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Basic FGF reduces stunning via a NOS2-dependent pathway in coronary-perfused mouse hearts

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Hampton, Thomas G., Ivo Amende, Jason Fong, Victor E. Laubach, Jian Li, Carolyn Metais, and Michael Simons. Basic FGF reduces stunning via an NOS2-dependent pathway in coronary-perfused mouse hearts. *Am J Physiol Heart Circ Physiol* 279: H260–H268, 2000.—Basic fibroblast growth factor (FGF-2) may protect the heart from ischemia-reperfusion injury (stunning) by stimulating nitric oxide (NO) production. To test this hypothesis, we pretreated coronary-perfused mouse hearts with 1 μg/ml FGF-2 or vehicle control before the onset of ischemia. Intracellular calcium (Ca_i^{2+}) was estimated by aequorin, and NO release was measured with an NO-selective electrode. Hearts perfused with FGF-2 maintained significantly better left ventricular (LV) function during ischemia than hearts perfused with vehicle. FGF-2 significantly delayed the onset of ischemic contracture and improved LV recovery during reperfusion. Ca_i^{2+} was similar in both groups at baseline during ischemia and reperfusion. L-N^6-(1-iminoethyl)lysine, a selective inhibitor of inducible NO synthase (NOS2), obliterated the protective effects of FGF-2. In transgenic hearts deficient in the expression of NOS2 (NOS2−/−), FGF-2 did not attenuate ischemia-induced LV dysfunction. Measurements of NO release demonstrated that FGF-2 perfusion significantly increased NO in wild-type but not in NOS2−/− hearts. We conclude that basic FGF attenuates myocardial stunning independent of alterations in Ca_i^{2+} by stimulating NO production via an NOS2-dependent pathway.

ischemia; nitric oxide; intracellular calcium; myocardial function

BASIC FIBROBLAST GROWTH FACTOR (FGF-2) has been shown in vivo to promote angiogenesis in the ischemic myocardium (see Ref. 43 for review). In animals treated with FGF-2, resting collateral flow values significantly exceeded those of controls, resulting in improved myocardial perfusion and performance (14, 25, 27, 38, 42). A number of studies, however, have suggested that the action of FGF-2 and other members of the FGF family of heparin-binding growth factors extend beyond stimulation of growth and proliferation of different cell types. These nonmitogenic actions of FGF-2 include induction of hypotension (8), alterations in intracellular ion homeostasis (20, 30, 31), and negative inotropy (20). Moreover, FGF-2 and FGF-1 administered to the heart have been shown to improve cardiac resistance to ischemia-reperfusion injury (17, 18, 34, 44). Although the mechanisms of these observations remain largely unknown, current data suggest that FGF-2 has physiological effects that are mediated, in part, through activation of nitric oxide synthase (NOS) and the subsequent generation of nitric oxide (NO) (5, 19, 23, 37). Because NO has been shown to play a significant role in mitigating ischemic stress (2–4, 40), we hypothesized that FGF-2 may provide cardioprotection via production of NO.

To determine whether nonmitogenic effects of FGF-2 could be beneficial to the heart during acute myocardial ischemia and reperfusion, FGF-2 was administered in a recently described murine model of myocardial stunning (12). The advantages of this mouse model are well-defined markers of ischemia-reperfusion injury, including ischemic contracture, alteration in calcium homeostasis, and prolonged ventricular dysfunction, occurring within a time window too short to activate the mitogenic properties of FGF-2. Transgenic mouse hearts deficient in the expression of the inducible isoform of NOS (NOS2−/−) (24) were used to further investigate the coupling of FGF-2 and NO during acute myocardial ischemia and reperfusion.

METHODS

Isolated Heart Preparation

The method for perfusion of the isovolumic mouse heart was described previously (12). Briefly, hearts were excised from adult C57/BL6 mice of either sex that had been anesthetized and heparinized (500 U/100 g body wt). The aorta was slipped over a 20-gauge blunt-tipped stainless steel needle through which oxygenated (95% O_2-5% CO_2) Krebs-Henseleit (KH) buffer (in mM: 118.0 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.5 $CaCl_2$, 1.2 $MgCl_2$, 23.0 $NaHCO_3$, 10.0 dextrose,

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and 0.3 EDTA, pH 7.4) was pumped at a rate of ~3 ml/min. An intraventricular balloon catheter system specially designed for the mouse heart (12) was passed through the mitral annulus into the left ventricle, and the distal end of the balloon catheter was connected to a Statham P23b (Gould, Cleveland, OH) transducer to record intraventricular pressure. Left ventricular (LV) pressure recordings were analyzed with regard to LV developed pressure (LVP), LV end-diastolic pressure, peak rate of pressure development (dP/dt_{max}), time to 90% pressure decline, and peak rate of pressure decline (dP/dt_{min}).

Measurement of Intracellular Ca^{2+}

In hearts in which intracellular Ca^{2+} (Ca_i^{2+}) was estimated, aequorin was injected into the apex of the heart as previously described (12, 13). Briefly, after the perfusate was modified to contain 0.5 mM $CaCl_2$, 0.6 mM $MgCl_2$, and 20 mM dextrose, 1–3 μl of aequorin were injected with a glass micropipette into a localized region of 2 mm^2 at the apex of the heart. The heart was positioned in an organ bath such that the aequorin-loaded region was ~2 mm from the bottom of the bath. The Ca^{2+} and Mg^{2+} concentrations of the perfusate were increased to 2.5 mM Ca^{2+} and 1.2 mM Mg^{2+} in a stepwise fashion over a period of 40 min. The entire isolated heart preparation was positioned in a light-tight box for collection of the aequorin light signal as previously described (12). Aequorin luminescence was detected by a photomultiplier tube and recorded as anodal current. For estimation of Ca_i^{2+}, Triton X-100 was injected into the coronary perfusate to quickly permeabilize the myocardial cell membranes and expose the remaining active aequorin to saturating Ca^{2+}. This resulted in a burst of light, the integral of which approximated the maximum light (L_{max}) against which light signals of interest (L) provided the fractional luminescence (L/L_{max}). L/L_{max} was referred to a calibration equation to estimate Ca_i^{2+} (12, 22).

