Acute protection of ischemic heart by FGF-2: involvement of FGF-2 receptors and protein kinase C

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Jiang, Zhi-Sheng, Raymond R. Padua, Haisong Ju, Bradley W. Doble, Yan Jin, Jianming Hao, Peter A. Cattini, Ian M. C. Dixon, and Elissavet Kardami. Acute protection of ischemic heart by FGF-2: involvement of FGF-2 receptors and protein kinase C. Am J Physiol Heart Circ Physiol 282: H1071–H1080, 2002.—We examined the effect of fibroblast growth factor (FGF)-2 on myocardial resistance to injury when administered after the onset of ischemia, in vivo and ex vivo, and the role of FGF-2 receptors and protein kinase C (PKC). FGF-2 was injected into the left ventricle of rats undergoing permanent surgical coronary occlusion leading to myocardial infarction (MI). After 24 h, FGF-2-treated hearts displayed significantly reduced injury, determined by histological staining and troponin T release, and improved developed pressure compared with untreated controls. An FGF-2 mutant with diminished affinity for the tyrosine kinase FGF-2 receptor 1 (FGFR1) was not cardioprotective. FGF-2-treated hearts retained improved function and decreased damage at 6 wk after MI. In the ex vivo heart, FGF-2 administration during reperfusion after 30-min ischemia improved functional recovery and increased relative levels of PKC subtypes α, ε, and ζ in the particulate fraction, in a cherythrine-preventable mode; it also decreased loss of energy metabolites. We conclude that intramyocardial FGF-2 administration shortly after the onset of ischemia confers protection from acute and chronic cardiac dysfunction and damage; FGF-2 delivered during reperfusion protects from ischemia-reperfusion injury; and protection by FGF-2 requires intact binding to FGFR1 and is likely mediated by PKC.

cardioprotection; growth factors; reperfusion injury; signal transduction; protein therapy

FIBROBLAST GROWTH FACTOR (FGF)-2, a prototypical member of the larger family of heparin-binding growth factors, is a multifunctional protein that stimulates cell growth and angiogenesis, affects gene expression and differentiation, and protects cells from apoptosis and/or necrosis (21). There is strong evidence that, as an angiogenic agent, FGF-2 may represent a promising new long-term treatment during remodeling after myocardial infarction (MI) (48). FGF-2, given intra-arterially or by pericardial implantation over a 4- to 6-wk period, has been shown to stimulate formation of collateral vessels in the chronic post-MI heart and thus improve perfusion (25, 47). Early clinical trials confirmed that FGF-2 is beneficial as a long-term treatment, although side effects were also noted (44).

In addition to its long-term effects on the circulatory system, FGF-2 affects adult cardiac myocytes directly; these cells express functional FGF-2 receptor 1 (FGFR1) (26) and respond to FGF-2 by changes in contractility (31) and hypertrophy (40). Furthermore, FGF-2 administered to the isolated rat or mouse heart before ischemia is clearly protective against subsequent ischemia and reperfusion injury (31, 32, 42). The acute protective effects of FGF pretreatment are proposed to engage a preconditioning-like mechanism (17, 31), and thus one theoretical therapeutic possibility for FGF-2 would be as an agent of primary injury prevention.

We now report on a third possibility for FGF-2 to be used as acute, local therapeutic treatment. We have examined the potential of FGF-2 to act as an agent of secondary injury prevention when administered locally after the onset of ischemia, in the presence or absence of reperfusion, and the role of FGFR1 and protein kinase C (PKC) in this context.

METHODS

Animals. Male Sprague-Dawley rats (200–250 g) were used in all experiments and were obtained from the Central Animal Care Facility at the University of Manitoba. The investigations followed the guidelines of the Canadian Council on Animal Care and were approved by the local Animal Care Committee of the National Research Council of Canada.

Materials. Recombinant rat wild-type (wt) or mutant (mt) FGF-2 produced in Escherichia coli bacteria and purified according to previously published procedures was used (22, 31). Water-soluble chelerythrine chloride was purchased from Research Biochemicals International (Natick, MA). Monoclonal antibodies to cardiac troponin T (TnT; no. CT3), developed by Jim Jung-Ching Lin (University of Iowa, Iowa City, IA), were obtained as a culture supernatant from the

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Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development, and maintained by the Department of Biological Sciences, University of Iowa. The supernatant was used at 1:200 dilution for Western blotting.

