Fibroblast Growth Factor-1 Improves Cardiac Functional Recovery and Enhances Cell Survival After Ischemia and Reperfusion

A Fibroblast Growth Factor Receptor, Protein Kinase C, and Tyrosine Kinase-Dependent Mechanism

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OBJECTIVES	We sought to investigate the role of fibroblast growth factor (FGF)-1 during acute myocardial ischemia and reperfusion.
BACKGROUND	The FGFs display cardioprotective effects during ischemia and reperfusion.
METHODS	We investigated FGF-1–induced cardioprotection during ischemia and reperfusion and the intracellular signaling pathways responsible for these effects in an ex vivo murine setup of myocardial ischemia and reperfusion.
RESULTS	Cardiac-specific human FGF-1 overexpression was associated with enhanced post-ischemic hemodynamic recovery and decreased lactate dehydrogenase release during reperfusion. Inhibition of the FGF receptor, protein kinase C (PKC), and tyrosine kinase (TK) resulted in blockade of FGF-1-induced protective effects on cardiac functional recovery and cell death.
CONCLUSIONS	The overexpression of FGF-1 induces cardioprotection through a pathway that involves the FGF receptor, PKC, and TK. (J Am Coll Cardiol 2004;44:1113–23) © 2004 by the American College of Cardiology Foundation

Fibroblast growth factor-1 (FGF-1) or acidic FGF is a multifunctional peptide that belongs to the large family of FGFs. Fibroblast growth factor-1 is produced by a myriad of cell types, including cardiomyocytes (1,2), endothelial cells (1,2), macrophages (1), and fibroblasts (1). In the heart, FGF-1 and its most important receptor, FGF-R1, play a pivotal role in the regulation of cardiac morphogenesis (3), angiogenesis (4), arteriogenesis (5), and cardiac remodeling after myocardial infarction (6). Recently, it was reported that FGFs mediate direct cardioprotective effects during acute ischemia and reperfusion (I/R) by the induction of an ischemic preconditioning (IPC)-like state (7–9). Both the administration (10) and overexpression (11) of FGF-2 result in cardioprotection. Furthermore, these studies demonstrated that FGF-2-mediated protection was dependent on mitogen-activated protein kinases (MAPKs), tyrosine kinases (TKs), and protein kinase C (PKC) isoform activation.

Fibroblast growth factor-1 also is considered to be protective in ischemic heart disease. Pre-ischemic administration of FGF-1 decreases cardiomyocyte apoptosis rates after ischemia and reperfusion in vitro, thereby enhancing cell survival (7). Moreover, Htun et al. (8) showed that pretreatment with FGF-1 induced a state of IPC in a porcine model of regional myocardial ischemia. Finally, Buehler et al. (9)

showed that constitutive cardiac-specific FGF-1 overexpression delayed myocardial infarct formation in vivo.

Several intracellular mediators that are considered to play a pivotal role in IPC and cardioprotection have been delineated. One of the crucial steps in IPC involves the activation of distinct PKC isoforms, most notably, the ε-isoform. Upon activation, PKC isoforms translocate to distinct subcellular localizations, where they mediate their protective effects and initiate gene transcription. (12) Hence, PKC activation induces cardioprotection in several animal models (10,12–14), including the mouse (15–17). In addition to PKC isoforms, TK have been implicated in IPC-mediated protection. During IPC, the TK isoforms Src and Lck become activated in a PKC-dependent fashion (13,18).

Although much is known about the pathways involved in the long-term effects of FGF-1 on mitogenesis and protein synthesis, which are mediated through high-affinity binding to the FGF-R1 receptor, very little is known about the mechanisms of growth factor-mediated cardioprotection and the intracellular signaling pathways involved. Whether or not reported FGF-induced cardioprotective effects are receptor (FGF-R1) mediated or depend on interaction of FGFs with yet-unknown intracellular targets is still a point of debate in literature. Although FGF-1 and -2 lack a leader sequence, which is necessary to exteriorize the protein, FGFs are released during the contraction of cardiac myocytes, allowing a receptor-mediated mechanism (19). This was demonstrated by Htun et al. (8), who showed that exogenous FGF-1–mediated protection is receptor-mediated.

Abbreviations and Acronyms

AOF	=	aortic flow
DMSO	=	dimethyl sulfoxide
dP/dt_{max}	=	maximal rate of positive pressure development (in mm Hg/s^2)
dP/dt_{min}	=	minimal rate of negative pressure development (in mm Hg^2)
FGF	=	fibroblast growth factor
HW/BW	=	heart weight/body weight
IPC	=	ischemic preconditioning
I/R	=	ischemia and reperfusion
LDH	=	lactic dehydrogenase
LVDP	=	left ventricular developed pressure
LVEDP	=	left ventricular end diastolic pressure
LVP_{dia}	=	diastolic left ventricular pressure
LVP_{sys}	=	systolic left ventricular pressure
MAPK	=	mitogen-activated protein kinase
PKC	=	protein kinase C
TG	=	transgenic
TK	=	tyrosine kinase
WT	=	wild type

However, several reports suggest that exogenous FGF-1 and -2 are internalized by the formation of a complex with FGF-R1 [20], which might indicate the existence of an intracellular target for this growth factor complex.

In this study, we investigated the effects of constitutive cardiac-specific human isoform of FGF-1 overexpression on cardiac functional recovery and cell survival after acute, global myocardial ischemia and reperfusion in an isolated, working mouse heart set-up. A marked cardioprotective effect of FGF-1 overexpression was demonstrated, which critically depended upon the activation of intracellular signaling pathways using FGF-R1 and the activation of PKC and TK.

METHODS

Adult, male transgenic (TG) mice with cardiac-specific overexpression of the human isoform of FGF-1 were described previously [5]. We used the fourth and fifth generation of the TG founders. The transgene construct is composed of a cytomegalovirus enhancer, a 2.2-kb MLC-2v promotor driving the 2.1-kb human FGF-1 cDNA, and is completed by a SV40 polyadenylation site. This transgene resulted in a two- to three-fold cardiac-specific FGF-1 overexpression, as confirmed by Northern, Western blotting and immunohistochemistry (Fig. 1) [5]. All experiments described in this study were approved by the Institutional Animal Care and Use Committee at Maastricht University. Experiments were performed by researchers who were blinded to treatment group and genotype.

Perfusion system. All the chemicals used for the Krebs-Henseleit solution were of the highest grade available and purchased from Merck (Darmstadt, Germany), except for the D(+)-glucose and pyruvate (Sigma, St. Louis, Missouri) and the (human) insulin (Actrapid, Novo Nordisk Farma B.V., Alphen a/d Rijn, the Netherlands). For this study, a mouse heart perfusion system as described by de Windt et al [21,22] was used. This murine "assisted-mode" isolated heart model closely resembles the in vivo situation in the mouse regarding both the generation of cardiac output and left ventricular pressure curves [21].

