calmodulin-dependent constitutive enzyme, by which the activity of eNOS is predominantly controlled (10). Recent studies showed that the activity of eNOS could also be regulated through the Akt-dependent phosphorylation of Ser1179 on eNOS (P-eNOS) (7, 11). Furthermore, it is not clear whether the phosphorylation of eNOS functions as a compensatory mechanism to promote NO production when eNOS expression is lower in CHF and CR. In addition, the cGMP/cGMP-dependent protein kinase (cGK)-I pathway is downstream of NO signaling in vascular smooth muscle and plays a key role in the regulation of vascular tone (18, 33). In response to NO, soluble guanylyl cyclase (sGC) produces the second messenger cGMP, leading to the activation of cGK-I, which mediates vascular relaxation via phosphorylation of several proteins regulating intracellular Ca2+ mobilization and cytoskeleton organization (18). However, it is also unclear whether an increase in cGMP/cGK-I signaling could lead to a compensatory enhancement of the vascular relaxation response to NO in CHF and CR when NO bioavailability is reduced.

To address these questions, we conducted this longitudinal study using a well-characterized canine model of pacing-induced CHF, in which CHF was induced by 3- to 4-wk rapid ventricular pacing and CR was created by termination of cardiac pacing (27, 30). In this way, we performed in vivo assessments of coronary vascular response to the selective endothelium-dependent vasodilators bradykinin (BK) and acetylcholine (ACH) and to an endothelium-independent vasodilator, nitroglycerin (NTG), in the same group of dogs over three stages: control, CHF, and CR. Furthermore, using myocardial biopsy specimens collected from three groups of dogs with normal, failing, or recovered hearts, respectively, we conducted molecular analysis to provide direct evidence of alterations of eNOS in translational and posttranslational regulation, as well as the adaptive changes of cGMP/cGK-I signaling. The combination of in vivo and molecular data

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demonstrates the alterations of NO/cGMP/cGK-I involved in both coronary vascular endothelial dysfunction and NO deficiency in CHF and the restoration of endothelium-dependent regulation of coronary blood flow (CBF) during CR.

MATERIALS AND METHODS

Animal and Surgical Preparation

Male adult mongrel dogs (25–30 kg) were anesthetized with isoflurane in oxygen and ventilated with a respirator after induction with acepromazine (0.03 mg/kg im) and propofol (5.5 mg/kg iv). A left thoracotomy was performed through the fifth intercostal space under sterile conditions. Tygon catheters were implanted in the descending thoracic aorta and left atrial appendage for measuring pressures. A solid-state miniature pressure transducer (model P6, Koningsberg, Pasadena, CA) was placed into the left ventricular (LV) chamber via an apical stab incision for recording LV pressure (LVP). A coronary catheter was implanted in the base of the left circumflex artery for intracoronary infusions of BK and ACh, and a transonic transit time flow probe (Transonic Systems, Ithaca, NY) was placed around the left circumflex artery distal to the coronary catheter for measurement of CBF. A pair of piezoelectric ultrasonic crystals was implanted on opposing anterior and posterior endocardial surfaces of the LV for measuring LV internal diameter (LVID). The position of the crystals was confirmed at autopsy. A screw-in pacing lead was attached to the right ventricular free wall, and a pair of stainless steel pacing wires was placed on the left atrium. All catheters and lead wires from instruments were externalized, the thoracotomy was closed in layers, and the intrapleural space was evacuated. An antibiotic (cephalexin, 500 mg) was administered postoperatively for 7 days after surgery. The indwelling catheters were flushed daily with heparinized saline to maintain patency. The study was initiated 2–3 wk after surgery when the dogs had recovered from surgery. The study was approved by the Lilly Institutional Animal Care and Use Committee, and all animals were maintained in accordance with the guidelines in The Guide for Care and Use of Laboratory Animals [DHHS Pub. No. (NIH) 83-23, revised 1985].

Experimental Recordings and Analysis

All hemodynamic signals were collected online and analyzed on a beat-to-beat basis with a digital data acquisition system (Ponemah, Gould Instrument System). The sampling rate was 250 Hz for arterial pressure (AP) and CBF and 500 Hz for LVP and LVID signals.