Experimental Protocol

Drugs. Recombinant bovine FGF-2 (rFGF-2) was obtained from Chiron (Sunnyvale, CA). N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS, was obtained from RBI (Natick, MA). L-N^6-(1-iminoethyl)lysine (L-NIL), a selective inhibitor of NOS2, was obtained from Sigma (St. Louis, MO). All studies were conducted at 30°C, and hearts were paced at 6 Hz to minimize consumption of aequorin. After a 15-min equilibrium period, baseline conditions were recorded. Subsequently, hearts were divided into the following perfusion groups: perfusion with KH for 40 min (control, $n = 10$), perfusion with KH for 20 min followed by perfusion with KH plus 1 μg/ml rFGF-2 for 20 min (rFGF-2, $n = 10$), perfusion with KH plus 400 μM L-NAME for 20 min followed by perfusion with KH plus 400 μM L-NAME plus 1 μg/ml rFGF-2 for 20 min (L-NAME + rFGF-2, $n = 6$), and perfusion with KH plus 400 μM L-NIL for 20 min followed by perfusion with KH plus 400 μM L-NIL plus 1 μg/ml rFGF-2 for 20 min (L-NIL + rFGF-2, $n = 5$). To test the effect of perfusion with the NOS inhibitors in the absence of rFGF-2, the following two additional perfusion groups were studied: perfusion with KH for 20 min followed by perfusion with KH plus 400 μM L-NAME for 20 min (L-NAME, $n = 5$), and perfusion with KH for 20 min followed by perfusion with KH plus 400 μM L-NIL for 20 min (L-NIL, $n = 5$).

Ischemia and reperfusion. The hearts were subjected to no-flow ischemia for 15 min. The organ bath was evacuated of its oxygenated solution and refilled with nitrogen-saturated perfusate. Pacing was maintained during ischemia. LV pressure was monitored throughout ischemia and reperfusion. All hearts ceased to contract within 3 min. The time for LVP to fall to 10% of baseline (T_{LVP10}) was measured to quantify differences in LV function during early ischemia. Mean ischemic Ca_i^{2+} was calculated as the mean Ca_i^{2+} recorded from the 2nd through the 14th minute of ischemia. Contracture was defined as an abrupt and sustained rise in intraventricular pressure above 4 mmHg. Contracture time was measured as the time from the onset of ischemia to the onset of contracture. At the end of 15 min of ischemia, the nitrogen-saturated bath was replaced by the original bath maintained at 30°C. Flow was recommenced. Mean Ca_i^{2+} during early reflow was calculated as the mean of the peaks of Ca_i^{2+} recorded during the 1st minute of reperfusion. After 20 min of reperfusion, Ca_i^{2+} and functional parameters were again measured.

NOS2−/− mice hearts. Additional hearts were excised from NOS2−/− mice (24) and age-matched homozygous littermate controls (NOS2+/+). These hearts were instrumented and perfused as described in *Isolated Heart Preparation* and divided into two perfusion groups: perfusion with KH for 40 min ($n = 7$), and perfusion with KH for 20 min followed by perfusion with KH plus 1 μg/ml rFGF-2 for 20 min ($n = 7$) before 15 min of ischemia and 20 min of reperfusion. Aequorin was injected into control and NOS2−/− hearts for estimation of Ca_i^{2+}.

Measurement of NO

Additional NOS2+/+ ($n = 5$) and NOS2−/− hearts ($n = 5$) were used to measure NO concentration in the coronary effluent using an amperometric sensor (ISO-NO, World Precision Instrument, Sarasota, FL), according to a technique previously described (29). Briefly, after 20 min of perfusion with either vehicle or 1 μg/ml rFGF-2, the electrode was positioned in the effluent to measure the amount of NO released from the coronary sinus. Electrode calibration was performed before each experiment with NO generated from the reaction of *S*-nitroso-*N*-acetylpenicillamine (Sigma) with cupric sulfate (Sigma) and acidic solution.

Quantification of NOS Gene Expression

To determine NOS2 and NOS3 mRNA levels in FGF-2-treated compared with control hearts, we performed 30 cycles of RT-PCR on equal amounts of total RNA from six control and six rFGF-2-treated hearts using primers corresponding to human NOS3 and NOS2 sequences. For NOS3, primers were as follows: 5′ (sense), 5′-CAGTGTCCAACATGCTGCTGGAAA-TTG-3′ (bases 1,050–1,076); antisense, 5′-TAAAGGTCTTCTT-GGTGATGCC-3′ (bases 1,511–1,535). For NOS2, primers were as follows: 5′ (sense), 5′-GCCTCGCTCTGGAAAGA-3′ (bases 1,425–1,441); antisense, 5′-TCCATGCAGACAACCTT-3′ (bases 1,908–1,924). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified from the same amount of RNA at the same time to correct for variation between different samples. The PCR products, separated on 1% agarose gels, were scanned and quantitated using Image-Quant software (Molecular Dynamics).

For Northern analysis of NOS1 and NOS3 mRNA levels in hearts of NOS2−/− and wild-type mice, total RNA was prepared from freshly excised hearts as previously described (26), subjected to electrophoresis on 1% paraformaldehyde-agarose gel, transferred to the GeneScreen Plus membrane (Dupont), and probed with random-primed mouse NOS1 and NOS3 cDNA probes. GAPDH cDNA probe was used to control for loading. Quantification was achieved using Image-Quant software.

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Table 1. *Left ventricular function at baseline conditions*

	Control	rFGF-2	L-NAME + rFGF-2	L-NIL + rFGF-2
n	10	10	6	5
LVP, mmHg	55 ± 6	50 ± 5	56 ± 3	57 ± 5
LVEDP, mmHg	5 ± 1	4 ± 1	6 ± 1	4 ± 1
dP/dt_{max}, mmHg/s	2,204 ± 278	2,055 ± 242	2,205 ± 152	2,608 ± 238
$T_{90\%P}$, ms	63 ± 3	60 ± 2	61 ± 3	57 ± 1
dP/dt_{min}, mmHg/s	1,360 ± 208	1,387 ± 190	1,589 ± 116	1,640 ± 121

Values are means ± SE; n = no. of mouse hearts. Control, wild-type hearts; rFGF-2, recombinant bovine basic fibroblast growth factor; L-NAME, N^G-nitro-L-arginine methyl ester; L-NIL, L-N^6-(1-iminoethyl)lysine; LVP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; dP/dt_{max}, peak positive pressure derivative; $T_{90\%P}$, time to 90% pressure decline; dP/dt_{min}, peak negative pressure derivative.

Statistical Analysis

Observations made before and after drug administration were compared using Student's two-tailed paired t-test. Observations made before and after the ischemia-reperfusion protocol within a group were compared using Student's two-tailed paired t-test. Between-group comparisons were made using analysis of variance. When an overall significance was observed, multiple comparisons were performed using the Bonferroni-modified t-test. A value of $P < 0.05$ was considered significant. Data are expressed as means ± SE.