Isolated ejecting mouse heart preparation. Mice were genotyped by polymerase chain reaction and anesthetized with pentobarbital (Nembutal, Sanofi Sante, Maassluis, the Netherlands, 50 mg/kg i.p.). After thoracotomy, the heart was excised quickly and placed immediately in ice-chilled perfusion buffer. The hearts were perfused with recirculating, prefiltered Krebs-Henseleit (KH) solution consisting of (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 3.0, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, Na-EDTA 0.5, D(+)-glucose 10, and pyruvate 1.5, which was gassed with carbogen (95% O_2, 5% CO_2). In the presence of 0.5 mM ethylenediamine tetraacetic acid, nominal free $[Ca^{2+}]$ was 2.5 mM. Insulin levels in the perfusate were 0.15 U/l. PO_2 pressure exceeded 650 mm Hg, and pH values ranged between 7.40 and 7.45. The temperature of the heart was maintained at 38.5°C.

The native ascending aorta was connected to the aortic cannula, mounted in the perfusion system, and immediately perfused retrogradely at a pressure of 50 mm Hg, after which the heart resumed spontaneous beating. A specially designed aortic cannula was used, matching the hemodynamic impedance characteristics of the murine ascending aorta [21]. Subsequently, a PE-50 catheter (Clay Adams, Parsippany, New Jersey) was introduced via the apex to measure left ventricular pressure. The left atrium was cannulated through a pulmonary vein using a 20-G cannula at a fixed preload of 10 mm Hg. Mean arterial pressure was monitored just downstream from the aortic cannula by using an identical pressure transducer. Finally, a water jacket (38.5°C) was placed around the heart to avoid temperature fluctuations. The hearts were paced at a frequency of 450 beats/min (except during ischemia).

Hemodynamic data acquisition. All hemodynamic parameters were recorded continuously using specialized software (Hemodynamic Data Acquisition System, Technical Department, Maastricht University) allowing online measurement, calculation, and presentation of aortic flow (AOF), cardiac output, systolic left ventricular pressure (LVP_{sys}), diastolic left ventricular pressure (LVP_{dia}), left ventricular end diastolic pressure (LVEDP), mean arterial pressure, and left ventricular maximal rate of positive pressure development (dP/dt_{max}) and minimal rate of negative pressure development (dP/dt_{min}). Left ventricular developed pressure (LVDP) was defined as the difference between LVP_{sys} and LVEDP. Cardiac output was defined as the sum of AOF and coronary flow. Coronary flow was determined from the difference between left atrial filling flow, as measured by the 2 N inline flow probe, and AOF, as measured by the 1 N inline flow probe. Before each experiment, flow probes and pressure transducers were calibrated.

Experimental protocol. After positioning the left atrial inflow catheter and pacing electrodes, antegrade perfusion and online data acquisition were started, after which the heart was allowed to stabilize for 20 min. In the last minute of this period, coronary effluent was collected, and baseline hemodynamic measurements were calculated. Subsequently, both left atrial filling flow and the aortic outflow tract were closed using an occluder, thereby initiating the no-flow normothermic ischemic period. In separate pilot experiments, we investigated the optimal duration of the ischemic period in three mice. The most pronounced effects on cardiac function were observed after 25 min of ischemia, and this period was used in the subsequent experiments. After completion of normothermic ischemia, the aortic clamp was released, allowing retrograde reperfusion of the coronaries for 10 min. After reperfusion in the Langendorff (retrograde) mode, the left atrial inflow was reopened to allow antegrade perfusion. After additional 60 min of reperfusion, the heart was removed quickly from the system, flash frozen, and stored at $-80°C$ for further analysis. Coronary effluent was collected at 5, 10, 15, 25, 35, and 60 min of reperfusion and directly frozen in liquid nitrogen. To stabilize lactate dehydrogenase (LDH) in the effluent, bovine serum albumin solution was added to a final concentration of 3%.

Biochemical analysis. Lactate and LDH concentrations were measured in the coronary effluent as markers of anaerobic metabolism and myocardial cell death. Lactate and LDH were assessed spectrometrically using a Cobas Bio autoanalyzer (Roche, Gipf-Oberfrick, Switzerland) according to the method of Apstein et al. (23) and Bergmeyer and Bernt et al. (24).

Pharmacological interventions. To obtain pharmacological inhibition of several intracellular signaling peptides, inhibitors (lavendustin A [LA], 0.5 μM, Sigma, St. Louis, Missouri; chelerythrine chloride [CHE], 5 μM, Sigma; and SU5402 [SU], 25 μM, Calbiochem, Darmstadt, Germany) were dissolved in either dimethyl sulfoxide (DMSO) or dH$_2$O, according to the guidelines of manufacturer, diluted in KH solution, and administered at final doses known to be much greater than the IC$_{50}$ values to wild-type (WT) or TG littermates (n = 6 per group).

Lavendustin A, a cell-permeable general inhibitor of cytoplasmic TKs, was dissolved in 0.01% DMSO (10). Chelerythrine chloride (5 μM) was used to inhibit PKC and was dissolved in dH$_2$O. SU5402 (25 μM) was dissolved in DMSO (0.01%) (10). SU5402 selectively inhibits the intracellular kinase of the FGF-R1, thereby preventing receptor dimerization (8,25).

Baseline hemodynamic values were obtained after 15 min of stabilization. Subsequently, the system was switched from the ejecting mode to the Langendorff mode for drug administration to reduce the amounts of inhibitor used and to prevent the inhibitor from entering the perfusing system. The inhibitors were administered during 10 min via an aortic cannula side hole, located 3 mm above its entrance. A perfusor pump was used to assure constant inhibitor levels. The inhibitor/KH solution was concentrated eight times to limit perfusion with unsaturated buffer. After completion of drug delivery, the heart was subjected to 25 min of ischemia, followed by 60 min of reperfusion. Coronary effluent was sampled after hemodynamic stabilization, during drug delivery, and during the reperfusion phase. The remaining part of the protocol was executed as described before.

Pilot experiments (n = 3 for both WT and TG) demonstrated that switching from ejecting mode to Langendorff mode and back again did not influence cardiac function or cardiac functional recovery. Because certain inhibitors were dissolved in DMSO (0.01%), we tested whether either inhibitor or DMSO alone influenced cardiac function (n = 3, data not shown), and no effect was demonstrated.

Immunoblotting and immunoprecipitation. Ventricular tissue samples (subjected to I/R) were powdered with a mortar and pestle and further homogenized at 4°C in a buffer containing 0.5% Triton, 0.5% NP-40, 10 mM Tris (pH 7.5), 2.5 mM KCI, 150 mM NaCl, 20 mM β-glycerolphosphate, 50 mM NaF, 1 mM orthovanadate, 10 μg/ml leupeptin, 1 mM dithiothreitol, 10 μg/ml soytrypsin inhibitor, and 200 mM benzamine using a polytron tissue homogenizer (Kinematica AG, Littau, Switzerland), and centrifuged for 20 min at 7,000 g. Samples containing equal amounts of protein (20 to 40 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% to 12%) and western blotting was performed as described previously (26). Antibodies and other supplies were obtained from the following sources: polyclonal PKC-ϵ antibody was from Upstate Biotechnology (Lake Placid, New York); and New England Biolabs (Beverly, Massachusetts) supplied polyclonal anti-phosphospecific antibodies against extracellular responsive kinase (ERK)1/2, c-Jun NH$_2$-terminal kinase (JNK)1/2, and p38 and polyclonal ERK1/2, JNK, and p38 antibodies. Data were quantified by assessment of the integrated optical density.