AP and left atrial pressure (LAP) were measured with the fluid-filled aortic and left atrial catheters connected to Statham strain gauge transducers (P23ID, Statham, Newark, NJ), which were calibrated with a mercury manometer. Mean arterial pressure (MAP) and mean LAP signals were recorded. LVP was measured with a solid-state miniaturized pressure gauge and was cross-calibrated in vivo against measurements of systolic AP and LAP. LV dp/dt was the first derivative of LVP, and LV dp/dtmax, the maximum positive value of LV dp/dt, was calculated online by the data acquisition system. LVID was measured with ultrasonic transit time dimension gauges (Crystal Biotech, Houston, TX). LV end-diastolic dimension (LVEDD) and LV end-systolic dimension (LVESD) were measured, and LV fractional shortening (LVFS, %) was calculated as 100 × (LVEDD − LVESD)/LVEDD. LV pressure-dimension loops were constructed from LVP and LVID data obtained simultaneously. LV stroke work was measured as the integral area of the pressure-dimension loops, and cardiac work (per minute) was calculated as the product of LV stroke work and heart rate (HR). CBF was recorded and mean CBF was measured online. Mean coronary vascular resistance was calculated as MAP/mean CBF. These methods have been published previously (26–28).

Development of Heart Failure and Recovery From Heart Failure

CHF was induced by chronic rapid right ventricular pacing with a programmable pacemaker (Pace Medical, Waltham, MA) at 240 beats/min for 3–4 wk (27, 28). Cardiac pacing was stopped after the development of CHF, and the animal was allowed to recover for 3–4 wk (cardiac recovery, CR) (26). After the in vivo measurements were completed, the dogs were killed with an overdose of pentobarbital sodium, and LV biopsy specimens were excised, frozen in liquid nitrogen, and stored at −80°C until being analyzed.

In Vivo Assessment of Coronary Vascular Relaxation

All in vivo measurements were made with conscious animals lying in a right lateral recumbent position after an intramuscular injection of morphine sulfate (0.2 mg/kg).

Determination of in vivo endothelium-dependent coronary vascular relaxation. CBF responses to BK and ACh, endothelium-dependent vasodilators, were examined at all three phases of the experiment: control, CHF, and CR. BK (0.1 and 0.5 μg/min) and ACh (1 and 5 μg/min) were administered by intracoronary infusion. To avoid infusion-related change of CBF baseline, the infusion procedure was conducted with a pump-driven syringe at the constant rate of 1 ml/min. Infusions started with 1 ml of saline, followed by 2 ml of BK or ACh solution. The order of the infusion of vasodilators was randomized, and at least 10–15 min intervened between each infusion to permit recovery to predrug baseline values before the subsequent infusion.

Determination of in vivo endothelium-independent coronary vascular relaxation. CBF response to NTG, an endothelium-independent vasodilator, was examined at each of the three phases of the experiment: control, CHF, and CR. NTG (25 μg/kg) was administered by an intravenous injection.

Determination of role of NO in endothelium-dependent and -independent coronary vascular relaxation. CBF responses to the vasodilators BK, ACh, and NTG were examined immediately after a treatment with nitro-L-arginine (NLA), a specific NOS inhibitor. NLA was administered by an intracoronary infusion for 20 min (2 mg/min).

In Vitro Biochemistry and Molecular Analysis

LV myocardial biopsy samples collected from normal dogs (n = 5), dogs with pacing-induced heart failure (n = 5), and dogs recovered from CHF (n = 5) were used for in vitro biochemistry and molecular analysis.

Western blot analysis. The protein levels of eNOS, P-eNOS, cGK-I, vasodilation-stimulated phosphoprotein (VASP), and phosphorylated VASP (P-VASP) were examined by Western blot analysis. Calsequestrin was used as an internal control. LV myocardial biopsy specimens (~150 mg) were pulverized to a fine powder. Aliquots (50 mg) were transferred to a precooled Lysing Matrix D tube (Qbiogene) containing 300 μl of extraction buffer and homogenized with the Fast Prep FP 20 (Bio101). Samples were microcentrifuged at 14,000 rpm at 4°C for 15 min, and the protein concentration in the supernatants was analyzed with the Bio-Rad Protein Assay. Protein samples were subjected to SDS-PAGE (10–12% gels), transferred to Hybond-P membrane (Amersham Pharmacia Biotech) or nitrocellulose, blocked in 5–7% milk, and incubated with primary antibodies against eNOS, P-eNOS, cGK-I, VASP, and P-VASP. Primary antibodies were incubated overnight in 3% milk at 4°C. Secondary antibody IgGs (alkaline phosphatase-conjugated anti-mouse, -rabbit, or -goat) were incubated for 1 h at room temperature in 0.5–3% milk. Chemiluminescent detection was performed directly with the Vistra Chemiluminescent kit (Amersham Pharmacia Biotech) and scanned with a PhosphorImager. Bands were quantified by densitometry.

