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The cardioprotective effect of the low molecular weight isoform of fibroblast growth factor-2: the role of JNK signaling

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Abstract

BACKGROUND: Our laboratory showed that overexpression of fibroblast growth factor-2 (FGF2) protected the heart against ischemia-reperfusion injury. FGF2 has different protein isoforms (low [LMW] and high [HMW] molecular weight isoforms) produced from alternative translation start sites. However, which FGF2 isoform(s) mediates this cardioprotection, and which signaling pathway (i.e., mitogen-activated protein kinase (MAPK)) elicits FGF2 isoform-induced cardioprotection remains to be elucidated.

METHODS AND RESULTS: Wildtype, *Fgf2* KO (absence of all FGF2 isoforms) and FGF2 LMWKO (absence of LMW isoform) hearts were subjected to an *ex vivo* work-performing heart ischemic model of 60 minutes ischemia and 120 minutes reperfusion. There was a significant decrease in the recovery of post-ischemic contractile function as well as in creatine kinase (CK) release ($p < 0.05$) in *Fgf2* KO and FGF2 LMWKO mouse hearts compared to wildtype hearts. Following ischemia-reperfusion injury, MKK4/7, JNK, and c-Jun were significantly phosphorylated (i.e., activated), and the levels of TUNEL-positive nuclei and caspase 3 cleavage were significantly increased in vehicle-treated *Fgf2* KO and FGF2 LMWKO compared to wildtype hearts ($p < 0.05$). A novel JNK pathway inhibitor, CEP11004 (50nM), significantly restored the post-ischemic contractile function and reduced myocardial cell death, as measured by CK release and apoptotic markers, compared to DMSO-treated cohorts ($p < 0.05$). Overall, our data indicate that the LMW isoform has an important role in restoring cardiac function after ischemia-reperfusion (I/R) injury. These results provide unequivocal evidence that inhibition of JNK signaling is involved in FGF2 LMW isoform-mediated cardioprotection and that the potential mechanism may be through inhibition of the apoptotic process.

Keywords

fibroblast growth factor; JNK signaling; apoptosis; genetically altered mice; ischemia-reperfusion injury; cardioprotection; low molecular weight isoform

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Introduction

Cardiovascular disease is the leading cause of death in the United States and developing world. Experimental and clinical studies have demonstrated that a number of interventions including brief periods of ischemia or hypoxia and certain endogenous factors such as fibroblast growth factor 2 (FGF2) or pharmacological agents are capable of protecting the heart against post-ischemic contractile dysfunction, arrhythmias and myocardial infarction [1].

FGF2, a prototypic member of the larger family of heparin-binding growth factors, is a multifunctional protein that stimulates cell growth and angiogenesis as well as affects cell differentiation, cell survival and cell death [2], activities which affect vascular tone control, blood pressure, cardiac hypertrophy, bone and brain development and wound healing in mice [3–7]. FGF2 is encoded by a single-copy gene and different isoforms (low molecular weight, LMW and high molecular weight, HMW) of FGF2, produced by alternative translation start sites, have been identified. The HMW isoforms (34, 24, 22.5 and 22 kDa found in human and 21 and 21.5 kDa found in mouse) are encoded by the CUG codons; whereas, the LMW isoform (18 kDa protein for both human and mouse) starts at the AUG codon [8]. HMW isoforms contain a nuclear localization signal that targets the HMW isoforms to the nucleus while the LMW FGF2 isoform is predominantly cytoplasmic [9]. Due to the different cellular localizations of HMW and LMW isoforms, it has been suggested that LMW FGF2 acts in an autocrine-paracrine mechanism via the extracellular cell surface receptor and that HMW FGF2 isoforms function in an intracrine manner. *In vitro*, the high and low molecular weight FGF2 isoforms display different biological properties in the heart such that all isoforms increased proliferation of neonatal rat cardiac myocytes, but only the HMW isoforms are associated with a significant increase in binucleation [10]. In the rat heart, the LMW isoform is the predominant isoform in the adult while the HMW isoforms are mostly expressed during neonatal development [11]. Our laboratory has recently reported that overexpressing FGF2 in the heart can protect against contractile dysfunction and myocardial infarction caused by ischemia-reperfusion (I/R) injury, and this cardioprotective effect is independent from its angiogenic activity [12]. Although FGF2 is a known cardioprotective molecule [2,12–14], the function(s) of the LMW and HMW protein isoforms in I/R injury and cardioprotection remain to be elucidated.

Some of the biological functions of FGF2 are mediated via binding with FGF receptors, a member of the receptor tyrosine kinase family. The activation of FGF receptors recruits particular target proteins such as Ras, a MAPK kinase kinase (MAPKKK) that triggers the mitogen activated protein kinase (MAPK) pathway [15], which includes extracellular signal-regulated kinases (ERK1/2), the p38 MAPKs and c-Jun N-terminal kinases (JNK1/2/3). Our laboratory showed that p38 MAPK and ERK were involved in FGF2-mediated cardioprotection [13]. Many studies have demonstrated that activation of JNK signaling is critical for the pathogenesis of apoptotic cell death occurring in I/R injury [16,17]. Whether the JNK signaling pathway is involved in FGF2 function, especially in FGF2 isoform-mediated cardioprotection, remains to be elucidated.

The present study investigated the role of the FGF2 LMW isoform in cardioprotection and the involvement of JNK signaling in FGF2 LMW isoform-mediated cardioprotection. Our results demonstrate that the LMW FGF2 isoform has an important role in protecting the heart against post-ischemic contractile dysfunction. Furthermore, post-ischemic contractile dysfunction is restored and the degree of myocardial infarction is reduced upon inhibition of the JNK pathway, suggesting that FGF2 LMW isoform-mediated cardioprotection involves JNK signaling.