**Myocardial infarction.** MI was produced by permanent ligation of the left coronary artery as described previously (7, 19). Briefly, rats were anesthetized with 2–2.5% isoflurane inhalation. The chest was opened by a left-side thoracotomy at the level of the fourth rib, the pericardium was incised, and the left coronary artery was ligated with a silk suture. Vehicle (saline), wtFGF-2 (0.2 μg), or E104A mtFGF-2 (2 μg) was then injected (in a total volume of 100 μl) into three sites at the lower half of the (ischemic, akinetic) left front ventricular wall, within 10 min of coronary ligation. The chest was then evacuated and closed; animals were placed in an incubated chamber and allowed to recover for 24–48 h (as required). Coronary ligation resulted in 30% mortality, similar in all groups. At various time points after ligation (4–24 h, 1 wk, or 6 wk), animals were anesthetized with ketamine-xylazine cocktail (90 mg/kg-10 mg/kg ip). Blood samples were collected from the carotid artery and centrifuged to obtain plasma. Rats were euthanized by decapitation, and hearts were harvested for determination of hemodynamic function or infarct size (n = 6–8).

**Determination of relative plasma TnT levels.** Concentration of plasma TnT was determined by Western blotting with monoclonal anti-cardiac TnT antibodies. Five microliters of plasma was diluted with one hundred microliters of SDS-PAGE (24) loading buffer; 15 μl of the diluted sample was loaded per gel (15% acrylamide) lane. After transfer, the polyvinylidene difluoride membrane was blocked with 10% plasma to be 1:200 dilution for Western blotting.

**Determination of relative plasma TnT levels.** Concentration of plasma TnT was determined by Western blotting with monoclonal anti-cardiac TnT antibodies. Five microliters of plasma was diluted with one hundred microliters of SDS-PAGE (24) loading buffer; 15 μl of the diluted sample was loaded per gel (15% acrylamide) lane. After transfer, the polyvinylidene difluoride membrane was blocked with 10% milk-Tris-buffered saline with 0.05% Tween 20 (TBST) for 1 h, incubated with anti-TnT antibody (1:200) for another hour, washed with 1% milk-TBST for 6 × 5 min and incubated with anti-mouse horseradish peroxidase (1:10,000, Bio-Rad) for 1 h, rinsed with 1 × TBST for 6 × 5 min, and finally processed for chemiluminescence (enhanced chemiluminescence). Standard TnT was purchased from Sigma.

**Determination of infarct size.** Extent of MI was estimated as described previously (3). Briefly, the heart was cut into horizontal slices (2-mm vertical length) and incubated with 1% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 10 min. Infarct tissue appears pale because of the absence of critical tissue factors necessary to interact with TTC to form the dye. Viable tissue stains red because of formation of formazan dye in the cell. Extent of infarction was estimated as the ratio of the area that remained unstained by TTC over the total ventricular area, in all slices, with Sigma ScanPro photographic analysis software. Because rats do not possess cardiac collateral circulation, the infarcted area is expected to be very similar to area at risk.

**Immunofluorescence.** Hearts were processed for cryosectioning and simultaneous immunolocalization of FGF-2 and vinculin with polyclonal anti-FGF-2 and monoclonal anti-vinculin antibodies, exactly as described by us previously (30). Staining without the primary antibodies was used to control for nonspecific fluorescence.