To detect PKC translocation, separate cytosolic and particulate fractions were prepared from ventricular tissue (27). Briefly, tissue samples were homogenized in 25 mM Tris-Cl, pH 7.5; 4 mM ethylenebis(oxyethylenenitrilo)-tetraacetic acid, 2 mM ethylenediamine tetraacetic acid, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin and incubated on ice for 30 min. Afterwards, samples were spun at 100,000 g for 30 min (4°C). The supernatant was used as cytosolic fraction, whereas the remaining pellet was rehomogenized with the same lysis buffer, now containing 1% Triton X-100, and incubated on ice for 30 min. After spinning the samples for 30 min at 100,000 g, the supernatant was saved as the particulate fraction.

To investigate the effects of genotype and inhibitors on MAPK activation and PKC translocation during ischemia
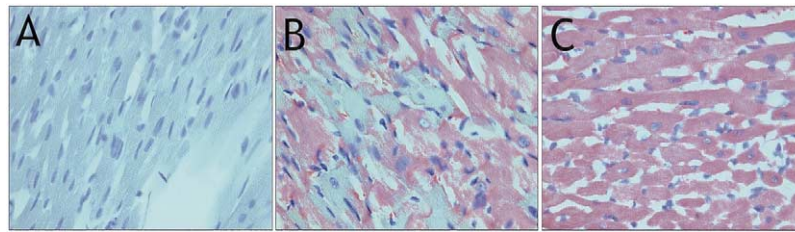
Figure 1. Left ventricular tissue of fibroblast growth factor-1 (FGF-1) transgenic and wild-type mice was stained with polyclonal antibody against FGF-1 (Promega, Madison, Wisconsin), and Vector red was applied as the secondary antibody. The diffuse staining of cardiomyocytes and extracellular matrix was observed in FGF-1 transgenic mice. Negative control shows background staining with only the secondary antibody Vector red. Note the low expression level of FGF-1 in the non-transgenic littermates.

alone, three animals per group underwent 25 min of ischemia, omitting reperfusion. After the ischemic period had been completed, hearts were flash-frozen and tissue samples were prepared as described above, followed by western blotting and densitometry.

Statistics. The results are presented as means ± SEM. The effect of genotype was evaluated using the Mann-Whitney U test or Kruskall-Wallis test, depending on the number of groups to be compared. The influence of time and genotype and their interactions on hemodynamic parameters and enzyme release was assessed using a two-way analysis of variance. A level of $p < 0.05$ was considered to be of statistical significance.

RESULTS

General. The average body weight was similar in both groups (WT [n = 36]; 29.6 ± 0.8 g, FGF [n = 39]; 28.3 ± 0.9 g, p = NS), as were heart weights and heart weight/body weight (HW/BW) ratios (WT: HW = 153 ± 10 mg, HW/BW = 5.3 ± 0.3 × 10^{-3}; FGF: HW= 151 ± 8 mg, HW/BW = 5.1 ± 0.4 × 10^{-3}, p = NS). To correct for differences in the site of genomic incorporation and copy number of the transgene construct, we investigated two distinct founder lines (lines 1 and 2), both of which carried the same transgene construct. No differences between both lines were found in HW and HW/BW ratios, basal cardiac function, or response to I/R or to inhibitor compounds. Because effects were similar in both lines, the experiments for this study were performed only in the line 1 animals.

Pre-ischemic hemodynamics. No differences were observed in pre-ischemic, intrinsic heart rates between both genotypes (TG vs. WT) and amounted to ±380 beats/min. Hemodynamic parameters did not change during the 15-min stabilization, indicating a stable baseline hemodynamic situation. No hemodynamic differences were observed between both genotype groups, indicating an equal pre-ischemic hemodynamic situation (Table 1).

Fibroblast growth factor-1 protects cardiac functional recovery and improves cell survival after ischemia and reperfusion. After an ischemic period of 25 min, cardiac function deteriorated in both transgenic mice and non-transgenic litter mates. In WT mice, AOF decreased dramatically from 10.0 ± 1.2 ml/min antegrade to only retrograde flow (−114%), indicating severe systolic dysfunc-

tion, whereas in FGF transgenic hearts, recovery of AOF was partially preserved (a 44% decrease from 9.7 ml/min to 5.2 ml/min, antegrade flow, p < 0.001) (Fig. 2A). Similarly, LVP_{sys} (−73%) and LVDP (−91%) (Fig. 2B) decreased to very low values in WT hearts, whereas in TG hearts, only a modest decrease was observed (11% decrease for LVP_{sys} [data not shown] and 22% decrease in LVDP, p < 0.001). In addition, LV dP/dt_{max} (Fig. 2C) and dP/dt_{min} values decreased dramatically in WT mice (−90% and −87%, respectively), whereas in TG mice, only a minor decrease was observed (−15% and −7%, respectively, p < 0.001 compared with WT). In addition, post-ischemic diastolic function was largely preserved in TG hearts compared with their WT counterparts. In WT mice, LVP_{dia} increased six-fold after I/R, indicating severe diastolic dysfunction, whereas in TG mice, near-complete preservation of LVP_{dia} (+28%, p < 0.001 compared with WT) was observed (Fig. 2D). No differences in LVEDP were found between both genotypes (data not shown). No differences between both genotypes were found for coronary flow values for each of the different time points measured, indicating that coronary flow was not increased as the result of FGF-1.

Baseline, pre-ischemic LDH values were similar in both experimental groups (WT: 27.1 ± 6.8 U/l·min vs. FGF-1: 31.5 ± 6.1 U/l·min; p = NS). Induction of global cardiac ischemia resulted in increased LDH release in both genotypes during the first 5 min of reperfusion (78.5 ± 23.7 U/l·min vs. 72.8±12.8 U/l·min, p < 0.05, compared with baseline) In WT mice, LDH release further increased (maximum of 3.2-fold) and remained elevated throughout the 60 min of reperfusion, indicating ongoing myocardial cell damage. In contrast, in the FGF-1 group, LDH release quickly dropped to baseline levels after an initial washout peak and remained at baseline levels throughout the remainder of the reperfusion period (Fig. 3A).

Pre-ischemic lactate release was equal in both experimental groups (WT: 836 ± 169 μmol/l·min vs. FGF: 798 ± 150 μmol/l·min; p = NS, Table 1). After ischemia, a washout peak for lactate was observed in both groups during the first minutes of reperfusion (WT: 1,297 ± 190 μmol/l·min vs. FGF: 1,535 ± 175 μmol/l·min), after which lactate release returned to baseline values. No significant differences were observed between both genotypes throughout the protocol (Fig. 3B).