RESULTS

Baseline Conditions and Effects of Ischemia

Baseline parameters of cardiac function including myocardial Ca_i^{2+} were similar at baseline in all groups (Table 1) and were not affected by administration of L-NAME, L-NIL (not shown), or rFGF-2. Interruption of coronary flow led to an abrupt fall in LV pressure in all hearts. This fall in LV pressure during early ischemia was significantly attenuated in hearts pretreated with rFGF-2 compared with control hearts (Fig. 1). Pretreatment with rFGF-2 prolonged T_{LVP10} (124 ± 9 vs. 74 ± 5 s, rFGF-2 vs. control, $P < 0.05$) and significantly delayed the onset of contracture (893 ± 7 vs. 819 ± 36 s, rFGF-2 vs. control, $P < 0.01$) (Table 2).

To explore the role of NO in mediation of this cardioprotective effect of FGF-2, we used L-NAME to inhibit all isoforms of NOS in the heart. Pretreatment with L-NAME completely blocked the cardioprotective effects of rFGF-2 during ischemia, significantly reducing T_{LVP10} (79 ± 2 vs. 124 ± 9 s, L-NAME + rFGF-2 vs. rFGF-2, $P < 0.05$) and accelerating the onset of ischemic contracture (674 ± 24 vs. 893 ± 7 s, L-NAME + rFGF-2 vs. rFGF-2, $P < 0.05$). However, perfusion with L-NAME alone (in the absence of rFGF-2) did not affect either T_{LVP10} [69 ± 3 vs. 74 ± 5 s, L-NAME vs. control, P = not significant (NS)] or the onset of ischemic contracture (820 ± 24 vs. 819 ± 36 s, L-NAME vs. control, P = NS).

To further define the type of NOS enzyme involved in this FGF-2 response, we utilized a NOS2-selective inhibitor, L-NIL. Similarly to L-NAME, L-NIL fully inhib-

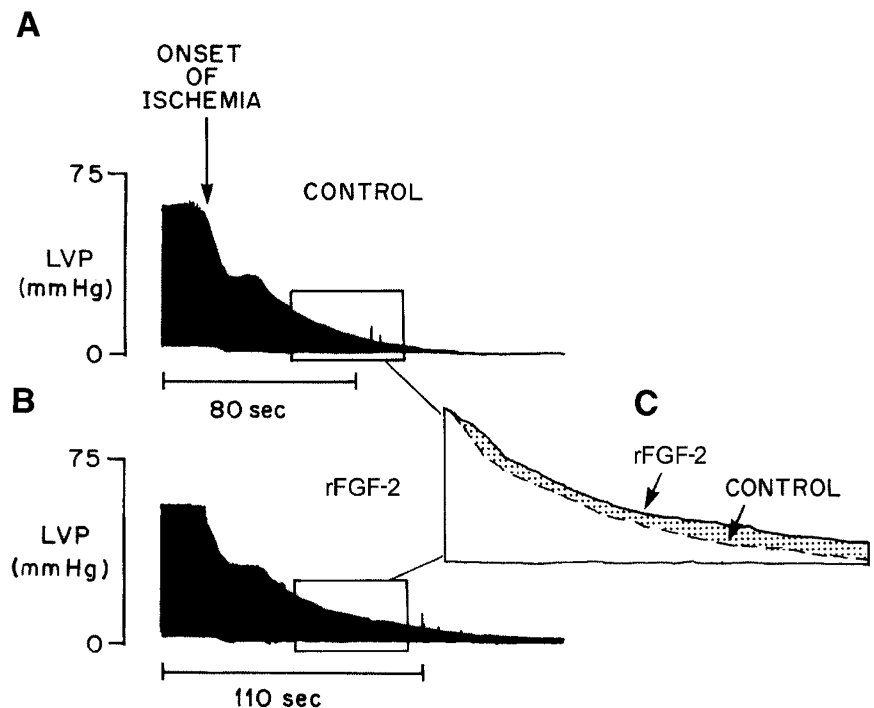

Fig. 1. Strip chart recordings of time course of fall in left ventricular (LV) pressure during ischemia. *A*: recording from a heart perfused with vehicle. *B*: recording from a heart perfused with 1 μg/ml recombinant bovine fibroblast growth factor (rFGF-2). *C*: recordings are superimposed, illustrating the delayed fall in LV pressure during early ischemia in hearts perfused with rFGF-2. LVP, LV developed pressure.

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Table 2. *Effects of rFGF-2 pretreatment on Ca_i^{2+} and function at baseline, during ischemia, and on reperfusion*

	Control	rFGF-2	NOS2−/−	NOS2−/− + rFGF-2
T_{LVP10}, s	74 ± 5 (10)	124 ± 3* (10)	65 ± 4 (7)	72 ± 5 (7)
Contracture onset time, s	819 ± 37 (10)	894 ± 7* (10)	771 ± 45 (7)	733 ± 58 (7)
Peak baseline Ca_i^{2+}, μM	0.86 ± 0.05 (5)	0.81 ± 0.01 (5)	0.87 ± 0.06 (4)	0.89 ± 0.08 (4)
Mean ischemic Ca_i^{2+}, μM	0.73 ± 0.04 (5)	0.68 ± 0.05 (5)	0.79 ± 0.11 (4)	0.82 ± 0.10 (4)
Mean Ca_i^{2+} during early reflow, μM	1.14 ± 0.09 (5)	1.17 ± 0.08 (5)	1.01 ± 0.16 (4)	1.19 ± 0.14 (4)

Values are means ± SE; nos. in parentheses indicate number of mouse hearts. Ca_i^{2+}, intracellular Ca^{2+}; T_{LVP10}, time for LVP to fall to 10% of baseline after onset of ischemia; contracture onset time, time from onset of ischemia to onset of contracture; mean ischemic Ca_i^{2+}, average Ca_i^{2+} during 15 min of ischemia; mean Ca_i^{2+} during early reflow, average Ca_i^{2+} recorded during first minute of reperfusion. *$P < 0.05$ vs. control.

ited the cardioprotective effects of rFGF-2, significantly reducing T_{LVP10} (62 ± 3 vs. 124 ± 9 s, L-NIL + rFGF-2 vs. rFGF-2, $P < 0.05$) and accelerating the onset of ischemic contracture (652 ± 16 vs. 893 ± 7 s, L-NIL + rFGF-2 vs. rFGF-2, $P < 0.05$). Similarly to perfusion with L-NAME, perfusion with L-NIL alone, in the absence of rFGF-2, did not affect either T_{LVP10} (67 ± 6 vs. 74 ± 5 s, L-NIL vs. control, P = NS) or the onset of ischemic contracture (740 ± 39 vs. 819 ± 36 s, L-NIL vs. control, P = NS).