Materials and Methods

Animals and exclusion criteria

Mice were housed in a pathogen-free facility and handled in accordance with standard use protocols, animal welfare regulations, and the NIH *Guide for the Care and Use of Laboratory Animals*. All protocols were approved by the University of Cincinnati Institutional Animal Care and Use Committee. Wildtype (Wt) mice, *Fgf2* knockout (KO) mice (all FGF2 isoforms were deleted) and FGF2 LMWKO mice (FGF2 LMW isoform was removed) were randomly assigned to the present study. All mice had a 50% Black Swiss/50% 129 background. Fifty four Wt, 43 *Fgf2* KO and 48 FGF2 LMWKO mouse hearts completed the DMSO- and CEP11004-treated ischemia-reperfusion injury study. 5 Wt, 5 *Fgf2* KO and 6 FGF2 LMWKO mouse hearts completed the U0126-treated studies, 6 Wt, 6 *Fgf2* KO and 5 FGF2 LMWKO mouse hearts completed the SB203580 studies, and 5 Wt, 4 *Fgf2* KO and 5 FGF2 LMWKO mouse hearts completed the anisomycin, p38 activator, studies. Ten Wt, 10 *Fgf2* KO and 10 FGF2 LMWKO mouse hearts completed the time-course study. Exclusion from the study was based on the signs of aortic or pulmonary vein leak in the working heart preparation. A total of 15 mice were excluded from the study.

Generation of FGF2 LMW knockout (LMWKO) mice

FGF2 LMWKO mice were generated in the laboratory of Dr. Thomas Doetschman as previously described [18]. A “Tag and Exchange” strategy [19] was employed, utilizing both the positive and negative selectibility of the *Hprt* gene, to introduce a 4 bp change that removed the ATG translational start site and NcoI site and introduced a PstI site (Figure 1A).

Isolated work-performing heart preparation [12]

Age-(10–12 weeks) and sex-matched Wt, *Fgf2* KO and FGF2 LMWKO mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and heparinized (5000U/kg, i.p.) to protect the heart against microthrombi. The aorta was cannulated, preserving the aortic valve and coronary artery ostia. The intraventricular catheter was inserted into the left ventricle to measure intraventricular systolic and diastolic pressures. A cannula was also inserted into the left pulmonary vein, thereby allowing the direction of perfusate to be switched from retrograde (Langendorff mode) to anterograde (working mode). Aortic pressure, atrial pressure and left ventricular pressure were measured with COBE pressure transducers and data was recorded using a Grass polygraph and digital acquisition system.

Model of global, low-flow ischemia

The hearts were equilibrated for 30 minutes at a basal workload of 250mmHg*mL/min. After 30 minutes of equilibration, the venous return was reduced to a flow of 1 mL/min for 60 minutes to elicit low-flow ischemia which lead to a 90% of reduction in coronary flow similar to that of patients suffering from severe coronary artery disease. Following 60 minutes of low-flow ischemia, venous return was increased to a flow of 5 mL/min and reperfusion occurred for 120 minutes. Cardiac function parameters represented by $\pm dP/dt$, left ventricular systolic pressure (LVSP) and left ventricular end diastolic pressure (LVEDP), perfusate gases and coronary effluent were obtained at designated time points of baseline, ischemia, and reperfusion (Figure 2). Percent of post-ischemic functional recovery was calculated from the data of contractile function ($+dP/dt$) at 120 minutes of reperfusion (R_{120}) versus the baseline (B) $+dP/dt$ (%) Recovery = $(+dP/dt R_{120}/+dP/dt B) * 100$.

Pharmacological agents

CEP11004, a JNK pathway inhibitor which inhibits mixed lineage kinase (MLK3), an upstream kinase involved in JNK activation, was generously donated as a gift from Cephalon Inc., West

Chester, PA. 1,4-Diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio) butadiene (U0126), an inhibitor of MEK1/2 (upstream kinase of ERK activation [20]), was purchased from Promega, and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole HCl (SB203580), a p38 MAPK inhibitor [21], and 2-(*p*-Methoxybenzyl)-3,4-pyrrolidinediol-3-acetate (anisomycin), a p38 activator [22], were purchased from Calbiochem. CEP11004, U0126, and anisomycin were dissolved in DMSO and SB203580 was dissolved in water; all were diluted in Krebs's solution to obtain final concentrations. CEP11004 (50nmol/L), U0126 (2.5μmol/L), SB203580 (2μmol/L), or anisomycin (5μmol/L) was administered 15 minutes prior to and for the first 15 minutes of ischemia and the last 15 minutes of ischemia and first 15 minutes of reperfusion. DMSO was given at the same length and time points for vehicle treatment (Figure 2).

Creatine kinase (CK) release

The coronary effluent was collected at designated time points of baseline, ischemia and reperfusion (see Figure 2) and protease inhibitor tablets (SIGMA) were added to the collected coronary effluent. The amount of CK was determined using a CK Reagent Set (International Bio-Analytical Industries, Inc.) and a modified manufacturer's instructions as previously described [12]. Creatine kinase release was normalized to coronary flow (ml/min) and heart weight (g) and represented as U/min*g.

Vascular bed staining

Paraffin-embedded non-ischemic hearts from Wt, *Fgf2* KO and FGF2 LMWKO mice were treated with pepsin (DAKO Corporation, Carpinteria, CA) for 10 minutes at 37°C and 3% H₂O₂ for 5 minutes. Primary antibodies against smooth muscle α-actin (1:400, SIGMA) and von Willebrand's factor (1:100, DAKO Corporation) were utilized to stain vascular smooth muscle cells and endothelial cells, respectively. To assess the level of smooth muscle-containing blood vessels, a total of 192 fields (4 fields/section, 6 sections/heart) from each group (8 hearts/group) were counted at a magnification of 200X. Quantification of capillary levels was performed by counting a total of 240 fields (5 fields/section, 6 sections/heart) from each group (8 hearts/groups) at a magnification of 1000X.