Table 1. Basal Hemodynamics and Enzyme Release for All Experimental Groups

	CO	AOF	CF	SV	LVDP	LVP$_{sys}$	LVP$_{dia}$	LVEDP	dP/dt$_{max}$	−dP/dt$_{min}$	LDH	Lactate
WT (n = 6)	13.4 ± 0.7	10.2 ± 1.2	3.3 ± 0.6	30.6 ± 1.6	84.1 ± 5.1	97.5 ± 5.5	3.9 ± 1.0	13.5 ± 2.3	6903 ± 818	3457 ± 187	27.1 ± 6.8	836 ± 169
FGF (n = 9)	12.2 ± 1.5	9.7 ± 1.2	2.5 ± 0.6	27.2 ± 3.3	70.7 ± 7.1	89.6 ± 6.5	7.1 ± 1.5	20.1 ± 1.6	6429 ± 818	3080 ± 262	31.5 ± 6.1	798 ± 150
WT/LA (n = 6)	12.2 ± 1.1	7.5 ± 0.9	4.7 ± 0.7	26.9 ± 2.4	85.8 ± 7.3	102.9 ± 7.4	6.7 ± 2.6	17.2 ± 2.6	7217 ± 571	3826 ± 367	27.1 ± 4.3	781 ± 163
FGF/LA (n = 6)	11.1 ± 0.9	7.2 ± 0.8	4.0 ± 0.2	23.6 ± 2.5	88.8 ± 4.8	100.9 ± 5.9	5.1 ± 1.0	12.9 ± 1.2	7715 ± 642	3819 ± 291	28.2 ± 4.2	926 ± 219
WT/che (n = 6)	9.7 ± 1.3	6.5 ± 1.3	3.1 ± 0.4	24.1 ± 2.9	76.0 ± 4.9	93.6 ± 3.8	8.7 ± 1.1	14.8 ± 1.2	6823 ± 369	3366 ± 279	25.1 ± 7.6	487 ± 67
FGF/che (n = 6)	10.6 ± 0.7	7.1 ± 0.8	3.5 ± 0.6	24.4 ± 1.7	87.5 ± 4.8	105.1 ± 3.9	8.8 ± 1.4	17.7 ± 1.6	8189 ± 462	4334 ± 250	23.1 ± 5.1	482 ± 24
WT/SU (n = 6)	13.9 ± 1.2	11.2 ± 0.9	2.8 ± 0.4	31.5 ± 2.5	81.7 ± 3.3	105.1 ± 3.4	7.2 ± 1.7	23.2 ± 3.2	7128 ± 149	3669 ± 90	30.6 ± 3.6	676 ± 56.8
FGF/SU (n = 6)	11.5 ± 1.8	9.0 ± 1.4	2.6 ± 0.5	26.0 ± 4.0	76.1 ± 7.9	101.7 ± 6.3	10.5 ± 2.1	22.6 ± 1.6	7165 ± 599	3471 ± 207	28.1 ± 8.1	509 ± 57

LDH values in U/1·min, lactate values in μmol/l·min.

AOF = aortic flow in ml/min; CF = coronary flow in ml/min; CO = cardiac output in ml/min; LDH = lactate dehydrogenase; LVDP = left ventricular developed pressure; LVEDP = left ventricular end diastolic pressure; dP/dt$_{max}$ = maximal rate of pressure development in mmHg/s^2; dP/dt$_{min}$ = maximal rate of ventricular relaxation in mmHg/s^2; LVP$_{dia}$ = left ventricular diastolic pressure; LVP$_{sys}$ = left ventricular systolic pressure; SV = stroke volume in μl.

Unraveling the signaling pathways involved in FGF-1–mediated cardiac protection. Cardiac-specific FGF-1 overexpression results in a marked cardioprotective effect on both functional recovery and cell survival after global myocardial ischemia and reperfusion. Our next goal was to assess the relative activation status of various intracellular signaling pathways that may underlie this phenomenon.

In TG control hearts not subjected to I/R (WT and TG, n = 3), both phosphorylated and nonphosphorylated ERK-1/2 were increased, resulting in a normal phospho-ERK/ERK ratio (Fig. 4A). In addition, in TG control hearts, PKC-ϵ translocation to the particulate fraction was constitutively enhanced. No differences in p38 and JNK phosphorylation were found between WT and TG control groups (Figs. 4C and 4D). No differences were found in amount of the nonphosphorylated isoform of each of the MAPKs in all experimental groups (data not shown).

Because the differences in cardiac functional recovery were already apparent directly after initiation of reperfusion, we decided to perform a separate experiment in which WT and TG hearts were subjected to 25 min of ischemia alone and investigated the effects on MAPK and PKC activation. Twenty-five minutes of ischemia resulted in enhanced phosphorylation levels of all three MAPKs (ERK-1/2, JNK-1/2, p38). Compared with WT hearts, FGF-1 TG hearts showed higher phospho-ERK/ERK ratios (Fig. 4B). No differences were found for JNK (Fig. 3C) and p38 (Fig. 4D) activation between both genotypes after induction of ischemia. In addition, PKC-ϵ translocation to the particulate fraction was enhanced after ischemia in both experimental groups. The level of PKC-ϵ translocation in WT hearts increased to the preischemic level of TG (Fig. 4A). This was associated with a decrease in cytosolic PKC-ϵ.

When hearts were subjected to 60 min of reperfusion, after the ischemic stimulus, the genotype-dependent differences in ERK-activation and PKC translocation completely disappeared (data not shown).