Real-time PCR. The RNA level of eNOS was analyzed by quantitative real-time PCR. LV myocardial biopsy specimens (~150 mg) were homogenized in 1 ml of TRIZol. Total RNA was purified with
RNeasy columns (Qiagen, Valencia, CA) before DNase treatment (DNA-free; Ambion, Austin, TX) and reverse-transcribed with the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Gene-specific primers were designed with Primer Express 1.5 software (Applied Biosystems, Foster City, CA) for eNOS (F: GC-CAACGTGGAGATCAGTA; R: GTTTCGCCGCAAAGGA). PCR reactions containing template, each primer at 100 nM, and 2× SYBR Green PCR Master Mix (Applied Biosystems) were incubated at 50°C for 2 min and denatured for 10 min at 95°C, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Relative quantitation was performed according to the comparative threshold cycle (CT) method, with 18s RNA as the normalization gene (ABI User Bulletin 2).

Measurement of NOx content in cardiac tissue. Total NOx (nitrate/ nitrite) content was measured with the Nitrate/Nitrite Fluorometric Assay Kit (Cayman Chemical). In brief, the tissue samples were rinsed, homogenized in buffer, and centrifuged at 14,000g for 20 min. Tissue homogenates were ultracentrifuged through 10-kDa molecular mass cutoff filters. The assay is performed by first converting nitrate to nitrite with nitrate reductase and then adding 2,3-diaminonaphthalene followed by NaOH, which converts nitrite into a fluorescent compound.

Statistical Analysis

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). For all comparisons, data within and among the protocols were analyzed with one-way or two-way ANOVA with post hoc test for statistical significance.

RESULTS

Hemodynamics and Cardiac Function Measured in the Conscious State

Table 1 summarizes hemodynamic and cardiac functional changes in conscious dogs during the control, CHF, and CR phases of the experiment. After rapid ventricular pacing for 3–4 wk, all dogs developed severe heart failure accompanied by exertional dyspnea and ascites. Compared with control, there were significant increases in HR and LV end-diastolic pressure (LVEDP) and decreases in MAP, LV systolic pressure, LV dP/dtmax, LVFS, and cardiac work. Cardiac recovery was initiated by terminating the ventricular pacing. Three to four weeks later cardiac systolic functional parameters, LV dP/dtmax, LVFS, and cardiac work, were restored to ~80–95% of their control level, while LVEDP and HR were reduced.

CBF Response to BK and ACh

To examine endothelium-dependent coronary vasodilation in CHF and CR, the peak CBF responses to BK and ACh were repeatedly assessed and compared in the same dogs over the three phases: control, CHF, and CR.

Since BK could potentially cause pain, dogs received a pretreatment of morphine (0.2 mg/kg im) before each experiment, and they were quiet during intracoronary infusions of BK without any observed pain response.

Intracoronary infusions of BK and ACh resulted in dose-dependent increases in CBF without significant change in hemodynamics or cardiac parameters (Table 2). BK (0.5 μg/min ic) caused a marked increase in peak CBF response by 109 ± 13 ml/min in the control. The peak CBF response to BK was significantly depressed (50 ± 5 ml/min, P < 0.05) in heart failure and was completely restored (101 ± 14 ml/min) after 3- to 4-wk cardiac recovery (Fig. 1, Table 2). ACh (5 μg/min ic) produced a significant increase in peak CBF response by 136 ± 14 ml/min in the control. The peak CBF response to ACh was reduced (76 ± 8 ml/min, P < 0.05 vs. control) in CHF and completely recovered (120 ± 19 ml/min, P > 0.05 vs. control) after 3- to 4-wk CR (Fig. 2, Table 2). Our data show that the endothelium-dependent coronary vasodilation in heart failure was significantly depressed and was completely restored after CR from heart failure.