**Perfusion of isolated hearts.** After removal from the animals and arrest in cold buffer, hearts were perfused in the Langendorff mode at 37°C as described previously (31). Briefly, a water-filled compliant balloon, connected to a pressure transducer (Stratham P23 ID) was inserted into the left ventricle via the mitral valve. Mechanical function was assessed as developed pressure (DP), end-diastolic pressure (EDP), and rate of contraction/relaxation. Functional data were acquired and analyzed with a PC-based system (Harvard Apparatus, Saint Laurent, PQ, Canada). Hearts were perfused at a constant pressure of 80 mmHg with a modified Krebs-Henseleit (K-H) buffer as described previously (31). For the ex vivo ischemia-reperfusion experiments, left ventricular EDP was adjusted to 5–10 mmHg by inflating the balloon. Global ischemia was induced by interrupting the flow under normothermic conditions. Reperfusion was achieved by reestablishing flow. At the beginning of reperfusion, FGF-2-supplemented (10 μg in 12 ml K-H buffer) or standard K-H buffer was infused directly into the perfusion buffer with a peristaltic pump at the point of entry to the heart. Four groups (n = 6–8/group) were used. All groups (1–4) were equilibrated with K-H buffer for 30 min. Group 1 was perfused with K-H buffer for another 5 min and then subjected to 30-min global ischemia and 60-min reperfusion in K-H buffer. Group 2 was subjected to 5 min of perfusion in K-H buffer-chelerythrine (5 μM) followed by 30 min global ischemia and 60-min reperfusion in K-H buffer-chelerythrine. Group 3 was perfused with K-H buffer for 5 min and subjected to 30-min ischemia, 12-min reperfusion in K-H buffer-FGF-2, and 48-min reperfusion in K-H buffer. Group 4 was subjected to 5 min of perfusion in K-H buffer-chelerythrine, followed by 30-min ischemia, 12-min reperfusion in K-H buffer-FGF-2-chelerythrine, and 48-min reperfusion in K-H buffer-chelerythrine. For assessment of contractile function after permanent coronary ligation in vivo, rats were killed at 4–24 h and 1–6 wk after MI and hearts were placed in a Langendorff apparatus. Hearts were maintained at constant pressure of 80 mmHg. ATP and creatine phosphate (CP) concentrations were determined as described previously (28).

**Subcellular distribution of PKC subtypes α, ε, and ζ.** Cytosolic (Cyt) and Triton X-100-soluble membrane (M) fractions were obtained from hearts (n = 3) subjected to 30-min ischemia and 30-min reperfusion in the presence or absence of FGF-2 exactly as described previously (45). The remaining Triton X-100-insoluble (pellet, P) fractions were solubilized directly in SDS-PAGE buffer (24). Cyt and total particulate fractions were also obtained from hearts treated with FGF-2 in the presence or absence of chelerythrine (n = 3). The different fractions (20 μg/lane) were analyzed by SDS-PAGE and Western blotting, probing for PKC subtypes α, ε, and ζ, exactly as we described previously (31).

**FGF-2(S) mutagenesis.** The plasmid FGF-2(S)pET19b, containing the AUG-initiated 18-kDa form of rat FGF-2, was used as the template for PCR site-directed mutagenesis. The oligonucleotide primers synthesized by BioSynthesis corresponding to sequences within FGF-2 but bearing single-base pair mismatches were used for amplification of mutated subfragments in separate reactions. PCR mutagenesis was carried out in 2 steps. 1) The primer 5’-TGTAGTTATTGACCTCAGGGGTCCAAAGAGAAAC-3’ (Glu104-Ala antisense strand primer) was used in combination with the FGF-2(S) sense strand primer 5’-GGATTGAGACTCCATATGGCTGCCGGGCAAGGCTTTCCGTGCGCTCAGCATCTCAGCCAGC-3’ to generate the upstream half of FGF-2 fragments termed GluM1. The primer 5’-GTAGTTATTGACCTCAGGGGTCCAAAGAGAAAC-3’ (Glu104-Ala sense primer) was used in combination with a T7 terminator primer 5’-GCTGGTATTGCTCAGCGGCGA-3’ (corresponding to vector sequences) to generate the downstream half mutated FGF-2 fragment termed GluM2. After amplification, the PCR products were analyzed in 1% agarose gels and isolated for subsequent PCR. 2) To generate the full-length mtFGF-2 cDNA, the subfragments GluM1 and GluM2 were mixed and followed by PCR amplification by two cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 10 min in a
30-μl reaction with 10 mM Tris, pH 8.3, 2.5 mM MgCl₂, 50 mM KCl, 0.2 μg/μl gelatin, 85 μM dNTP, and 1 U of Taq polymerase. After two cycles, 20 μl of fresh solution containing the above-described buffer was added and further PCR reactions were carried out in 39 cycles consisting of denaturation at 95°C for 1 min, annealing at 60°C for 45 s, and extension at 72°C for 1 min. Full-length FGF-2 mutant fragment was ligated into TA(pCR2.1) cloning vector (Invitrogen) to generate pFGFm. The mtFGF-2 cDNA was released from pGFm by NdeI-XhoI, gel isolated, and used to replace the full-length wtFGF-2 cDNA in the pET19 expression vector. The mutant’s sequence was confirmed by the dyeoxy method (μ-mol sequencing kit; Promega, Madison, WI).