TK activation underlies FGF-1–mediated cardioprotection. No intrinsic effects of the agents or DMSO (0.01%) were found on cardiac function and LDH/lactate release, which were tested during 85 min of antegrade perfusion. No significant differences were found between experimental groups for both pre-ischemic systolic (AOF, LVP$_{sys}$, LVDP, LV dP/dt$_{max}$) and diastolic (LVP$_{dia}$, LVEDP, and LV dP/dt$_{min}$) function (Figs. 5A to 5D). Ischemia resulted in a dramatic decrease in both systolic and diastolic cardiac function in WT animals, similar to the non-treated WT animals (data not shown). The administration of LA did not influence WT parameters (data not shown) but completely blocked the hemodynamic protective effects of FGF-1 in TG animals, whereas post-ischemic LDH release was comparable with non-treated TG hearts. This indicated that the cardioprotective effect of FGF-1 on functional recovery, but not necrosis, was dependent on TK activity (Fig. 6). Finally, the addition of LA reduced the activation
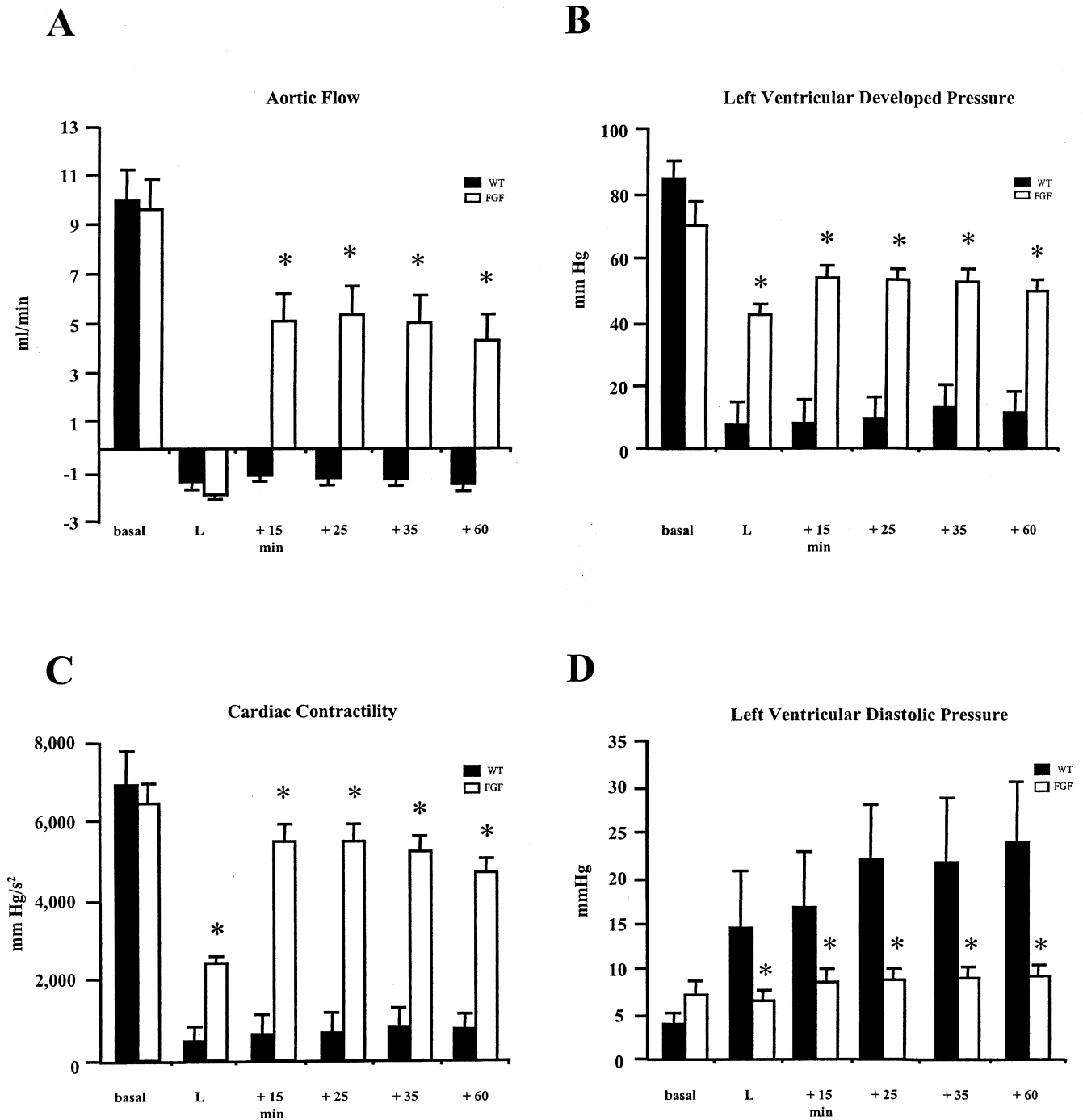
A

B

C

D

Figure 2. Cardiac function and cardiac functional recovery after global ischemia and reperfusion in fibroblast growth factor (FGF) and wild-type (WT) control hearts: **(A)** Aortic flow in ml/min. **(B)** Left ventricular developed pressure in mm Hg. **(C)** Maximal rate of positive pressure development (dP/dt$_{max}$ in mm Hg/s^2). **(D)** Left ventricular diastolic pressure (in mm Hg). *p < 0.05 compared with WT. "Basal" indicates the pre-ischemic period, "L" indicates Langendorff reperfusion, and "+15, +25, +35, +60 min" indicate 15, 25, 35, and 60 min of antegrade reperfusion, respectively.

of ERK (expressed as phospho-ERK-1/2/ERK-1/2 ratios) in FGF transgenic hearts to the level of untreated WT controls (Fig. 4B), indicating that attenuation of cardioprotection in FGF hearts may be accompanied by a decreased activation of pERK in these hearts. Treatment with LA also reduced pJNK activation (Fig. 4C), although no differences were observed between both genotypes. Surprisingly, the

administration of LA resulted in a marked upregulation of p38 activation in both genotypes (Fig. 4D).

Pharmacologic inhibition of PKC results in partial abrogation of FGF-1–induced protection. Compared with the non-treated FGF-1 group, the CHE-treated group (FGF/CHE) showed a moderate but significant decrease in cardiac functional recovery after ischemia and reperfusion,
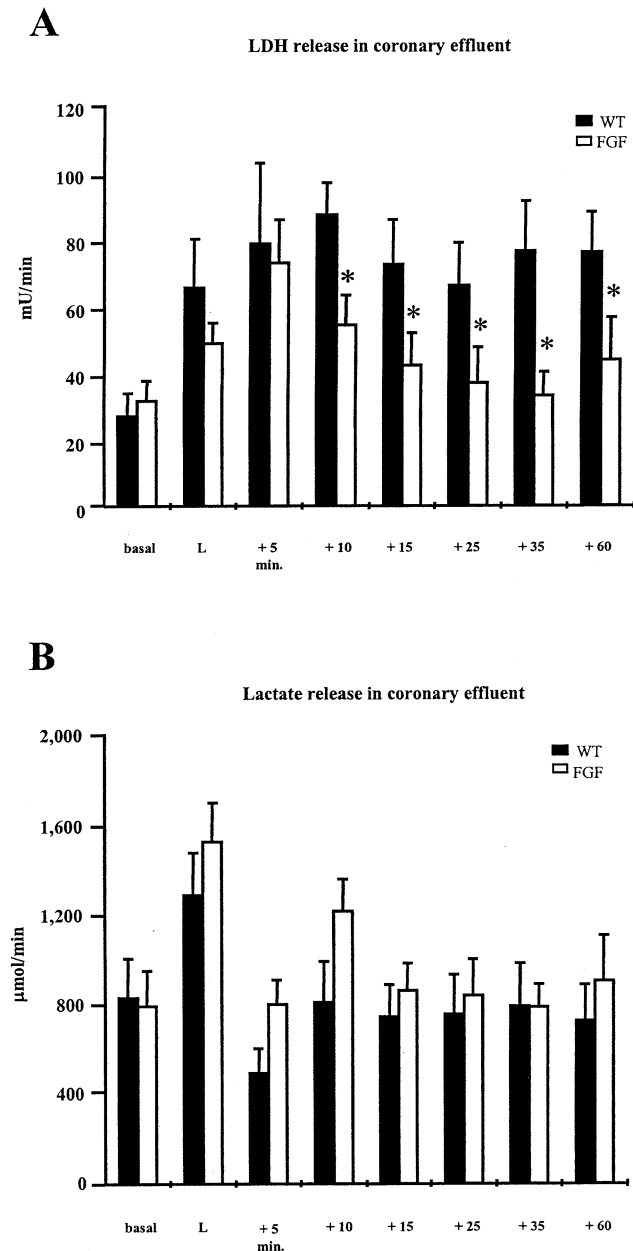
A