CBF Response to BK and ACh After Pretreatment With NLA

To identify the role of NO in the endothelium-dependent CBF response, the effect of NO blockade with NLA on the CBF response to BK or ACh was repeatedly assessed in the phases of control, CHF and CR (Figs. 1 and 2). Treatment with NLA (2 mg/min, 20 min) did not significantly alter systemic hemodynamics, cardiac functional parameters, or the baseline of CBF.

Before heart failure (control), treatment with NLA significantly decreased the peak CBF response to BK (0.5 μg/min ic) by 47 ± 14 ml/min (P < 0.05) and to ACh (5 μg/min ic) by 60 ± 9 ml/min (P < 0.05), confirming the role of NO in the endothelium-dependent coronary vasodilation response to BK and ACh. The inhibitory effect of NLA on the peak CBF response to BK and ACh was significantly reduced (~23 ± 3 and ~32 ± 4 ml/min, both P < 0.05 vs. control) in CHF, indicating an impairment of endogenous NO-mediated coronary vasodilation. After cardiac recovery for 3–4 wk, the inhibitory effect of NLA on the peak CBF response to BK and ACh was restored (~42 ± 9 and ~43 ± 14 ml/min), suggesting a recovery of endogenous NO-mediated coronary vasodilation.

CBF Response to NTG

To determine the response of coronary vascular smooth muscle to NO, the CBF response to the endothelium-independent vasodilator NTG was examined before and after treatment with NLA. This assessment was repeated in five dogs in the control, CHF, and CR phases of the experiment.

Intravenous injection of NTG resulted in a substantial increase in CBF (63.2 ± 7.0 ml/min) in the control phase and a

Table 1. Hemodynamics, LV contractile function, and coronary blood flow in conscious dogs in control, heart failure, and cardiac recovery

<table>
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<tr>
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<th>Control</th>
<th>Heart Failure</th>
<th>Recovery</th>
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<tbody>
<tr>
<td>HR, beats/min</td>
<td>90 ± 5</td>
<td>125 ± 5*</td>
<td>65 ± 3†</td>
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<tr>
<td>MAP, mmHg</td>
<td>104 ± 5</td>
<td>79 ± 2*</td>
<td>90 ± 2†</td>
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<tr>
<td>LV systolic pressure, mmHg</td>
<td>127 ± 2</td>
<td>94 ± 3*</td>
<td>117 ± 2†</td>
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<tr>
<td>LVEDP, mmHg</td>
<td>7.9 ± 1.2</td>
<td>21.1 ± 0.5*</td>
<td>11.9 ± 1.7†</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>43.2 ± 3.1</td>
<td>48.4 ± 3.2*</td>
<td>48.6 ± 3.4*</td>
</tr>
<tr>
<td>LV dP/dtmax, mmHg/s</td>
<td>2.705 ± 104</td>
<td>1.257 ± 53*</td>
<td>2.032 ± 95†</td>
</tr>
<tr>
<td>LV fractional shortening, %</td>
<td>16.9 ± 2.7</td>
<td>8.3 ± 2.5*</td>
<td>16.3 ± 3.0†</td>
</tr>
<tr>
<td>Stroke work, dyn/cm (×10³)</td>
<td>99 ± 6</td>
<td>36 ± 2*</td>
<td>110 ± 3†</td>
</tr>
<tr>
<td>Minute work, dyn·cm⁻²·min⁻¹ (×10⁶)</td>
<td>9.0 ± 0.9</td>
<td>4.3 ± 1.0*</td>
<td>7.2 ± 1.1†</td>
</tr>
<tr>
<td>CBF, ml/min</td>
<td>36.9 ± 4.4</td>
<td>38.5 ± 3.4</td>
<td>38.1 ± 3.5</td>
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Values are means ± SE; n = 6. HR, heart rate; MAP, mean arterial pressure; LVEDP, left ventricular (LV) end-diastolic pressure; LVEDD, LV end-diastolic dimension; LV dP/dtmax, maximum positive value of 1st derivative of LV pressure; CBF, coronary blood flow. *P < 0.05 vs. control, †P < 0.05 vs. congestive heart failure (CHF).
similar response (64.7 ± 7.6 ml/min) in CHF. Interestingly, after cardiac recovery, the CBF response to NTG was significantly enhanced by 71% compared with the response in the control, with similar changes in hemodynamics and cardiac parameters (Fig. 3 and Table 3). The CBF response to NTG was not affected by treatment with NLA. These results suggest an augmented relaxation of coronary vascular smooth muscle in response to NO after cardiac recovery.