RESULTS

Effect of wt- and mtFGF-2 on injury and contractile dysfunction after MI. wtFGF-2, the (Glu¹⁰⁴-Ala) FGF-2 mutant (mtFGF-2), or saline was injected directly into the ischemic left ventricle within 10 min of permanent coronary ligation. At 4 and 24 h after MI, animals were killed, hearts were removed, and the degree of myocardial damage was assessed with the tetrazolium method for staining followed by morphometric analysis. Results are shown in Fig. 1. Injection of wtFGF-2 (2 μg) resulted in a significantly smaller infarct area compared with saline-injected controls at both time points. A reduced wtFGF-2 dose (0.2 μg) also produced a smaller infarct area compared with saline, although the effect was smaller than with the higher wtFGF-2 dose. The extent of infarction in the group injected with mtFGF-2 (2 μg) was similar to that in the saline-treated group, signifying the lack of protection.

The degree of myocardial damage was also examined by determining relative levels of cardiac TnT present in the serum. We used Western blot analysis of total serum proteins probed with an anti-cardiac TnT antibody; incubation without the primary antibody was used as control for nonspecific binding. Relative TnT levels were estimated by densitometry and found to be significantly lower (by ~40%) in the blood of wtFGF-2-treated rats compared with saline-treated controls (P < 0.05, n = 6). As seen in Fig. 1, B and C, the effects of wtFGF-2 were dependent on dose, and TnT levels in the plasma from mtFGF-2-treated rats were not significantly different from those of untreated controls.

To examine retention and localization of wt- or mtFGF-2 in the heart, the injected left ventricular ischemic myocardium was sectioned 6 h after surgery and examined for FGF-2 localization by immunofluorescence. Double immunostaining for the cytoskeletal protein vinculin was used to identify irreversibly injured myocardocytes (46). Transverse sections from both wt- and mtFGF-2-treated hearts contained regions of intense anti-FGF-2 pericellular labeling, showing a basement membrane-like distribution (Fig. 2). Intense pericellular staining was never seen in noninjected hearts and is presumed to represent exogenous FGF-2 retained by heparan sulfate proteoglycans (HSPGs) of the basement membrane and cell surface (“low-affinity” FGF-2 receptors). Exogenous wt- or mtFGF-2 localized in association with both necrotic and viable cells. Figure 2C
shows pericellular localization of exogenous mtFGF-2 in viable myocytes (identified by intact, plasma membrane-associated antivinculin staining; Refs. 30, 46; Fig. 2D) as well as irreversibly injured myocytes that have lost their antivinculin staining. No differences were evident in the staining pattern of wt- or mtFGF-2, as expected from their similar affinity to heparin (50).

Contractile properties of the infarcted hearts were assessed ex vivo in the Langendorff mode 24 h, 1 wk, and 6 wk after MI. Hearts were perfused with K-H buffer; DP measurements were obtained under increasing preload values (EDP 0–15 mmHg). Results are shown in Fig. 3. At a preload of 5 mmHg, sham-operated noninfarcted hearts displayed a DP of 87.85 ± 4.29 mmHg, whereas saline-treated infarcted hearts had a significantly reduced DP of 53.77 ± 2.30 mmHg, indicating that the heart retained only 61% of contractile function (P < 0.05; n = 6). At the same preload, the DP of FGF-2-treated infarcted hearts reached 72.24 ± 4.55 mmHg, significantly higher than that of the saline-treated hearts, indicating that now the heart retained 82% of its function (P < 0.05; n = 6). Positive as well as negative change in pressure with time at a preload of 5 mmHg was also significantly improved in FGF-2-treated hearts. Significant improvement of DP recovery in the FGF-2-treated hearts was seen under all preload conditions tested.