Cardiac preparation and immunoblotting for detection of FGF2

Snap-frozen non-ischemic hearts were powdered and homogenized in homogenization buffer (20mM Tris, 2mM EDTA, 2M NaCl, 1% NP40, PMSF), and FGF2 was extracted as previously described¹². Western blot analysis of the extracted FGF2 was performed with a rabbit polyclonal antibody to FGF2 (1:1000, Santa Cruz Biotechnology). Levels of FGF2 protein isoforms were visualized by chemiluminescence (Amersham Bioscience). Densitometry of protein bands was performed using a Fluorchem 8800 gel imager (Alpha Innotech).

Time course of MAPK kinase 4 and 7 (MKK4/7) and JNK phosphorylation (i.e., activation)

Wt, *Fgf2* KO and FGF2 LMWKO hearts were arrested at different time points of ischemia-reperfusion injury to elucidate the time frame of MKK4, MKK7 and JNK phosphorylation. The time points include: sham, 5 minutes ischemia, 60 minutes ischemia and 60 minutes ischemia followed by either 5 or 15 minutes of reperfusion. Administration of CEP11004 was similar to that of hearts subjected to 60 minutes of ischemia and 120 minutes of reperfusion. Hearts were snap frozen in liquid nitrogen and stored in -80°C until further analysis.

Whole heart preparation for MAPK immunoblotting [13] and caspase 3 detection

Snap-frozen non-ischemic hearts and ischemic or ischemic-reperfused hearts from DMSO- and CEP11004-treated Wt, *Fgf2* KO and FGF2 LMWKO groups were powdered and homogenized in homogenization buffer (25mM HEPES, pH7.5, 1% glycerol, 150mM NaCl,

1% Triton X-100, 5mM EDTA, 1mM sodium orthovanadate, 25mM β -glycerolphosphate, 50mM sodium fluoride solid, 0.5 μ M okadaic acid, diisopropylfluorophosphate, 100 μ M calpain inhibitor, Pefabloc stock 1 and 2, SIGMA phosphatase inhibitor). The homogenate was centrifuged at 13,000g for 15 minutes and the supernatant collected. Protein concentration was determined via Lowry protein assay.

Immunoblotting of MAPK pathway

Cardiac samples were loaded onto a 10% SDS polyacrylamide gel and transferred onto nitrocellulose membrane. The blots were blocked with 5% non-fat milk and then incubated with primary antibody (1:1000) against phospho-ERK1/2 (Santa Cruz Biotechnology), phospho-p38 MAPK (Santa Cruz Biotechnology), phospho-JNK (Promega), phospho-MKK4 (Cell Signaling), phospho-MKK7 (Cell Signaling) and phospho-c-Jun-Ser73 (Cell Signaling). Total expression levels of ERK1, p38 MAPK, JNK2, MKK4, MKK7 and c-Jun were evaluated by stripping the blot and incubating with primary antibody (1:1000) against ERK1 (Signal Transduction), p38 MAPK (Santa Cruz Biotechnology), JNK2 (Santa Cruz Biotechnology), MKK4 (Cell Signaling), MKK7 (Cell Signaling) and c-Jun (Cell Signaling). The phosphorylation state or total level of MAPKs and c-Jun was visualized by chemiluminescence. Densitometry of protein bands was performed using a Fluorchem 8800 gel imager. The phosphorylation (i.e., activation) of MAPKs and c-Jun was represented as the ratio of phospho-MAPKs and phospho-c-Jun vs. total MAPKs and c-Jun, respectively.

Immunoblotting of caspase 3 activation

Cardiac samples were loaded onto a 15% SDS polyacrylamide gel and transferred onto nitrocellulose membrane. The blots were blocked with 5% non-fat milk and then incubated with primary antibody against cleaved caspase 3 (1:500 dilution, Cell Signaling). The total expression level of caspase 3 was performed by re-incubating the membrane with total caspase 3 antibody (1:500 dilution, Cell Signaling). The cleaved or total level of caspase 3 was visualized by chemiluminescence. Densitometry of protein bands was performed using a Fluorchem 8800 gel imager. The activation of caspase 3 was represented as the ratio of cleaved caspase 3 vs. total caspase 3.

TUNEL assay

The TUNEL assay was used to detect apoptotic cell death by enzymatic labeling of DNA strand breaks with fluorescein-dUTP and TdT (Promega) [23]. Briefly, heart sections from non-ischemic and ischemic-reperfused DMSO- and CEP11004-treated Wt, *Fgf2* KO and FGF2 LMWKO groups were fixed in 4% formaldehyde, permeabilized and stained with TUNEL-reaction mixture and DAPI. The total number of nuclei per unit area of the heart was determined by counting the number of DAPI-positive cell in a total of 96 fields (24 fields/heart) for each group (4 hearts/group) at a magnification of 200X. The number of apoptotic nuclei (TUNEL-positive) was determined by counting the same 96 fields from each group. The images were overlayed and the data were calculated as the percentage of apoptotic nuclei vs. total number of nuclei.

Statistics

All values in the text and figures are represented as mean $\pm$ SEM of *n* independent experiments. Percentage of contractile function, CK release and TUNEL assay were subjected to analysis of variance (ANOVA) followed by Students' *t*-test. Immunoblotting data were subjected to Students' *t*-test. Probabilities of 0.05 or less ($p < 0.05$) were considered statistically significant.