Stunning

Fifteen minutes of global ischemia followed by twenty minutes of reperfusion resulted in prolonged ventricular dysfunction characterized by reduced levels of LVP generation as well as significant decreases in dP/dt_{max} and dP/dt_{min}. Pretreatment with rFGF-2 significantly improved the extent of recovery of LVP compared with control (untreated) hearts (83 ± 5 vs. 61 ± 6%) and equally significant preservation of dP/dt_{max} and dP/dt_{min} (86 ± 3 vs. 65 ± 6% and 85 ± 5 vs. 60 ± 5%, respectively; Fig. 2). Stunning in hearts perfused with either NOS inhibitor by itself was not different from that in control hearts. Functional recovery of LVP in untreated control hearts (61 ± 6%) was not significantly different from that in hearts perfused with either L-NAME alone (59 ± 9%) or L-NIL alone (57 ± 6%). Depression of dP/dt_{max} and dP/dt_{min} (65 ± 6 and 60 ± 5%, respectively) in untreated hearts was similar to that in hearts perfused with L-NAME alone (60 ± 9 and 55 ± 8%, respectively) and hearts perfused with L-NIL alone (57 ± 9 and 67 ± 4%, respectively).

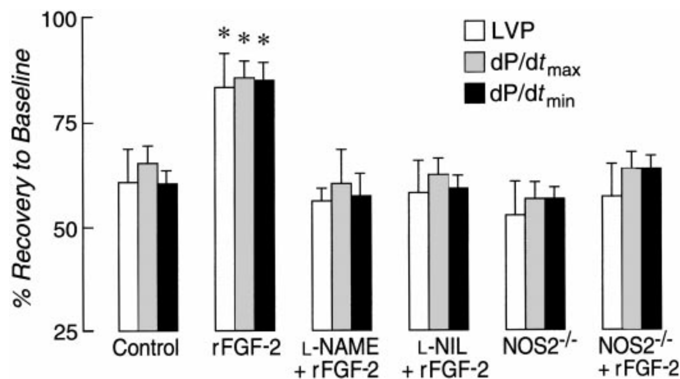

Fig. 2. Perfusion with rFGF-2 before 15 min of ischemia and 20 min of reperfusion improved recovery of ventricular function after 20 min of reperfusion compared with control hearts. L-NAME, N^G-nitro-L-arginine methyl ester; L-NIL, L-N^6-(1-iminoethyl)lysine; NOS2−/−, hearts deficient in the expression of the inducible isoform of nitric oxide synthase; dP/dt_{max}, peak positive pressure derivative; dP/dt_{min}, peak negative pressure derivative. *$P < 0.05$ compared with control hearts. Data are means ± SE.

Unlike initial pretreatment with rFGF-2, addition of the growth factor to the coronary perfusate after the onset of ischemia, immediately before reperfusion, did not improve LV function 20 min after reperfusion (LVP 60 ± 4%, dP/dt_{max} 62 ± 4%, and dP/dt_{min} 58 ± 4%, all P = NS vs. control). As in the case of acute ischemic changes, pretreatment with either L-NAME or L-NIL led to a complete inhibition of rFGF-2 effects (Figs. 2 and 3).

Role of NOS2

The preceding studies suggested that the NOS2 isoform was the primary NOS isoform responsible for FGF-2-induced preservation of myocardial function in this model. To further corroborate these results, we repeated the same studies in hearts from NOS2−/− mice using their NOS2+/+ littermates as controls. Continuous recordings of LV pressure at baseline and during ischemia and reperfusion in NOS2+/+ and NOS2−/− hearts pretreated with rFGF-2 or control buffer are illustrated in Fig. 3. As in the case of previous studies, ischemia in both NOS2+/+ and NOS2−/− hearts was characterized by an abrupt fall in LV pressure, a gradual onset of ischemic contracture, and prolonged ventricular dysfunction throughout 20 min of reperfusion. rFGF-2 pretreatment prolonged T_{LVP10}, reduced the onset of contracture, and improved LV recovery throughout reperfusion (Table 2 and Fig. 2). However, in NOS2−/− hearts, rFGF-2 failed to provide any protective effects against global ischemia and stunning as measured by changes in LVP, dP/dt_{max}, and dP/dt_{min} after 20 min of reperfusion (Table 2 and Fig. 2).

Myocardial Calcium Homeostasis

Changes in myocardial Ca_i^{2+} are thought to play an important role in ischemia-induced myocardial dys-

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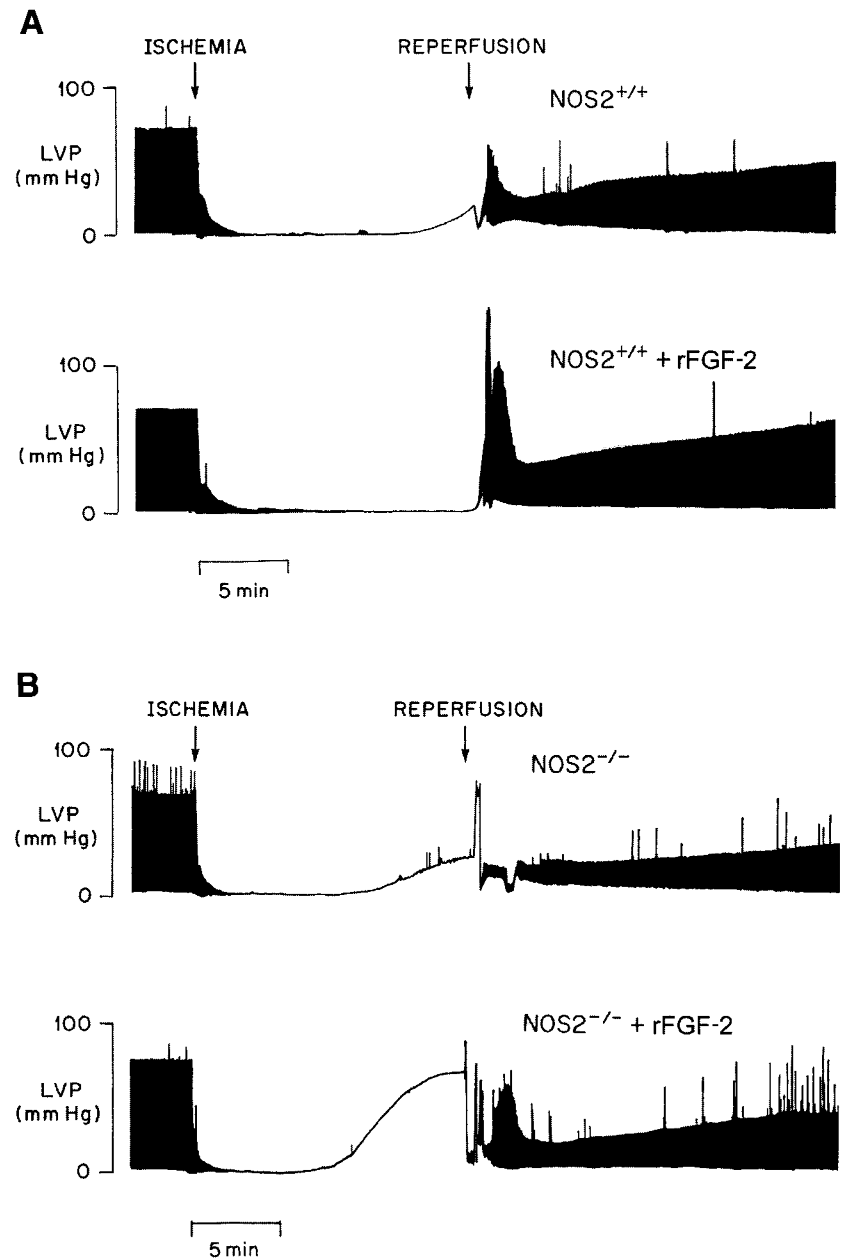