Cardiac function was also assessed 1 wk after coronary ligation and treatment with FGF-2 or vehicle. At 5-mmHg preload, saline-treated infarcted hearts (n = 5) displayed a DP of 52.22 ± 2.11 mmHg, whereas FGF-2-treated infarcted hearts (n = 5) had a significantly (P < 0.05) higher DP of 63.92 ± 3.52 mmHg (representing a 22% increase in function). Even at 6 wk after MI, FGF-2-treated, infarcted hearts displayed increased DP (by ~35%), compared with saline-treated controls, at the preload range tested (0–7.5 mmHg; Fig. 4A; P < 0.05, n = 4).

The extent of myocardial loss was also assessed at 6 wk after MI. Scar tissue was significantly reduced (by 38%) in the FGF-2-treated hearts compared with saline-treated controls (Fig. 4A; P < 0.05, n = 4).

Effect of FGF-2 administered during reperfusion on recovery: Involvement of PKC. The in vivo study examined the effect of FGF-2 on ischemic, nonreperfused myocardium. Current strategies for effective manage-
ment of an acute ischemic episode require prompt tissue reperfusion. Although restoration of blood flow is considered essential for salvaging myocardial function, it has been associated with a degree of exacerbation of myocardial injury (36). To investigate whether FGF-2 would be protective when administered during reperfusion, we used the ex vivo heart model of 30 min of global ischemia followed by 60 min of reperfusion as previously described (31, 32). In this model, FGF-2 is infused into the reperfusion medium during the first 12 min of reperfusion. A schematic representation of the different groups used for these studies is shown in Fig. 5.

At 20–60 min of reperfusion, recovery of DP in group 1 (untreated controls) and group 3 (FGF-2-treated) hearts was at 47 ± 4% and 74 ± 3%, respectively, of corresponding preischemic values (Fig. 6A). Furthermore, the FGF-2 group displayed only a fourfold increase in EDP, significantly less than the sevenfold increase in the control group (Fig. 6B). It would appear, therefore, that FGF-2, delivered during early reperfusion, induced a significant improvement of contractile function parameters (P < 0.05).

We then used the water-soluble PKC inhibitor chelerythrine to examine whether PKC activation was required for the manifestation of FGF-2 cardioprotection of ischemic myocardium during reperfusion. Pretreatment of hearts with chelerythrine alone, followed by the continuous presence of chelerythrine during reperfusion (group 2) produced no discernible difference in contractile recovery (DP = 50 ± 2%) compared with untreated controls (DP = 47% ± 4%) (Fig. 6A); fold increase in EDP was similarly not significantly different from that of group 1 (Fig. 6B). When FGF-2 was administered to the chelerythrine-pretreated hearts (group 4) it no longer improved contractile recovery: DP in the chelerythrine-FGF-2 group reached 52 ± 4% of preischemic values, significantly reduced compared with the FGF-2-treated group (74 ± 3%; P < 0.05) and not significantly different from either untreated controls or the chelerythrine-pretreated group (Fig. 6C). Similarly, the FGF-2-induced improvement in fold increases in EDP was completely prevented in the chelerythrine-pretreated hearts (Fig. 6D).

To determine whether FGF-2 affected the subcellular distribution of PKC subtypes α, ε, and ζ, we examined their relative levels in the Cyt, M, and P fractions. The M and P fractions represent further fractionation of the "particulate" fraction remaining after removal of cytosolic proteins and are enriched in membrane and cytoskeletal intercalated disk proteins, respectively. As shown in Fig. 7, FGF-2 significantly decreased the relative levels of all PKC subtypes tested in the cytosol. This was accompanied by significant increases in the relative levels of PKC-α in the M fraction and those of PKC-ε and -ζ in the P fraction. We also examined whether chelerythrine affected relative levels of PKC subtypes in FGF-2-treated hearts (Fig. 7). Relative levels of all PKCs in the cytosol of chelerythrine-FGF-2-treated hearts were significantly higher than those in FGF-2-only-treated samples. Conversely, relative