Results

Effect of ablation of FGF2 LMW isoform on the heart

No FGF2 LMW isoform was detected in *Fgf2* LMWKO mice (Figure 1B), and immunoblotting showed that cardiac expression of the FGF2 HMW isoforms are not FGF2 LMW isoform dependent (Figure 1B and C). There was no significant difference in heart-weight to body-weight ratio (mg/g) between the 3 groups (Wt=5.0±0.1mg/g, *Fgf2* KO=4.9±0.1mg/g, *Fgf2* LMWKO=5.3±0.2mg/g) indicating that ablation of the LMW or all isoforms did not alter cardiac growth.

No significant alteration in vessel number or defects in vasculogenesis or angiogenesis was detected in Wt, *Fgf2* KO and FGF2 LMWKO mouse hearts. There was no significant difference in the number of smooth muscle-containing blood vessels per square millimeter (mm²) in Wt (8.8±0.4/mm²), *Fgf2* KO (9.7±0.7/mm²) and FGF2 LMWKO (8.6±0.4/mm²) hearts. Also, in FGF2 LMWKO (51.8±5.1/mm²) and Wt (55.1±4.0/mm²) groups, the cardiac capillary density was similar to *Fgf2* KO groups as measured previously [12].

Alteration of MAPK activation in *Fgf2* KO and FGF2 LMWKO mouse hearts

MAPK activation (i.e., phosphorylation) is involved in ischemia-reperfusion injury, in either a beneficial or deleterious manner [24]. Also, a number of FGF2's actions occur via MAPK signaling [25,26]. However, whether manipulation of the cardiac expression of the FGF2 protein isoforms modulates MAPK activation (as measured by its phosphorylation state), thereby altering pathways that may aid the heart against ischemic insults, is unknown. A significant increase in phosphorylation of JNK ($p<0.05$, Figure 3A and 3D), a decrease in the p38 MAPK phosphorylation state ($p<0.05$, Figure 3A and 3C), but no difference in ERK phosphorylation (Figure 3A and 3B) was observed in *Fgf2* KO and FGF2 LMWKO hearts compared to Wt hearts. Protein expression of the MAPKs was not different among groups. To confirm that the LMW isoform of FGF2 modulated MAPK phosphorylation, FGF2 LMWKO hearts were treated with recombinant mouse LMW FGF2 (rmLMW FGF2, 10µg/heart) and arrested five minutes after administration. A significant decrease (53%) in JNK phosphorylation ($p<0.05$) and a marked increase (50%) in p38 MAPK phosphorylation ($p<0.05$) in rmLMW FGF2-treated vs. saline-treated FGF2 LMWKO hearts was observed (data not shown). Overall, these data indicate that manipulation of FGF2 protein isoform expression modulates the level of MAPK phosphorylation, in particular, p38 MAPK and JNK.

Activation of c-Jun terminal kinase (JNK) pathway following ischemia-reperfusion injury in Wt, *Fgf2* KO and FGF2 LMWKO mouse hearts

The JNK pathway is known to be activated during ischemia-reperfusion injury [17]. To elucidate whether manipulation of any FGF2 protein isoform(s) would influence JNK phosphorylation, indicative of activation, during ischemia-reperfusion injury, Wt, *Fgf2* KO and FGF2 LMWKO hearts were subjected to 5 or 60 minutes global low-flow ischemia or 60 minutes global low-flow ischemia followed by 5 or 15 minutes of reperfusion. Phosphorylation of JNK was significantly increased (2.2±0.3 and 1.8±0.2 fold, respectively) in sham-treated *Fgf2* KO and FGF2 LMWKO vs. sham-treated Wt hearts ($p<0.05$, Figure 4A), and this finding was consistent with that observed in non-ischemic hearts (Figure 3A and 3C). In addition, JNK phosphorylation was significantly elevated in *Fgf2* KO and FGF2 LMWKO vs. Wt hearts at 5 minutes of ischemia (*Fgf2* KO: 2.6±0.9 fold increase and FGF2 LMWKO: 2.2±0.1 fold increase), 60 minutes of ischemia and 15 minutes of reperfusion (*Fgf2* KO: 5.0±0.9 fold increase and FGF2 LMWKO: 4.8±1.2 fold increase), or 120 minutes of reperfusion (*Fgf2* KO: 6.6±1.3 fold increase and FGF2 LMWKO: 5.8±1.5 fold increase) ($p<0.05$, Figure 4A and 4B). At 5 minutes and 15 minutes of reperfusion, there was a significant increase (3.3±0.9 and 2.1±0.5 fold, respectively) in JNK phosphorylation in Wt hearts vs. sham-treated cohort ($p<0.05$,

Figure 4A and 4B) which is consistent with results from others [17]. Since JNK's phosphorylation and activation requires the upstream kinases MKK4 and MKK7 [27], the activation as measured by phosphorylation level of MKK4/7 during ischemia-reperfusion injury was assessed. Phosphorylation level of MKK7 was significantly increased (1.7 ± 0.6 and 1.7 ± 0.3 fold, respectively) in sham-treated *Fgf2* KO and FGF2 LMWKO vs. sham-treated Wt hearts ($p < 0.05$, Figure 4A and 4C), and was significantly increased in *Fgf2* KO and FGF2 LMWKO vs. Wt hearts at 60 minutes ischemia and 5 minutes of reperfusion (*Fgf2* KO: 3.7 ± 0.4 fold increase and FGF2 LMWKO: 5.9 ± 0.3 fold increase), 15 minutes of reperfusion (*Fgf2* KO: 2.6 ± 0.9 fold increase and FGF2 LMWKO: 2.2 ± 0.1 fold increase) or 120 minutes of reperfusion (*Fgf2* KO: 2.8 ± 0.4 fold increase and FGF2 LMWKO: 2.9 ± 0.9 fold increase) ($p < 0.05$, Figure 4A and 4C). MKK4 phosphorylation was significantly elevated (1.7 ± 0.1 and 1.8 ± 0.4 fold, respectively) in *Fgf2* KO and FGF2 LMWKO vs. Wt hearts at 60 minutes ischemia and 15 minutes of reperfusion (Figure 4A and 4D).