**Myocardial NOx Content**

To determine the alteration of NO production in failing and recovering hearts, we measured the myocardial NOx (nitrate/nitrite) content. As shown in Fig. 4, myocardial NOx content was significantly reduced (34 ± 14% of control) in failing hearts and partially restored (78 ± 14% of control) in recovery hearts.

**Changes in eNOS Expression and Protein Levels**

To determine the translational and posttranslational regulation of eNOS in failing and recovery hearts, we examined eNOS mRNA expression, total eNOS protein, and phosphorylation of eNOS. As illustrated in Fig. 5, compared with control, eNOS expression was reduced to 34 ± 12% (P < 0.05) of control, with a parallel reduction of total eNOS protein (57 ± 17%, P < 0.05) and P-eNOS (48 ± 20%, P < 0.05) in heart failure (Fig. 5, B–D). In recovery hearts, eNOS expression and total protein level remained at the same level as failing hearts, 41 ± 13% and 49 ± 15% of control hearts (both P < 0.05; Fig. 5, B and C), respectively. However, P-eNOS was completely restored to the control level (111 ± 30%, P > 0.05; Fig. 5D), and the ratio of P-eNOS to total eNOS was greatly increased by twofold (Fig. 5E), indicating a posttranslational regulation.

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**Fig. 1.** Peak coronary blood flow (CBF) responses to an intracoronary infusion of bradykinin (BK) at 0.1 (A) and 0.5 (B) μg/min before and after treatment with nitro-L-arginine (NLA) in conscious dogs at control, with congestive heart failure (CHF), and in cardiac recovery (CR). Peak CBF response decreased in CHF but was fully restored after CR. Values are means ± SE (n = 6). §P < 0.05 vs. pretreatment with NLA.

**Fig. 2.** Peak CBF responses to intracoronary infusion of acetylcholine (ACh) at 1 (A) and 5 (B) μg/min before and after treatment with NLA in conscious dogs at control, CHF, and CR. Peak CBF response decreased in CHF but was fully restored after CR. Values are means ± SE (n = 6). §P < 0.05 vs. control; *P < 0.05 vs. pretreatment with NLA.
Fig. 3. Peak CBF responses to an intravenous injection of nitroglycerin (NTG, 25 mg/kg) before and after treatment with NLA in conscious dogs at control, CHF, and during CR. Peak CBF response did not change in CHF but increased significantly after CR. Inhibition of endothelial nitric oxide synthase (eNOS) activity with NLA did not change CBF response to NTG. Values are means ± SE (n = 5). *P < 0.05 vs. control.

Alteration of cGMP/cGK-I Signaling Pathway

To determine the alteration of activity in the cGMP/cGK-I signaling pathway in CHF and cardiac recovery, we examined a novel biomarker for cGMP/cGK-I signaling, VASP and P-VASP at two different sites (Ser239 and Ser157). As illustrated in Fig. 6B, cGK-I protein levels were unaltered in CHF and recovery. However, activity of this pathway was altered in recovering hearts as indicated by the phosphorylation status of VASP (nearly a 3-fold increase) at Ser239 (Fig. 6D), although no change in protein expression was detected (Fig. 6F). The other phosphorylation site (Ser157), activated by PKA, was unchanged (Fig. 6E). All the data taken together indicate an increase in activity of cGK-I and an elevation of sensitivity of the cGMP/cGK-I signaling pathway to NO.