Fig. 3. Recordings of isovolumic LVP during ischemia and reperfusion in a wild-type heart (NOS2+/+) perfused with control buffer and a heart perfused with rFGF-2 before ischemia and reperfusion (*A*) and in an NOS2−/− heart perfused with control buffer and a heart perfused with rFGF-2 before ischemia and reperfusion (*B*). In NOS+/+ + rFGF-2 heart, contracture is absent and functional recovery is nearly 90%. In the NOS2−/− + rFGF-2 heart, ischemic contracture is apparent and functional recovery is barely >50%.

function. Therefore, additional experiments were carried out to assess the effect of rFGF-2 administration on myocardial ionized calcium levels. Myocardial Ca_i^{2+} measured at baseline was not different between NOS2+/+ and NOS2−/−hearts, and pretreatment with rFGF-2 had no effect on these levels (Table 2). Interruption of coronary flow produced abrupt alterations in Ca_i^{2+} in all hearts, with a gradual rise in diastolic and peak Ca_i^{2+} as ischemia progressed. Mean ischemic Ca_i^{2+}, Ca_i^{2+} averaged from the 2nd through the 14th minute of ischemia, was not affected by rFGF-2 pretreatment and was the same in NOS2+/+ and NOS2−/− hearts (Table 2). Restoration of coronary flow was followed by a marked increase in myocardial Ca_i^{2+}. Neither the extent of this increase nor peak Ca_i^{2+} levels were affected by rFGF-2 administration in NOS2+/+ or NOS2−/− hearts (Table 2).

Release of NO and FGF-2 Effects on NOS Gene Expression

To directly demonstrate the role of rFGF-2-induced NO release, we measured the concentration of NO in coronary effluent before and after rFGF-2 administration. NO concentration increased significantly after perfusion with rFGF-2 compared with measurements after perfusion with vehicle (236 ± 24 vs. 190 ± 25 nM/g, $P < 0.05$) in wild-type hearts. In contrast, perfusion with rFGF-2 did not increase NO concentration in NOS2−/− hearts compared with NO values measured after perfusion with vehicle (170 ± 24 vs. 154 ± 46 nM/g, P = NS) (Fig. 4*A*). To assess whether rFGF-2 increased NO production by stimulating NOS enzyme or increasing its gene expression, we carried out RT-PCR analysis of NOS2 and NOS3 mRNA levels before

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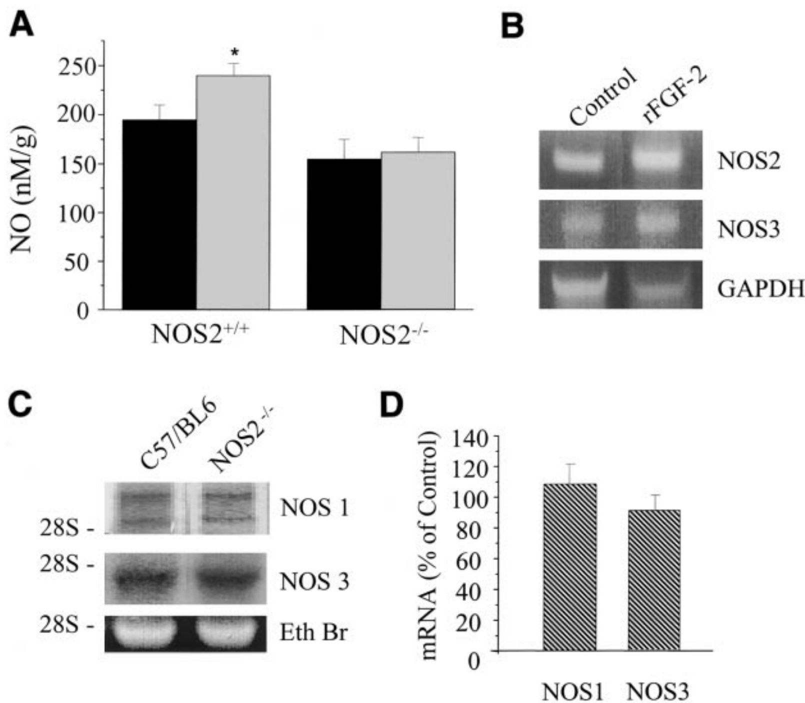

Fig. 4. *A*: nitric oxide (NO) release following perfusion with rFGF-2 and vehicle before ischemia and reperfusion in NOS+/+ and NOS−/− hearts. Production of NO was measured in the coronary effluent using an amperometric sensor. Data are means ± SE. *$P < 0.05$ compared with NOS+/+ control hearts. *B*: to assess the effect of rFGF-2 administration on NOS gene expression, we performed RT-PCR analysis of NOS2 and NOS3 mRNA levels before and after 40 min of exposure to rFGF-2. These studies showed no differences in NOS2 or NOS3 mRNA levels in these settings (mean of 6 experiments). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *C*: Northern analysis of NOS1 and NOS3 gene expression in hearts of NOS2−/− and wild-type (C57/BL6) mice. Eth Br, ethidium bromide. *D*: quantification of NOS1 and NOS3 mRNA levels. NOS1 and NOS3 levels in NOS2−/− mice are expressed as percentages of levels in control (C57/BL6) mice. Note the similar levels of expression of both genes in both NOS2 knockout and wild-type mice (mean of 4 experiments).

and after 40 min of exposure to rFGF-2. No differences in either NOS2 or NOS3 levels were detected (Fig. 4*B*).