Inhibition of JNK pathway involved in FGF2 LMW isoform-induced cardioprotection on post-ischemic recovery of cardiac function

Wt, *Fgf2* KO and FGF2 LMWKO hearts were subjected to 60 minutes of low-flow ischemia and 120 minutes of reperfusion, and treated with CEP-11004 to determine whether JNK signaling mediates FGF2 LMW isoform-induced cardioprotection. There were no differences in basal cardiac function in DMSO- and CEP11004-treated Wt, *Fgf2* KO and FGF2 LMWKO hearts (Table 1). Following ischemia-reperfusion injury, there was a significant decrease in post-ischemic recovery of contractile function in DMSO-treated *Fgf2* KO hearts ($39 \pm 4\%$) and FGF2 LMWKO hearts ($47 \pm 5\%$) vs. Wt ($60 \pm 7\%$) hearts ($p < 0.05$, Figure 5). There was significant systolic and diastolic dysfunction as measured by left ventricular systolic pressure (LVSP), $+dP/dt$ (derivative of change in contractile pressure over time), left ventricular end-diastolic pressure (LVEDP), and $-dP/dt$ (derivative of change in relaxation pressure over time) in ischemic-reperfused, DMSO-treated *Fgf2* KO and FGF2 LMWKO vs. DMSO-treated Wt hearts ($p < 0.05$, Table 1). Furthermore, there was no difference in the systolic and diastolic parameters between *Fgf2* KO and FGF2 LMWKO hearts (Table 1), suggesting that the FGF2 LMW isoform is most likely the isoform required for cardioprotection. After CEP11004 treatment, post-ischemic contractile function was significantly restored in all groups: Wt ($81 \pm 4\%$), *Fgf2* KO ($83 \pm 5\%$) and FGF2 LMWKO ($67 \pm 5\%$) ($p < 0.05$, Figure 5), indicating that inhibition of JNK signaling has a role in FGF2 isoform-induced cardioprotection. Furthermore, the percent increase in post-ischemic contractile function in *Fgf2* KO ($\sim 50\%$) and FGF2 LMWKO ($\sim 35\%$) hearts was significantly higher than in Wt hearts ($\sim 17\%$). Finally, there were significant increases in systolic (LVSP and $+dP/dt$) and diastolic (LVEDP and $-dP/dt$) parameters in CEP11004-treated groups compared to DMSO-treated groups ($p < 0.05$, Table 1), indicating that CEP11004 improved both contractile and relaxation function. Collectively, these results indicate that the cardioprotective effect of the FGF2 LMW isoform occurs, in part, through modulating JNK activation.

To determine whether p38 or ERK signaling was also important, the p38 MAPK inhibitor, SB 203580, p38 MAPK agonist, anisomycin, or the MEK1/ERK inhibitor, U0126, was administered. Inhibition of p38 MAPK or MEK1/ERK or activation of p38 MAPK did not improve contractile dysfunction (data not shown); whereas, JNK pathway inhibition abolished the cardiac dysfunction observed in *Fgf2* KO and FGF2 LMWKO hearts (Figure 5). Hence, JNK is the predominant MAPK pathway involved in modulating the cardioprotective actions of the FGF2 LMW isoform.

Inhibition of JNK signaling involved in FGF2 LMW isoform-induced cardioprotection on myocardial cell injury

Myocardial cell death was represented as CK release measured at designated time points from coronary effluent (Figure 2). There was a significant increase in CK release at early reperfusion compared to baseline values in all vehicle-treated groups ($p < 0.05$, Table 2). Following CEP11004 treatment, there was a significant decrease in CK release in all groups during ischemia compared to DMSO-treated hearts as well as during early reperfusion compared to DMSO-treated groups ($p < 0.05$, Table 2). p38 MAPK or MEK1/ERK inhibition also did not reduce CK release during reperfusion (data not shown). p38 activation resulted in a reduction in CK release during reperfusion; however, there was no difference in CK release in anisomycin-treated Wt, *Fgf2* KO and FGF2 LMWKO hearts, suggesting that the reduction of CK release was not FGF2 isoform dependent (data not shown). This result indicates that inhibition of the JNK signaling pathway is important in protecting the heart from myocardial cell injury.

Effect of CEP11004 on JNK signaling and its downstream targets

Following 60 minutes ischemia and 120 minutes reperfusion, JNK and MKK7 phosphorylation levels were significantly increased in DMSO-treated *Fgf2* KO and FGF2 LMWKO vs. DMSO-treated Wt hearts ($p < 0.05$, Figures 6A and 6B, 7A and 7B). This finding suggests that the FGF2 LMW isoform may have a partial role in modulating JNK signaling. Immunoblotting indicated that the phosphorylation state of JNK, MKK7 and MKK4 was inhibited in CEP11004-treated *Fgf2* KO and FGF2 LMWKO groups compared to vehicle-treated cohorts ($p < 0.05$, Figures 6A and 6B, 7A, 7B, and 7C) demonstrating that CEP11004 sufficiently inhibited MKK4/7 signaling. No significant difference in MKK4 phosphorylation was observed in DMSO-treated Wt, *Fgf2* KO, and FGF2 LMWKO hearts (Figures 7A and 7C). Moreover, p38 MAPK and ERK phosphorylation (i.e., activation) were not altered in the CEP11004-treated vs. DMSO-treated groups (Figures 6A, 6C, and D).