![Fig. 4. Effect of wtFGF-2 at 6 wk after coronary ligation.](http://ajpheart.physiology.org/)
levels of all PKCs in the particulate fraction (combined M and P fractions) of chelerythrine-FGF-2-treated hearts was significantly lower than in the FGF-2-treated samples (Fig. 7B). This is in agreement with previous studies that showed chelerythrine to prevent the translocation of PKC in cardiomyocytes (11). Our data show that, in hearts reperfused after ischemia, FGF-2 induced redistribution of PKCs from cytosolic to particulate fractions in a chelerythrine-preventable mode.

We examined the effect of FGF-2 on ATP and CP levels (Fig. 8) after 60-min reperfusion. Untreated hearts subjected to ischemia-reperfusion had significantly reduced levels of both ATP and CP, at 43% and 58% of preischemic values, respectively. ATP levels in FGF-2-treated hearts were significantly higher than those of untreated hearts, representing 67% of preischemic values ($P < 0.05; n = 6$). CP levels in FGF-2-treated hearts were also significantly higher than those of untreated hearts and were not significantly different from preischemic controls ($P < 0.05; n = 6$). Increased levels of energy metabolites in the FGF-2-treated hearts would be consistent with decreased myocardial damage.

**DISCUSSION**

The need for effective treatments that reduce the extent of an evolving MI cannot be understated. No clinically useful agents capable of protecting the ischemic myocardium, in the presence or absence of reestablishment of flow, are currently available (49).
therefore examined whether FGF-2 has a protective effect on the ischemic myocardium in two different experimental models. In vivo, FGF-2 was injected directly into the ischemic ventricle during irreversible surgical coronary ligation to examine its ability to salvage ischemic tissue from damage. In the Langendorff-perfused heart, FGF-2 was administered during the reperfusion phase to examine its effects on dysfunction and loss of energy metabolites subsequent to ischemia and reperfusion. In addition, we examined the participation of specific elements of the FGF-2 signal transduction pathway in relation to cardioprotection. We found that 1) protection against myocardial injury and contractile dysfunction is achieved through single-dose intramyocardial administration of FGF-2 into the ischemic rat myocardium in a model of permanent coronary occlusion and is effective acutely (4–24 h) as well as at 1–6 wk after MI; 2) FGF-2, given during reperfusion, protects against ischemia-reperfusion injury of the ex vivo heart; 3) the protective effects of FGF-2 on ischemic myocytes require intact binding to

Fig. 7. A: effect of FGF-2 administered during reperfusion of the ex vivo heart on relative protein kinase C (PKC) distribution after 30-min ischemia and 30-min reperfusion, as indicated. Inset, representative Western blot of subcellular fractions (Cyt, cytosol; M, Triton-soluble membrane; P, Triton-insoluble pellet; 20 µg/lane) probed for PKC-α, -ε, and -ζ as indicated. Densitometric values from control samples were arbitrarily converted to 100, and values of samples from FGF-2-treated hearts were normalized accordingly. *Significant (n = 3; P < 0.05, Student’s t-test) difference between FGF-2-treated and control values. B: effect of chelerythrine on relative PKC distribution after 30-min ischemia and 30-min reperfusion in the presence of FGF-2. Inset, representative Western blot of subcellular fractions (PRT, particulate; 20 µg/lane) probed for PKC-α, -ε, and -ζ as indicated. Densitometric values from FGF-2-treated samples were arbitrarily converted to 100, and values of samples from chelerythrine-treated samples were normalized accordingly.

Fig. 8. Effect of FGF-2 delivery during reperfusion of the ex vivo ischemic heart on ATP and creatine phosphate (CP) levels. Hearts were subjected to 30-min global ischemia followed by 60-min reperfusion under constant pressure conditions. FGF-2 (10 µg) was infused during the first 12 min of reperfusion. The y-axis shows ATP and CP levels in µmol/g wet weight, determined before ischemia and after 60-min reperfusion, as indicated. Levels of ATP and CP were both significantly increased (P < 0.05) compared with those of untreated hearts.
FGFR1; and 4) these effects are likely mediated by PKC.