DISCUSSION

Coronary vascular endothelial dysfunction and NO deficiency result in the depression of endothelium-dependent coronary vasodilation and contribute significantly to the cardiac abnormalities in CHF (4, 15, 21, 24, 25, 31, 34, 36). Therefore, identifying the reversibility of coronary vascular endothelial dysfunction and NO deficiency in CHF and related underlying mechanisms is clinically important. In the present study, we confirmed coronary vascular endothelial dysfunction and NO deficiency in CHF, as shown in previous reports (4, 25, 34, 36). Importantly, we demonstrated a complete restoration of endothelium-dependent coronary vasodilation in CR from CHF, which was mainly mediated through two mechanisms: 1) a complete recovery of P-eNOS from a posttranslational modification of eNOS that prompted a restoration of NO production and 2) an increase in the activity of the cGMP/cGK-I pathway signaling that resulted in an enhancement of coronary vascular smooth muscle relaxation in the response to NO.

Heart Failure and Recovery in Dogs

In the present study, rapid ventricular pacing led to heart failure that was associated with depression of myocardial contractility, increase in LV wall stress, and dilation of cardiac chambers. Termination of the ventricular pacing after the development of CHF allowed failing hearts to recover, leading to the normalization of LV contractile function and wall stress. Thus we established a unique model for this longitudinal investigation of endothelium-dependent and independent coronary vascular relaxation and their related molecular mechanisms during the development of CHF and CR.

Endothelium-Dependent CBF Response

BK stimulates B2 BK receptors and ACh activates muscarinic cholinergic receptors on vascular endothelial cells, and both cause the production of NO, resulting in endothelium-dependent relaxation of vascular smooth muscle (11, 16). In the present study using a chronically implanted intracoronary catheter technique, the in vivo endothelium-dependent CBF response was assessed through stimulations with BK and ACh in conscious dogs during three phases of the experiment: control, CHF, and CR. Either BK or ACh caused a significant increase in CBF, which was significantly reduced after specific NO blockade with NLA, indicating NO dependence in the CBF response. In CHF, the CBF response to BK or ACh was significantly depressed and the inhibitory effect of NLA was reduced. Our results confirmed previous observations (4, 25,
indicating endothelial dysfunction and NO deficiency in CHF. Importantly, we found the full recovery of coronary vasodilation with BK or ACh infusions and the inhibitory effect of NLA after 3- to 4-wk CR indicated a complete restoration of the depressed NO-dependent CBF response in the failing heart, which paralleled the cardiac contractile functional recovery. It is noteworthy that the endothelium-mediated BK and ACh relaxing response in coronary vessels also contains a NO-independent component, such as endothelium-derived hyperpolarizing factor (3), including prostaglandins and cytochrome P-450 products of arachidonic acid (1, 23). In the present study, the NO-independent component was mainly represented by the CBF response to BK and ACh after NLA treatment. Interestingly, the NO-independent component in the CBF response to BK and ACh was also reduced in CHF and fully restored in the dog recovered from CHF. The impairment of myocardial blood flow regulation, associated with vascular endothelial dysfunction and NO deficiency, contributes to the cardiac functional abnormalities in CHF (32). Thus the restoration of endothelium-dependent coronary vascular relaxation may be important for the improvement of cardiac function in failing heart during CR.

Transcriptional and Posttranscriptional Regulation of eNOS

Although eNOS is a constitutive enzyme, eNOS gene expression and protein levels in vascular endothelium can be modulated by many pathological conditions. It has been reported that, associated with the decrease in NO production, eNOS mRNA and protein levels were reduced in CHF (5, 29). Confirming earlier reports, we showed a significant reduction of eNOS expression and protein level in failing hearts, accompanied by depression of in vivo endothelium-dependent CBF response and myocardial NO content. Surprisingly, we found that eNOS expression and protein levels remained low during recovery from heart failure, despite a significant contractile functional rebound in these hearts. Our results suggested that recovery of impaired endothelial function, particularly the eNOS system, in CHF is a slower process. Currently the details of these transcriptional and posttranscriptional regulatory mechanisms remain unclear, and this is an area needing further investigation.