B

Figure 3. Pre- and post-ischemic lactate dehydrogenase (LDH) **(A)** and lactate **(B)** release in fibroblast growth factor (FGF) and wild-type (WT) control hearts (lactate in μmol/l·min and LDH in U/l; *p < 0.05 compared with WT). "Basal" indicates the pre-ischemic period, "L" indicates Langendorff reperfusion, and "+15, +25, +35, +60 min." indicates 15, 25, 35, and 60 min of antegrade reperfusion, respectively.

indicating partial abrogation of the FGF-1–induced cardioprotective effect (Figs. 5A to 5D). Similarly, CHE pretreatment in FGF-1 transgenic hearts resulted in an increased LDH release after I/R compared with non-treated FGF-1 TG and decreased PKC translocation to the particulate fraction (Fig. 4A). Induction of ischemia resulted in decreased cytosolic levels of PKC-ϵ in all experimental groups (data not shown).

Inhibition of FGF-R1 attenuates FGF-1–induced protection. SU5402 did not affect cardiac functional recovery in WT hearts (data not shown). When administered to

FGF-1 hearts, SU completely abrogated the FGF-1–induced protection of cardiac functional recovery that was observed in the non-treated FGF-1 group (Figs. 5A to 5D). As in treatment with LA, LDH release in SU-treated FGF TG hearts remained low after I/R and comparable with untreated FGF TG despite decreased cardiac functional recovery, indicating that FGF-R1 blockade did not prevent FGF-1–mediated protection of cell survival (Fig. 6).

DISCUSSION

In a murine ex vivo working heart model of ischemia and reperfusion, we found marked cardioprotective effects of cardiac-specific FGF-1 overexpression on cardiac functional recovery and cell survival. The protective effects on cardiac functional recovery are dependent on the activation of FGF-R1, PKC, and TK, as pharmacologic inhibition of these signaling peptides abolished the cardioprotective effect of FGF-1. The FGF-1–mediated protection of cell viability was partially dependent on PKC and completely independent of FGF-R1 and TK. To our knowledge, this is the first report directly linking FGF-1–mediated cardiac protection to a known IPC pathway.

Several groups have observed beneficial effects of FGF-1 during acute myocardial ischemia. Buehler et al. (9) demonstrated in an in vivo model of regional myocardial I/R that FGF-1 overexpression results in delayed infarct formation when compared with WT counterparts. This effect proved to be only temporary because the same infarct size was eventually reached, indicating a trophic effect of FGF-1 rather than cardioprotection due to increased collateral flow and limitation of infarct size. This is in agreement with data from our group, demonstrating that coronary artery ligation in these FGF-1 transgenic mice does not result in attenuation of infarct size one week after permanent coronary artery ligation (6). The trophic effects of FGF-1 were previously reported by Htun et al. (8), who demonstrated that the administration of FGF-1 to pigs before myocardial ischemia mimicked a state of ischemic preconditioning (8).