The "knockout" of the NOS2 gene may have affected expression of NOS1 or NOS3 genes in these mice. To evaluate this possibility, we performed Northern analysis of NOS1 and NOS3 gene expression in hearts from C57/BL6 NOS2+/+ and NOS2−/− mice. No significant changes in expression of either gene compared with that in control mice were detected (Fig. 4, *C* and *D*).

DISCUSSION

Myocardial stunning is the phenomenon described by Braunwald and Kloner (6) whereby an ischemic insult interferes with normal cardiac function, cellular processes, and ultrastructure for prolonged periods. Numerous mechanisms of myocardial stunning have been proposed, the most probable of which include generation of oxygen-derived free radicals, metabolic impairment, and calcium overload (see Ref. 3 for review). Recently, a number of pharmacological agents and physiological manipulations have been shown to induce early or late ischemic preconditioning, a state characterized by reduced susceptibility to postischemic decline in myocardial function. In particular, FGF-2 has been demonstrated to improve myocardial function in the setting of acute myocardial ischemia both in vivo (17, 44) and in isolated rat heart studies (34). The well-known angiogenic effects of FGF-2, however, occur too gradually to be relevant in such settings (43). The purpose of this study, therefore, was to study the potential role of NO release in FGF-2-mediated cardioprotection and to define the NOS isoform responsible for FGF-2-induced NO release.

We chose a recently described murine model of myocardial stunning, characterized by a rise in Ca_i^{2+}, myocardial contracture during ischemia, Ca_i^{2+} overload during early reperfusion, and prolonged ventricular dysfunction (12). In this model, pretreatment with FGF-2 before the onset of ischemia significantly delayed the fall of LV pressure during ischemia, reduced ischemic contracture, and improved functional recovery following reperfusion. Interestingly, administration of FGF-2 at the time of reperfusion did not improve myocardial recovery. This would suggest that FGF-2 activates a signaling pathway that is protective before the onset of ischemia, akin to other instances of ischemic preconditioning. Perfusion with FGF-2 did not alter LV function or Ca_i^{2+} at baseline (Tables 1 and 2). The inotropic state of the heart before the induction of ischemia, therefore, could not account for the differences in recovery.

A number of experimental observations suggest that NO generation before ischemia may ameliorate ischemic stress (9, 33, 34). In particular, enhanced NO release appears to be an important mechanism in development of late preconditioning against myocardial stunning (4, 36). Although the specific isoform of NOS involved in this response has not been identified, a recent study implicated NOS2 as a potential key enzyme (40). The levels of NO production that we report in isolated mouse hearts are similar to those reported in isolated rat hearts (29). Moreover, previous studies (10) showed that augmenting endogenous NO production before ischemia and reperfusion significantly improved functional recovery. However, others (28) showed that NO augmentation may increase postischemic injury and that NOS antagonists may improve recovery from ischemia and reperfusion. Moreover, increased NO production by cardiomyocytes may contribute to cytokine-induced contractile dysfunc-

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tion characteristic of systemic sepsis and some cardiomyopathies (1).

The antagonism of FGF-2 benefits by pretreatment with L-NAME in the current study would suggest that NO generation was a factor in resistance to ischemic injury in our model. To test whether the NOS2 isoform is a primary mediator of FGF-2-induced ischemic preconditioning, we examined the effects of the selective NOS2 inhibitor L-NIL in a concentration shown to be effective in selectively blocking NOS2 (7, 11). Whereas pretreatment with L-NIL by itself did not alter any of the baseline parameters or change the extent of recovery, it completely abrogated the cardioprotective effects of FGF-2. To confirm this conclusion, we employed a mouse strain with deletions of both copies of the NOS2 gene. In these hearts, unlike those of NOS2+/+ littermate controls, pretreatment with FGF-2 was completely ineffective in either increasing NO production before ischemia (Fig. 4) or reducing ischemia-induced myocardial stunning (Figs. 2 and 3). Taken together, these results demonstrate that the cardioprotective effects of FGF-2 are mediated by NO and implicate NOS2 as the primary activator.

Given the relatively short time interval from FGF-2 treatment to the observed effect, it appears more likely that the growth factor stimulated the activity of the NOS2 enzyme rather than increasing its levels of expression. RT-PCR analysis of NOS2 mRNA 40 min after FGF-2 administration failed to demonstrate any change in NOS2 message level, suggesting that changes in enzyme activity, and not expression, are responsible for the observed effect.

The activity of the NOS2 enzyme is thought to be determined by the level of the NOS2 protein, by the availability of a cofactor, tetrahydrobiopterin (BH4), and by cellular levels of glutathione (39). In particular, BH4 levels, primarily determined by the activity of GTP cyclohydrolase I, play a critical role in regulation of inducible NOS activity. Expression of human NOS2 gene in rat smooth muscle cells that do not produce BH4 under normal conditions is ineffective in stimulating NOS2 activity. However, cotransfection of NOS2 with GTP cyclohydrolase I in the same cells resulted in significant increase of NOS2 activity (41). Similarly, glutathione presence is essential for maximal NOS2 enzyme activity (15). Reduction of glutathione levels, which would occur in the setting of ischemia, would lead to a significant reduction in NOS2 activity. Thus rFGF-2 could potentially induce NOS2 activity by increasing cellular levels of BH4 or preventing ischemia-induced reduction in glutathione concentration.

There may be multiple mechanisms by which NO provides protection from ischemia-reperfusion injury. NO may reduce reperfusion injury by acting as a scavenger of oxygen free radicals (29), whereas inhibition of NO synthesis may worsen myocardial stunning due to a mismatch between oxygen demand and supply (16). Furthermore, endogenous NO has an energy-sparing effect that reduces the amount of stunning after reperfusion (33). NO may also play a significant role in transmembrane signaling in the ischemic heart (21, 39), including the activation of protein kinase C-ε (35). Finally, the role of NO in mitigating stunning may be related to NO stimulation of guanylyl cyclase within ventricular myocytes (2). Furthermore, reduction of cGMP by NO (32) and consequent reduction in Ca^{2+} influx may attenuate the harmful increases in Ca_i^{2+} during ischemia and reperfusion.