eNOS was originally characterized as a calcium/calmodulin-dependent enzyme, by which the activity of eNOS is primarily controlled (9). Recently, it was found that the Akt-dependent phosphorylation of Ser1179 on eNOS resulted in a calcium-independent activation of eNOS and augmentation of NO.
The phosphorylation of eNOS could be regulated by shear stress (12) or enhanced by ACh and BK (10, 13). In this study, we showed that P-eNOS decreased significantly in the failing heart and was proportional to the decrease in the total eNOS protein. This suggests that, as a part of the regulatory mechanism for the activity of eNOS, the decrease in phosphorylation of eNOS could directly lead to the reduction of NO production in heart failure. More importantly, we found that P-eNOS was able to be completely restored in recovery hearts, despite the fact that total eNOS protein remained at a low level. It has been reported that P-eNOS could be 15–20 times more effective in producing NO than unphosphorylated eNOS (10, 12). Consistently, we showed that the NOx content in cardiac tissue recovered from 34% of control in failing hearts to 78% of control in recovery hearts, which was in agreement with a recent study of NO deficiency in the failing human heart unloaded by LV assist device (22). Thus the modification of eNOS protein by phosphorylation, as a post-translational regulation, could play an essential compensatory role contributing to the restoration of eNOS activity and NO production during CR from heart failure.

**Endothelium-Independent CBF Response**

NTG acts as a NO donor, resulting in coronary vascular relaxation. In the present study, we used NTG for the in vivo assessment of the endothelium-independent CBF response. In contrast to the marked decrease in the endothelium-dependent CBF response to BK and ACh, the endothelium-independent CBF response to NTG did not change significantly in CHF. This observation was also consistent with a previous report (4, 34). Interestingly, the coronary vasodilation response to NTG was significantly enhanced by ~70% after CR, representing enhanced relaxation of the coronary vascular smooth muscle in response to NO. It has been shown that both acute inhibition of endothelial NO formation (2, 7) and the chronic deficiency of NO in eNOS−/− mice (2, 8, 17) results in an increase in the sensitivity of vascular relaxation to NO donors. Our findings suggest that augmentation of vascular smooth muscle relaxation to NO could have a compensatory contribution to the restoration of endothelium-dependent coronary vasodilation under the condition of incomplete recovery of endothelial dysfunction and NO deficiency.

**P-VASP and cGMP/cGK-I Signaling**

The cGMP/cGK-I signaling pathway has been well established as a NO downstream target in vascular smooth muscle for the essential role in the regulation of vascular relaxation. NO stimulates the target enzyme, sGC, to produce the second messenger cGMP, causing further activation of cGK-I, which mediates vascular relaxation via phosphorylation of several proteins.
proteins regulating intracellular Ca\(^{2+}\) mobilization and cytoskeleton organization (18). Although sGC has been found to be widely distributed in vascular smooth muscle, endothelial cells, and myocytes, particularly associated with cellular membranes (35), immunoelectron microscopy demonstrated an overlapping cellular distribution of cGK-I and its substrate VASP, primarily localized in vascular endothelium and smooth muscle cells (19). Evidence from the study of cGK-I-deficient mice further indicated that VASP, a family member of proline-rich protein, was phosphorylated primarily by cGK-I at Ser\(^{239}\) (P-VASPSer\(^{239}\)) (20). Thus P-VASPSer\(^{239}\) has been proposed to be a sensitive biochemical marker for monitoring the activity of cGMP/cGK-I signaling pathway and endothelial integrity, associated with NO-related vascular relaxation. In the present study, we found that there was no appreciable change in the expression of cGK-I, VASP, and P-VASPSer\(^{239}\) in failing heart. In contrast, the expression of P-VASPSer\(^{239}\) increased nearly threefold in recovery hearts, even though the expression of cGK-I and VASP were unchanged. In addition, we examined the expression of P-VASPSer\(^{157}\) which is phosphorylated by PKA (14) and did not change in recovery hearts. Thus the selective augmentation of P-VASPSer\(^{239}\) expression in recovery hearts indicated an increase in the activity of cGK-I and cGMP/cGK-I signaling. This conclusion was further supported by our in vivo observation that the endothelium-independent CBF response to NTG was significantly enhanced in dogs after CR from CHF. Since the measurement of myocardial NOx (nitrate/nitrite) content suggested that the NO production in coronary vascular smooth muscle response to NO could play an important role in supporting the restoration of endothelium-mediated coronary vascular relaxation during CR from CHF. In summary, an increase in eNOS phosphorylation and enhancement of the activity of cGMP/cGK-I contributes to the restoration of endothelium-dependent coronary vascular relaxation during cardiac recovery from heart failure. Our results suggest that the promotion of eNOS phosphorylation and the elevation of activity of cGMP/cGK-I could be a potential therapeutic approach for heart failure.

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REFERENCES