JNK pathway inhibition on apoptosis signaling

To identify a potential mechanism that might underlie the cardioprotection mediated by the FGF2 LMW isoform after inhibition of the JNK pathway, the activation of apoptosis regulatory factors such as caspase 3, and key downstream targets such as c-Jun activation were evaluated. No differences were observed in the early stage of apoptosis as measured by caspase 3 cleavage in non-ischemic hearts (Figure 8A inset); however, an increase in TUNEL-positive cells, an indication of the late stage of apoptosis was seen in non-ischemic *Fgf2* KO and FGF2 LMWKO vs. Wt hearts ($p < 0.05$, Figure 8C inset). Activation of c-Jun and cleavage of caspase 3 were increased in DMSO-treated *Fgf2* KO and FGF2 LMWKO vs. DMSO-treated Wt hearts ($p < 0.05$, Figures 7A, 7D and 8A). In DMSO-treated groups, there was also a significant increase in TUNEL-positive cells in *Fgf2* KO ($26 \pm 1\%$) and FGF2 LMWKO ($31 \pm 5\%$) vs. Wt ($16 \pm 4\%$) hearts ($p < 0.05$, Figure 8B and C). These results suggest that the FGF2 LMW isoform may modulate apoptotic signaling during ischemia-reperfusion injury. After CEP11004 treatment, there was a significant decrease in the activation of c-Jun and caspase 3 cleavage in Wt, *Fgf2* KO and FGF2 LMWKO hearts compared to DMSO-treated hearts ($p < 0.05$, Figure 7C and 8A). Furthermore, the number of TUNEL-positive cells was significantly decreased in CEP11004-treated Wt ($8 \pm 1\%$), *Fgf2* KO ($5 \pm 1\%$) and FGF2 LMWKO ($8 \pm 2\%$) hearts compared to DMSO-treated hearts ($p < 0.05$, Figure 8B and C). Thus, inhibition of the JNK pathway resulted in decreased ischemia-reperfusion-induced apoptosis.

Discussion

Many studies have demonstrated that FGF2 is a cardioprotective molecule [2,12,14]. Our laboratory previously showed that FGF2 is very important in protecting the heart from

myocardial dysfunction and myocardial infarction following ischemia-reperfusion (I/R) injury [12]. FGF2 consists of multiple isoforms (LMW and HMW isoforms) which target to different cellular locations [9]. The role of FGF2 isoform(s) in cardioprotection remains to be elucidated. The current study demonstrated that the FGF2 LMW isoform is most likely responsible in protecting the heart against I/R injury, in particular against post-ischemic myocardial dysfunction and that modulation of the JNK pathway is an underlying mechanism for this cardioprotection.

The FGF2 LMW and HMW isoforms have been reported to evoke different biological functions in a variety of cell types [2,12,28]. Transfection of FGF2 isoforms in an osteoblastic cell line showed that neutralizing FGF2 antibodies blocked cell growth in FGF2 LMW overexpressing cells but not in FGF2 HMW isoforms overexpressing cells, suggesting that the FGF2 HMW isoforms modulate cell growth and DNA synthesis by a mechanism independent of its cell surface receptors [29]. In cardiomyocytes, both LMW and HMW isoforms increase proliferation while FGF2 HMW overexpression significantly increased binucleation [30]. Although FGF2 LMW and HMW isoforms have different biological functions in several *in vitro* studies, the question remains as to the role of specific FGF2 isoform(s) in I/R injury and cardioprotection. Kardami and colleagues demonstrated that treatment with the FGF2 LMW isoform improved cell survival and functional recovery in the ischemic rat heart [14] and also that overexpression of the FGF2 LMW isoform in mice reduced cardiomyocyte damage [9]. In the present study, the role of FGF2 LMW isoform in I/R injury was elucidated by subjecting novel mouse models to I/R injury. Hearts deficient in the FGF2 LMW isoform (FGF2 LMWKO) displayed a significant decrease in percent recovery of post-ischemic contractile function (Figure 5) compared to Wt hearts. Interestingly, FGF2 LMWKO mouse hearts, in which only the FGF2 HMW isoforms are present, had a percent recovery of post-ischemic contractile function similar to mice deficient in all LMW and HMW isoforms (Figure 5). This observation suggests that the LMW isoform is responsible for protecting the heart from myocardial dysfunction which is consistent with that reported by the Kardami group [9,14]. A question remains as to what roles, if any, do the HMW isoforms of FGF2 have in cardioprotection and ischemia-reperfusion injury. In the present study, we observed that there was no difference in CK release between the three vehicle-treated groups (Wt, *Fgf2* KO and FGF2 LMWKO; see Table 2), suggesting that the LMW and HMW FGF2 isoforms may be necessary to protect the heart against necrosis. Also, our previous work, where our laboratory showed that cardiac-specific overexpression of all FGF2 isoforms significantly reduced myocardial infarction as measured by CK release and histochemical analysis via H&E staining and triphenyltetrazolium chloride staining [12], suggests that both the LMW and HMW isoforms may be required for cardioprotection against myocardial infarction.