Our Ca_i^{2+} data, however, suggest that FGF-2 may play only a minor role in modulating levels of Ca_i^{2+} at baseline, during ischemia, and during early reperfusion. These observations contradict a recent report that FGF-2 has a negative inotropic effect on adult rat cardiac myocytes that may have resulted from alterations in Ca_i^{2+} homeostasis (20). No differences in Ca_i^{2+} overload during early reperfusion are apparent between NOS2−/− hearts perfused with or without rFGF-2. The energy-sparing effects provided by FGF-2-induced NO production, therefore, may be a predominant mechanism for cardioprotection during ischemia, because FGF-2 prolongs T_{LVP10} during ischemia and delays the onset of ischemic contracture in wild-type hearts. No such effects were observed in NOS2−/− hearts perfused with FGF-2. We speculate, therefore, that FGF-2 activation of NO may attenuate the effects of depletion of energy substrates during acute myocardial ischemia and reperfusion, thereby mitigating ischemic stress and reducing the extent of stunning after reperfusion.

In summary, FGF-2 attenuates myocardial stunning, independent of alterations in Ca_i^{2+}, by increasing NO production via NOS2-dependent pathways. Pretreatment with FGF-2 may be an effective way of modulating NO levels and limiting ischemia-induced alterations in myocardial function.

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REFERENCES

1. **Balligand JL, Ungureanu-Longrois D, Simmons WW, Kobzik L, Lowenstein CJ, Lamas S, Kelly RA, Smith TW, and Michel T.** Induction of NO synthase in rat cardiac microvascular endothelial cells by IL-1β and IFN-γ. *Am J Physiol Heart Circ Physiol* 268: H1293–H1303, 1995.
2. **Beresewicz A, Karwatowska-Prokopczuk E, Lewartowski B, and Cedro-Ceremuazynska K.** A protective role of nitric oxide in isolated ischaemic/reperfused rat heart. *Cardiovasc Res* 30: 1001–1008, 1995.
3. **Bolli R.** Basic and clinical aspects of myocardial stunning. *Prog Cardiovasc Dis* 40: 477–516, 1998.
4. **Bolli R, Bhatti ZA, Tang XL, Qiu Y, Zhang Q, Guo Y, and Jadoon AK.** Evidence that late preconditioning against myocardial stunning in conscious rabbits is triggered by the generation of nitric oxide. *Circ Res* 81: 42–52, 1997.