To our knowledge, FGF-2 is the only agent identified to date that exerts protection when administered locally to the ischemic heart and also during reperfusion. Our findings extend and are in broad agreement with previous reports of cardioprotection by systemic administration of FGF-1 (a factor belonging to the same family of heparin-binding growth factors as FGF-2) or FGF-2 in vivo (4, 5). Others, using intracoronary infusion of FGF-2 in a canine model, reported that FGF-2 significantly limited myocardial necrosis after acute coronary occlusion (15). A significant difference in our approach is that in the in vivo model we used local intramyocardial delivery to avoid overall systemic involvement, including potential side effects but also any uncertainty as to the direct involvement of heart muscle itself. In addition, we addressed the duration of the beneficial effects by assessing the hearts up to several weeks from treatment.

Injected FGF-2 induced protection of cardiomyocytes in vivo as indicated by infarct size estimates as well as relative TnT plasma levels (Fig. 1). FGF-2 was clearly retained by the myocardium and localized around myocytes, indicating its potential to directly influence these cells. We previously showed (26) that FGF-2 injected into the myocardium triggers tyrosine phosphorylation in the cardiac myocyte, a finding consistent with in situ activation of its tyrosine kinase (TK) FGFR1.

The biological effects of extracellular FGF-2 are mediated by binding to plasma membrane TK receptors (FGFR1–4) and HSPG “low”-affinity sites (27). The HSPG sites are considered to allow and/or facilitate binding to the TK receptors; they may also mediate independent signaling events (38). To examine whether the cardioprotective effects of FGF-2 required intact binding to FGFR1 (the only FGF-2 receptor expressed in adult rodent myocardium; Refs. 10, 18, 26, 33), we used mtFGF-2. Rat mtFGF contains a substitution of glutamine-104 with an alanine residue, equivalent to the glutamine-96 mutation on human FGF-2. This mutation produces FGF-2 that binds to the low-affinity HSPG sites with unchanged affinity but has diminished affinity for FGFR1 (50). Our own characterization confirmed that, although mtFGF-2 was retained by the heart (presumably by binding to HSPGs of the basement membrane and the extracellular matrix) and localized around cardiomyocytes (Fig. 2) in a manner identical to wtFGF-2, it was unable to displace $^{125}$I-labeled wtFGF-2 from the high-affinity plasma membrane sites (unpublished observations). mtFGF-2 had no cardioprotective properties. Thus it would appear that the effect of FGF-2 in the heart is dependent on its ability to bind to FGFR1 and that binding to HSPG sites is not sufficient for cardioprotection. Although FGFR1 is known to mediate cardiac growth in development, its role in the adult myocardium has been less clear. We propose that FGFR1 can mediate FGF-2 cardioprotection of the ischemic myocardium in vivo.

FGF-2 was injected in the middle of the lower half (nearer to the apex) of the left ventricle, an area that was rendered ischemic as judged by its changed color and development of akinesis after occlusion. Exogenous FGF-2 localized in association with viable myocardium surrounding the infarcted region, as well as with irreversibly injured myocytes (Fig. 2). It is therefore presumed that FGF-2 exerted its protection on cells suffering intermediate levels of ischemic damage, at or near the infarct border. FGF-2 protection is likely to have included immediate effects, as is discerned clearly in the ex vivo model, as well as effects on gene expression, possibly causing a response similar to that of delayed preconditioning. FGF-2 stimulates expression of nitric oxide synthase (29) as well as expression of the transcription factor nuclear factor-κB (16); both intermediates, as well as PKC-ε (also stimulated by FGF-2; Ref. 31), are strongly implicated in the induction of late preconditioning (1). FGF-2 protection was discernible at 1–6 wk after MI. Although this is likely a consequence of acute preservation of myocardium at the early time points, we cannot exclude the possibility (not examined here) that formation of new blood vessels may also have occurred, as has been reported by others (41) who used comparable FGF-2 treatment. An angiogenic response would contribute to improved cardiac function at the later (but not the early) time points.