Many of FGF2's actions are mediated through interaction with its low affinity receptor (heparin sulfate proteoglycan, HSPG) and its high affinity receptor tyrosine kinase FGF receptor (FGFR). The HSPG receptors regulate FGF bioavailability and improve FGF2's affinity to the FGFRs [31]. Ligand binding results in the dimerization and autophosphorylation of the receptor [8] and stimulation of the MAPK pathway [32]. The FGF2 LMW and HMW isoforms have different roles in MAPK activation. Evidence showed that administration of the FGF2 LMW isoform to a tumor cell line activated p38 MAPK and ERK, leading to cell death [33]. Overexpressing the FGF2 HMW isoform in pancreatic cells caused an increase in ERK activation [34]. Sheikh and group [9] demonstrated that overexpression of the FGF2 LMW isoform resulted in a significant increase in the phosphorylated form of JNK and p38 MAPK in the heart. However, in the present study, JNK activation (i.e., phosphorylation) was significantly increased and p38 activation was significantly decreased in non-ischemic *Fgf2* KO and FGF2 LMWKO vs. Wt hearts (Figure 3). Importantly, there was no difference in JNK and p38 MAPK phosphorylation between *Fgf2* KO and FGF2 LMWKO hearts, suggesting that the LMW isoform is responsible for regulating the activation of JNK and p38 MAPK in

non-ischemic murine hearts. This action of the LMW isoform may be a potential mechanism by which it elicits a cardioprotective effect.

Our data indicate that the FGF2 LMW isoform elicited its cardioprotective effect via modulating the MKK7/JNK pathway and not the MKK4/JNK pathway, providing novel evidence on the regulation MKK4/7. MKK4/7 are regulated by different MAPK kinase kinase (MEKK) such that MEKK1, 2, 3, 4 all have the potential to activate MKK4 while only MEKK1, 2, 3 can regulate MKK7 [35]. The concentration (50nM) of CEP11004 employed in the study did not inhibit the other MAPKs (ERK and p38 MAPK). It is well characterized that ERK acts as a cell survival molecule while p38 MAPK has dual effects (cell survival or cell death) during myocardial I/R injury [24]. Interestingly, regardless of the activation of strong survival proteins, ERK and p38 MAPK, during ischemia-reperfusion injury (Figure 6), only inhibition of JNK signaling caused almost full recovery of contractile function and significant reduction in cell injury/death in CEP11004-treated groups vs. DMSO-treated cohorts (Figure 5, Figure 8C). In fact, the present study provided evidence that inhibition of the MEK1/ERK or p38 pathway neither improved contractile function nor reduced CK release during I/R injury (data not shown), suggesting that the deleterious actions of JNK are a predominant event in I/R injury in our mouse models and that the JNK pathway is regulated by the FGF2 LMW isoform in cardioprotection.

A significant increase in c-Jun activation was observed in DMSO-treated *Fgf2* KO and FGF2 LMWKO hearts compared to DMSO-treated Wt hearts. This finding suggests that the FGF2 LMW isoform modulated c-Jun activation by regulating JNK pathway activation during ischemia-reperfusion injury. Upon JNK pathway inhibition, the activation of c-Jun in Wt, *Fgf2* KO and FGF2 LMWKO hearts was significantly decreased. Overexpression of only the 24 kDa FGF2 isoform in a cancer cell line resulted in increased c-Jun activation [35] suggesting that FGF2 isoforms had the ability to modulate c-Jun activation. Since numerous studies revealed that phosphorylation of c-Jun by JNK is necessary for the apoptotic response in certain cell types [36,37] as well as in several models of ischemia-reperfusion injury [38,39], the FGF2 LMW isoform may modulate apoptosis through inhibition of c-Jun activation, leading to the cardioprotective effect.

It has been well established that cardiomyocyte cell death during I/R injury can be induced both by apoptosis and necrosis [40]. FGF2 and JNK are both involved in regulating apoptosis [9,41,42]. FGF2 has been shown to inhibit endothelial cell apoptosis by a Bcl-2-dependent or -independent mechanism [43], and antisense inhibition of FGF2 induces apoptosis in vascular smooth muscle cells [44,45]. Furthermore, FGF2 can alter the level of Bcl-2 and Bax family proteins involved in apoptosis [46]. JNK activation has been reported to be a key factor in apoptosis, translocating to the nucleus and phosphorylating and activating transcription factors such as c-Jun, or translocating to mitochondria to phosphorylate the apoptotic-related proteins, Bax, Bad, and Bcl-2 [47]. Several hallmarks of apoptosis, including caspase 3 cleavage (early phase) and DNA strand breaks (late phase), are used for detection of apoptosis. Following JNK pathway inhibition, there was a significant decrease in TUNEL-positive nuclei as well as caspase 3 cleavage in Wt, *Fgf2* KO and FGF2 LMWKO mouse hearts (Figure 8). Interestingly, in DMSO-treated *Fgf2* KO and FGF2 LMWKO hearts, there was a significant increase in TUNEL-positive nuclei as well as caspase 3 cleavage, suggesting that in addition to ischemia-reperfusion injury's influence on apoptosis, the FGF2 LMW isoform also modulates apoptotic events.

In summary, we have shown that the FGF2 LMW isoform is important for protecting the heart from post-ischemic cardiac dysfunction. Furthermore, the LMW isoform modulates MKK7, JNK, and c-Jun activation as well as apoptotic processes prior to and during ischemia-reperfusion injury. A novel JNK pathway inhibitor, CEP11004, administered during ischemia-

reperfusion restored the recovery of post-ischemic cardiac function and reduced myocardial cell death. Overall, our findings have unequivocally demonstrated the importance of the FGF2 LMW isoform in cardioprotection and for the first time, demonstrated the relationship between the FGF2 LMW isoform and the JNK signaling pathway in cardioprotection.