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5. **Boussairi EH and Sassard J.** Cardiovascular effects of basic fibroblast growth factor in rats. *J Cardiovasc Pharmacol* 23: 99–102, 1994.
6. **Braunwald E and Kloner RA.** The stunned myocardium: prolonged, postischemic ventricular dysfunction. *Circulation* 66: 1146–1149, 1982.
7. **Bryk R and Wolff DJ.** Mechanism of inducible nitric oxide synthase inactivation by aminoguanidine and L-N^6-(1-iminoethyl)lysine. *Biochemistry* 37: 4844–4852, 1998.
8. **Cuevas P, Carceller F, Ortega S, Zazo M, Nieto I, and Gimenez-Gallego G.** Hypotensive activity of fibroblast growth factor. *Science* 254: 1208–1210, 1991.
9. **Draper NJ and Shah AM.** Beneficial effects of a nitric oxide donor on recovery of contractile function following brief hypoxia in isolated rat heart. *J Mol Cell Cardiol* 29: 1195–1205, 1997.
10. **Engelman DT, Watanabe M, Engelman RM, Rousou JA, Flack JE, Deaton DW 3rd, and Das DK.** Constitutive nitric oxide release is impaired after ischemia and reperfusion. *J Thorac Cardiovasc Surg* 110: 1047–1053, 1995.
11. **Griffiths MJ, Messent M, MacAllister RJ, and Evans TW.** Aminoguanidine selectively inhibits inducible nitric oxide synthase. *Br J Pharmacol* 110: 963–968, 1993.
12. **Hampton TG, Amende I, Travers KE, and Morgan JP.** Intracellular calcium dynamics in mouse model of myocardial stunning. *Am J Physiol Heart Circ Physiol* 274: H1821–H1827, 1998.
13. **Hampton TG, Kranias EG, and Morgan JP.** Simultaneous measurement of intracellular calcium and ventricular function in the phospholamban-deficient mouse heart. *Biochem Biophys Res Commun* 226: 836–841, 1996.
14. **Harada K, Grossman W, Friedman M, Edelman ER, Prasad PV, Keighley CS, Manning WJ, Sellke FW, and Simons M.** Basic fibroblast growth factor improves myocardial function in chronically ischemic porcine hearts. *J Clin Invest* 94: 623–630, 1994.
15. **Harbrecht BG, Di Silvio M, Chough V, Kim YM, Simmons RL, and Billiar TR.** Glutathione regulates nitric oxide synthase in cultured hepatocytes. *Ann Surg* 225: 76–87, 1997.
16. **Hasebe N, Shen YT, and Vatner SF.** Inhibition of endothelium-derived relaxing factor enhances myocardial stunning in conscious dogs. *Circulation* 88: 2862–2871, 1993.
17. **Horrigan M, MacIsaac A, Nicolini F, Vince D, Lee P, Ellis S, and Topol E.** Reduction in myocardial infarct size by basic fibroblast growth factor after temporary coronary occlusion in a canine model. *Circulation* 94: 1927–1933, 1996.
18. **Htun P, Ito WD, Hoefer IE, Schaper J, and Schaper W.** Intramyocardial infusion of FGF-1 mimics ischemic preconditioning in pig myocardium. *J Mol Cell Cardiol* 30: 867–877, 1998.
19. **Huang Z, Chen K, Huang P, Finkelstein S, and Moskowitz M.** bFGF ameliorates focal ischemic injury by blood flow-independent mechanism in eNOS mutant mice. *Am J Physiol Heart Circ Physiol* 272: H1401–H1405, 1997.
20. **Ishibashi Y, Urabe Y, Tsutsui H, Kinugawa S, Sugimachi M, Takahashi M, Yamamoto S, Tagawa H, Sunagawa K, and Takeshita A.** Negative inotropic effect of basic fibroblast growth factor on adult rat cardiac myocyte. *Circulation* 96: 2501–2504, 1997.
21. **Kelly RA, Balligand JL, and Smith TW.** Nitric oxide and cardiac function. *Circ Res* 79: 363–380, 1996.
22. **Kihara Y, Grossman W, and Morgan JP.** Direct measurement of changes in intracellular calcium transients during hypoxia, ischemia, and reperfusion of the intact mammalian heart. *Circ Res* 65: 1029–1044, 1989.
23. **Kostyk SK, Kourembanas S, Wheeler EL, Medeiros D, McQuillan LP, D'Amore PA, and Braunhut SJ.** Basic fibroblast growth factor increases nitric oxide synthase production in bovine endothelial cells. *Am J Physiol Heart Circ Physiol* 269: H1583–H1589, 1995.
24. **Laubach VE, Shesely EG, Smithies O, and Sherman PA.** Mice lacking inducible nitric oxide synthase are not resistant to lipopolysaccharide-induced death. *Proc Natl Acad Sci USA* 92: 10688–10692, 1995.
25. **Lazarous DF, Scheinowitz M, Shou M, Hodge E, Rajanayagam S, Hunsberger S, Robison WG Jr, Stiber JA, Correa R, Epstein SE, and Unger EF.** Effects of chronic systemic administration of basic fibroblast growth factor on collateral development in the canine heart. *Circulation* 91: 145–153, 1995.
26. **Li J, Hampton T, Morgan JP, and Simons M.** Stretch-induced VEGF expression in the heart. *J Clin Invest* 100: 18–24, 1997.
27. **Lopez JJ, Edelman ER, Stamler A, Hibberd MG, Prasad P, Caputo RP, Carrozza JC, Douglas PS, Sellke FW, and Simons M.** Basic fibroblast growth factor in a porcine model of chronic myocardial ischemia: a comparison of angiographic, echocardiographic and coronary flow parameters. *J Pharmacol Exp Ther* 282: 385–390, 1997.
28. **Matheis G, Sherman MP, Buckberg GD, Haybron DM, Young HH, and Ignarro LJ.** Role of L-arginine-nitric oxide pathway in myocardial reoxygenation injury. *Am J Physiol Heart Circ Physiol* 262: H616–H620, 1992.
29. **Maulik N, Engelman DT, Watanabe M, Engelman RM, Maulik G, Cordis GA, and Das DK.** Nitric oxide signaling in ischemic heart. *Cardiovasc Res* 30: 593–601, 1995.
30. **Merle PL, Feige JJ, and Verdetti J.** Basic fibroblast growth factor activates calcium channels in neonatal rat cardiomyocytes. *J Biol Chem* 270: 17361–17367, 1995.
31. **Merle PL, Usson Y, Robert-Nicoud M, and Verdetti J.** Basic FGF enhances calcium permeable channel openings in adult rat cardiac myocytes: implication in the bFGF-induced increase of free Ca^{2+} content. *J Mol Cell Cardiol* 29: 2687–2698, 1997.
32. **Mery PF, Pavoine C, Belhassen L, Pecker F, and Fischmeister R.** Nitric oxide regulates cardiac Ca^{2+} current. Involvement of cGMP-inhibited and cGMP-stimulated phosphodiesterases through guanylyl cyclase activation. *J Biol Chem* 268: 26286–26295, 1993.
33. **Node K, Kitakaze M, Kosaka H, Komamura K, Minamino T, Inoue M, Tada M, Hori M, and Kamada T.** Increased release of NO during ischemia reduces myocardial contractility and improves metabolic dysfunction. *Circulation* 93: 356–364, 1996.
34. **Padua RR, Sethi R, Dhalla NS, and Kardami E.** Basic fibroblast growth factor is cardioprotective in ischemia-reperfusion injury. *Mol Cell Biochem* 143: 129–135, 1995.
35. **Ping P, Takano H, Zhang J, Tang XL, Qiu Y, Li RC, Banerjee S, Dawn B, Balafonova Z, and Bolli R.** Isoform-selective activation of protein kinase C by nitric oxide in the heart of conscious rabbits: a signaling mechanism for both nitric oxide-induced and ischemia-induced preconditioning. *Circ Res* 84: 587–604, 1999.
36. **Qiu Y, Rizvi A, Tang XL, Manchikalapudi S, Takano H, Jadoon AK, Wu WJ, and Bolli R.** Nitric oxide triggers late preconditioning against myocardial infarction in conscious rabbits. *Am J Physiol Heart Circ Physiol* 273: H2931–H2936, 1997.
37. **Sellke FW, Wang SY, Friedman M, Harada K, Edelman ER, Grossman W, and Simons M.** Basic FGF enhances endothelium-dependent relaxation of the collateral-perfused coronary microcirculation. *Am J Physiol Heart Circ Physiol* 267: H1303–H1311, 1994.
38. **Simons M and Laham RJ.** Therapeutic angiogenesis in myocardial ischemia. In: *Angiogenesis and Cardiovascular Disease*, edited by Ware JA and Simons M. New York: Oxford Univ. Press, 1999.
39. **Stoclet JC, Muller B, Gyorgy K, Andriantsiothaina R, and Kleschyov AL.** The inducible nitric oxide synthase in vascular and cardiac tissue. *Eur J Pharmacol* 375: 139–155, 1999.
40. **Takano H, Manchikalapudi S, Tang XL, Qiu Y, Rizvi A, Jadoon AK, Zhang Q, and Bolli R.** Nitric oxide synthase is the mediator of late preconditioning against myocardial infarction in conscious rabbits. *Circulation* 98: 441–449, 1998.
41. **Tzeng E, Yoneyama T, Hatakeyama K, Shears LL, 2nd, and Billiar TR.** Vascular inducible nitric oxide synthase gene

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therapy: requirement for guanosine triphosphate cyclohydrolase I. *Surgery* 120: 315–321, 1996.

42. **Unger EF, Banai S, Shou M, Lazarous DF, Jaklitsch MT, Scheinowitz M, Correa R, Klingbeil C, and Epstein SE.** Basic fibroblast growth factor enhances myocardial collateral flow in a canine model. *Am J Physiol Heart Circ Physiol* 266: H1588–H1595, 1994.
43. **Ware JA and Simons M.** Angiogenesis in ischemic heart disease. *Nat Med* 3: 158–164, 1997.
44. **Yanagisawa-Miwa A, Uchida Y, Nakamura F, Tomaru T, Kido H, Kamijo T, Sugimoto T, Kaji K, Utsuyama M, and Kurashima C.** Salvage of infarcted myocardium by angiogenic action of basic fibroblast growth factor. *Science* 257: 1401–1403, 1992.

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