Unlike FGF-1, which has been reported to prevent ischemia-reperfusion-induced apoptosis (6), we do not think it likely that FGF-2 protection in our in vivo system involved effects on apoptosis. Examination of several sections per heart revealed a negligible number of terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL)-positive nuclei in muscle or nonmuscle cells (unpublished observations), in agreement with reports linking apoptosis predominantly to reperfusion events (20).

In our previous studies (32) in which FGF-2 was administered to isolated rat hearts before ischemia, we noted that FGF-2 was retained by the myocardium even after 30 min of ischemia followed by 1 h of reperfusion. As a result, FGF-2 might be expected to continue exerting effects on myocytes during the reperfusion phase. Indeed, as presented here, FGF-2 administration during the reperfusion stage elicited a significant improvement in recovery of function. The extent of this recovery was similar to that induced by FGF-2 given before ischemia. Both preischemic (31, 32) and posts ischemic (Fig. 2) administration of FGF-2 resulted in hearts displaying a 1.6-fold increase in DP over that of untreated controls, after 30-min ischemia and 60-min reperfusion. Thus it would appear that, irrespective of whether it induces a preconditioning response in nonischemic cells, FGF-2 is cardioprotective when administered during reperfusion on ischemic myocytes. Increased contractile recovery accompanied by increased levels of energy metabolites in the FGF-2-treated ex vivo ischemic hearts is consistent with decreased myocyte injury. Preservation against contractile dysfunction or “stunning” (14) or direct effects on energy...
metabolism may also have contributed to improved functional recovery and merit further investigation.

To address the mechanism of FGF-2 cardioprotection during reperfusion, we examined the role of PKC. PKC, in particular the ε-isofrom, mediates the early and late preconditioning responses and cardioprotection (35, 37, 43). FGF-2-triggered signal transduction in cardiomyocytes includes FGFR1-mediated activation of phospholipase C and PKC-ε in cardiomyocytes (9, 31). In the present investigation, involvement of PKC signaling was supported by the FGF-2-induced redistribution of PKC to particulate compartments (Fig. 7). A recent paper showed that PKC activity redistribution during reperfusion serves to preserve myocytes from reperfusion injury (45). PKC activation by FGF-2 during reperfusion therefore would be expected to enhance cardiac resistance to injury, exactly as we found (Fig. 6). The inhibition of FGF-2-induced cardioprotection (Fig. 6) and PKC translocation (Fig. 7B) by chelerythrine supports the notion that PKC activation during reperfusion is required for the cardioprotective effects of FGF-2.

FGF-2 affected all three PKC subtypes examined, representing all three classes of PKC. Because there is evidence that PKC-ε mediates protection during reperfusion (45), it is likely that at least this subtype contributed to the protective effect of FGF-2. It is of interest that FGF-2 caused translocation of PKC-ε to the P fraction, containing the majority of at least one of its established targets and interacting proteins, the gap junction protein connexin43 (2, 9, 34). We showed previously (8, 9) that FGF-2 stimulates PKC-ε-dependent phosphorylation of the gap junction protein connexin43, leading to decreased coupling between cardiomyocytes. Myocyte uncoupling caused by other agents such as anesthetics is considered to be protective by preventing the spreading of hypercontracture via gap junctions (12, 39). We therefore speculate that FGF-2 cardioprotection during reperfusion may include PKC-mediated effects on cardiac gap junctions.

In conclusion, FGF-2 is an excellent candidate for secondary prevention of evolving ischemic myocardial damage, at least in our animal model. The safety and efficacy of intramyocardial and/or local delivery of therapeutic agents is the subject of intense interest (23, 44). Recent studies using pig models indicated that fluoroscopy-guided intramyocardial injection is a feasible and safe procedure (13). It is thus reasonable to suggest that acute “protein therapy” with FGF-2 formulations may not be technically forbidding. Assuming that safety issues have been fully addressed, FGF-2 might be delivered intramyocardially, by catheterization, through the coronary artery, during performance of angiograms, and last, but not least, as an additional treatment during reestablishment of blood flow.

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