Intracellular signaling pathways involved in FGF-mediated protection. Several mechanistic studies have been conducted, mainly focusing on the denomination of the signaling pathways involved in FGF-2–mediated protection. In these studies, strong involvement of TK and PKC was demonstrated. Previously, Padua et al. (10) showed in an ex vivo rat heart model of global myocardial I/R that pretreatment with FGF-2 improved cardiac functional recovery in a PKC- and TK-dependent manner. Recently, Sheikh et al. (11) elegantly demonstrated that FGF-2 overexpression increased cardiomyocyte viability in an ex vivo cardiac ischemia and reperfusion model. FGF-2 overexpression was associated with constitutively enhanced phosphorylation levels of p38 and pJNK, in addition to enhanced expression of PKC-α and -ϵ. Surprisingly, no effects on cardiac functional recovery after I/R were reported, whereas exogenous FGF-2 administration did result
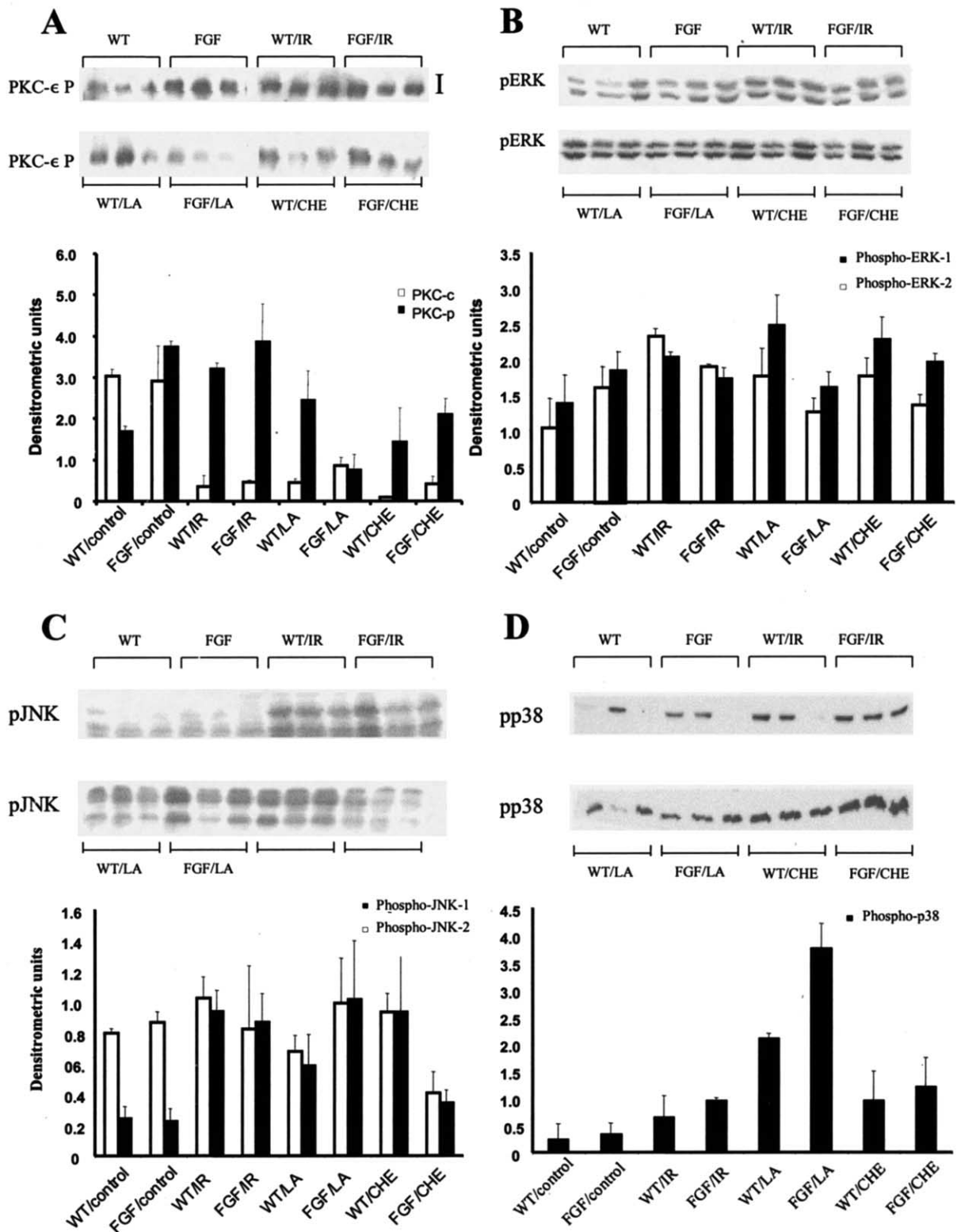
Figure 4. Western blot analysis demonstrating the activation of mitogen-activated protein kinases (MAPKs) (ERK-1/2, JNK-1/2, p38) and protein kinase C (PKC)-ε translocation to the particulate fraction. Each lane was loaded with equal amounts of protein. Polyclonal antibodies were used to detect bands. The **upper panel of each figure** is a representative blot, whereas the **lower panels** depict grouped densitometric data (mean ± SEM) from three experiments. For MAPKs, we used the ratio of phosphorylated MAPK/non-phosphorylated MAPK. **(A)** The **upper panel** shows a representative blot of the amount of PKC-ε translocation to the particulate fraction, whereas the **lower panel** demonstrates the quantitative densitometric data of PKC-ε translocation to the particulate fraction. **(B to D)** The **upper panels** show a representative blot of the activated form of each MAPK subtype, whereas the **lower panel** depicts the quantification of densitometric data. **(B)** phospho-ERK, **(C)** phospho-JNK1/2, **(D)** phospho-p38. CHE = chelerythrine; FGF = fibroblast growth factor transgenic littermates; IR = ischemia and reperfusion; LA = lavendustin A; PKC = protein kinase C; WT = wild-type littermates.
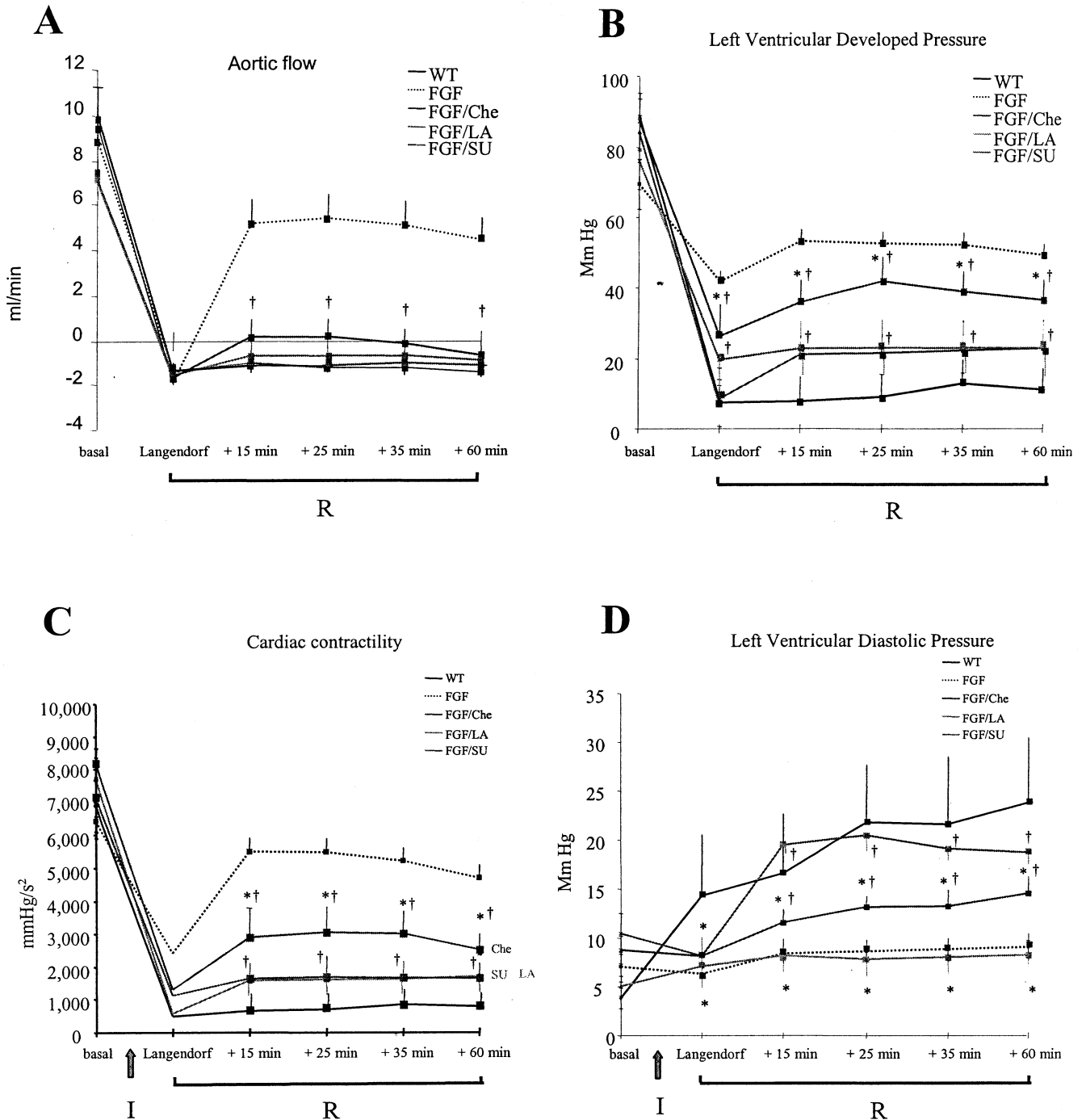
Figure 5. Cardiac functional recovery in untreated fibroblast growth factor (FGF) and wild-type (WT) control hearts. In addition, this figure demonstrates the hemodynamic effects of pretreatment of FGF hearts with selective inhibitors of tyrosine kinases (FGF/LA), PKC (FGF/CHE), and FGF-R1 (FGF/SU). **(A)** Aortic flow. **(B)** Left ventricular developed pressure. **(C)** Maximal rate of positive pressure development (dP/dt$_{max}$ in mm Hg/s^2). **(D)** Left ventricular diastolic pressure. *p < 0.05 compared with WT, †p < 0.05 compared with FGF. Che = chelerythrine chloride; LA = lavendustin A; SU = SU5402. "Basal" indicates the preischemic period, "L" indicates Langendorff reperfusion, and "+15, +25, +35, +60 min" indicates 15, 25, 35, and 60 min of antegrade reperfusion, respectively.

in moderately enhanced functional recovery. In addition, Bogoyevitch et al. (28) demonstrated PKC-α, -δ, and -ϵ upregulation after FGF-1 administration to isolated cardiac myocytes.

We and others showed in different models that the protective effects of FGF-1 and -2 (10) can be abrogated by

selective PKC inhibition. Although this indicates that PKC is required for FGF-1–mediated cardioprotection, it does not necessarily imply that PKC translocation is depending on activation of the FGF-R1. It merely suggests that PKC translocation is a prerequisite and indispensable for the preconditioning phenomenon to occur.
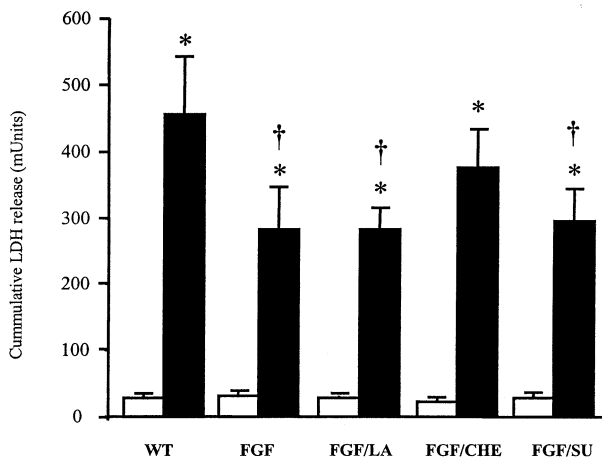
Figure 6. Pre- and post-ischemic lactate dehydrogenase (LDH) release in non-treated and inhibitor-treated fibroblast growth factor (FGF) hearts and wild-type (WT) control hearts. LDH release in U/l (*p < 0.05 compared with WT, †p < 0.05 compared with FGF). CHE = chelerythrine; LA = lavendustin A; SU = SU5402. **Open bars** = pre-ischemia; **closed bars** = post-ischemia.

There is some evidence that FGF-1-induced protection is mediated by TK, as is FGF-2–mediated protection (10). Htun et al. (8) showed that FGF-1 administration and its binding to FGF-R1 induces TK activation. We demonstrated that FGF-1–induced protection of cardiac functional recovery could be completely abrogated by the administration of LA, indicating that TK activation is necessary for FGF-1–induced cardioprotection. However, the FGF-1–induced protection of cell viability during I/R is not dependent on TK because the inhibition of TK did not affect LDH release in TG hearts, which could be accounted for by species-dependent differences in TK-mediated cell death.

We demonstrate that administration of SU, an FGF-R1 blocker, resulted in complete abrogation of protection of function. This is in agreement with Htun et al. (8), who suggested that FGF-1–induced protection was receptor mediated. Surprisingly, the protective effects of FGF-1 on cell survival could not be inhibited by FGF-R1 blockade, indicating that prevention of I/R-induced cell death is, at least partially, dependent on intracellular FGF-1.

Effects of FGF-1 on cardiac cell survival during ischemia and reperfusion. In the FGF-1 group, LDH release during reperfusion was attenuated when compared with WT animals. However, as already shown previously (21), this release accounts for only 8% of the total LDH content of the heart. Therefore, in this model, only a minor part of the total cardiomyocyte population becomes necrotic due to ischemia. The relatively small reduction in necrosis due to FGF-1 overexpression may therefore not explain the large difference in cardiac functional recovery that was observed between both genotypes. Interestingly, inhibition of PKC translocation, but not inhibition of FGF-R1 and TK activation, enhanced cell death in TG animals, suggesting that FGF-1–dependent protection on cell survival after I/R

is partially dependent on PKC, but completely independent of FGF-R1 and TK.

Fibroblast growth factor-1 is known to decrease apoptosis in an in vitro model of hypoxia and reoxygenation (7). However, it seems unlikely that delayed activation of programmed cell death is the mechanism underlying the protective effects on cell damage and function that we observed because the protective effects of FGF-1 became apparent immediately after starting reperfusion and completion of the sequential activation of the cascade involved in the process of apoptosis would take longer than 25 min. In addition, we performed a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end labeling assay on FGF-1 TG and WT hearts (data not shown), which showed no signs of apoptosis after 60 min of reperfusion. However, Dumont et al. (29) showed annexin-V labeling early after initiation of reperfusion in an in vivo murine model of myocardial I/R. Therefore, we cannot completely rule out the occurrence of early stages of apoptosis in this model.

Because myocyte necrosis or apoptosis cannot account for the large functional differences in cardiac functional recovery due to FGF-1 overexpression, we hypothesized that FGF-1 attenuates myocardial stunning, a process occurring in the reperfused heart, in which certain areas of the heart that were previously ischemic are still viable but not contracting (30). Several groups demonstrated that stunning could be prevented by IPC (31,32). Furthermore, Htun et al. (8) demonstrated that FGF-1 induces a state that mimics IPC. The fact that TK and FGF-R1 inhibition did not attenuate the FGF-1–mediated protection of cell survival further underlines this hypothesis.

Effects of FGF-1 on anaerobic metabolism. In our model, we observed a lactate washout peak 5 min after the start of reperfusion, indicating anaerobic glycolysis during the ischemic phase. Lactate release returned to baseline within 10 min after the start of reperfusion. This indicates that after 10 min, little anaerobic metabolism is present in the reperfused heart and that the myocardium is adequately perfused in both genotypes. One might hypothesize that lactate release would remain elevated longer in the WT group when compared with FGF-1 animals because FGF-1 acts as a vasodilator (33), facilitating the reopening of coronary arterioles and capillaries. However, both at baseline and throughout the rest of the experimental protocol, no differences in lactate release between both genotype groups could be observed. In addition, no effects on lactate release could be observed in inhibitor-treated WT and TG hearts.

Possible implications for future growth factor therapy. In the majority of both animal and human studies, FGF-1 and -2 overexpression or exogenous administration were demonstrated to be beneficial for the heart in situations of acute ischemia (7,8). The results of this study suggest that pre-ischemic FGF-1 administration might protect the heart from subsequent ischemic damage. In addition, one could speculate that administration of FGF-1 to cardioplegia

solutions during cardiac surgery might further reduce ischemia and reperfusion-induced damage.

One of the limitations of this model is that FGF-1 is present before the ischemic period is commenced, which does not resemble the human situation of reperfusion during myocardial infarction. Additional studies are required to investigate whether FGF-1 also protects cardiac function and reduces reperfusion damage when administered only during restoration of flow. If this hypothesis could be confirmed, new treatment options are within reach. One of these is to administer FGF-1 simultaneously with thrombolytic therapy in patients suffering from acute myocardial infarction, with the purpose of reducing infarct size and improving cardiac functional recovery.

Conclusions. Cardiac-specific FGF-1 overexpression results in augmentation of cardiac functional recovery and attenuation of reperfusion-induced myocardial cell necrosis during I/R. The protective effects attributed to FGF-1 were linked to a known IPC pathway by demonstrating that protection is dependent on FGF-R1, PKC, and TK. These findings might have important implications for future growth factor therapy and for the prevention of reperfusion-induced damage.

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