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References

1. Yellon DM, Downey JM. Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol Rev* 2003;83:1113–51. [PubMed: 14506302]
2. Detillieux KA, Sheikh F, Kardami E, Cattini PA. Biological activities of fibroblast growth factor-2 in the adult myocardium. *Cardiovasc Res* 2003;57:8–19. [PubMed: 12504809]
3. Zhou M, Sutliff RL, Paul RJ, Lorenz JN, Hoying JB, Haudenschild CC, Yin M, Coffin JD, Kong L, Kranias EG, Luo W, Boivin GP, Duffy JJ, Pawlowski SA, Doetschman T. Fibroblast growth factor 2 control of vascular tone. *Nat Med* 1998;4:201–7. [PubMed: 9461194]
4. Ortega S, Ittmann M, Tsang SH, Ehrlich M, Basilico C. Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. *Proc Natl Acad Sci U S A* 1998;95:5672–7. [PubMed: 9576942]
5. Dono R, Texido G, Dussel R, Ehmke H, Zeller R. Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. *Embo J* 1998;17:4213–25. [PubMed: 9687490]
6. Schultz JE, Witt SA, Nieman ML, Reiser PJ, Engle SJ, Zhou M, Pawlowski SA, Lorenz JN, Kimball TR, Doetschman T. Fibroblast growth factor-2 mediates pressure-induced hypertrophic response. *J Clin Invest* 1999;104:709–19. [PubMed: 10491406]
7. Coffin JD, Florkiewicz RZ, Neumann J, Mort-Hopkins T, Dorn GW 2nd, Lightfoot P, German R, Howles PN, Kier A, O'Toole BA, et al. Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice. *Mol Biol Cell* 1995;6:1861–73. [PubMed: 8590811]
8. Okada-Ban M, Thiery JP, Jouanneau J. Fibroblast growth factor-2. *Int J Biochem Cell Biol* 2000;32:263–7. [PubMed: 10716624]
9. Sheikh F, Sontag DP, Fandrich RR, Kardami E, Cattini PA. Overexpression of FGF-2 increases cardiac myocyte viability after injury in isolated mouse hearts. *Am J Physiol Heart Circ Physiol* 2001;280:H1039–50. [PubMed: 11179045]
10. Pasumarthi KB, Doble BW, Kardami E, Cattini PA. Over-expression of CUG- or AUG-initiated forms of basic fibroblast growth factor in cardiac myocytes results in similar effects on mitosis and protein synthesis but distinct nuclear morphologies. *J Mol Cell Cardiol* 1994;26:1045–60. [PubMed: 7799445]
11. Liu L, Doble BW, Kardami E. Perinatal phenotype and hypothyroidism are associated with elevated levels of 21.5- to 22-kDa basic fibroblast growth factor in cardiac ventricles. *Dev Biol* 1993;157:507–16. [PubMed: 8500657]
12. House SL, Bolte C, Zhou M, Doetschman T, Klevitsky R, Newman G, Schultz Jel J. Cardiac-specific overexpression of fibroblast growth factor-2 protects against myocardial dysfunction and infarction in a murine model of low-flow ischemia. *Circulation* 2003;108:3140–8. [PubMed: 14656920]
13. House SL, Branch K, Newman G, Doetschman T, Schultz Jel J. Cardioprotection induced by cardiac-specific overexpression of fibroblast growth factor-2 is mediated by the MAPK cascade. *Am J Physiol Heart Circ Physiol* 2005;289:H2167–75. [PubMed: 16040717]
14. Padua RR, Sethi R, Dhalla NS, Kardami E. Basic fibroblast growth factor is cardioprotective in ischemia-reperfusion injury. *Mol Cell Biochem* 1995;143:129–35. [PubMed: 7596347]

15. Powers CJ, McLeskey SW, Wellstein A. Fibroblast growth factors, their receptors and signaling. *Endocr Relat Cancer* 2000;7:165–97. [PubMed: 11021964]
16. Ham J, Babij C, Whitfield J, Pfarr CM, Lallemand D, Yaniv M, Rubin LL. A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron* 1995;14:927–39. [PubMed: 7748560]
17. Bogoyevitch MA, Gillespie-Brown J, Ketterman AJ, Fuller SJ, Ben-Levy R, Ashworth A, Marshall CJ, Sugden PH. Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. p38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion. *Circ Res* 1996;79:162–73. [PubMed: 8755992]
18. Garmy-Susini B, Delmas E, Gourdy P, Zhou M, Bossard C, Bugler B, Bayard F, Krust A, Prats AC, Doetschman T, Prats H, Arnal JF. Role of fibroblast growth factor-2 isoforms in the effect of estradiol on endothelial cell migration and proliferation. *Circ Res* 2004;94:1301–9. [PubMed: 15073041]
19. Askew GR, Doetschman T, Lingrel JB. Site-directed point mutations in embryonic stem cells: a gene-targeting tag-and-exchange strategy. *Mol Cell Biol* 1993;13:4115–24. [PubMed: 8391633]
20. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* 1998;273:18623–32. [PubMed: 9660836]
21. Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett* 1995;364:229–33. [PubMed: 7750577]
22. Barros LF, Young M, Saklatvala J, Baldwin SA. Evidence of two mechanisms for the activation of the glucose transporter GLUT1 by anisomycin: p38(MAP kinase) activation and protein synthesis inhibition in mammalian cells. *J Physiol* 1997;504(Pt 3):517–25. [PubMed: 9401960]
23. Gorczyca W, Gong J, Darzynkiewicz Z. Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res* 1993;53:1945–51. [PubMed: 8467513]
24. Abe J, Baines CP, Berk BC. Role of mitogen-activated protein kinases in ischemia and reperfusion injury : the good and the bad. *Circ Res* 2000;86:607–9. [PubMed: 10746992]
25. Bao YL, Tsuchida K, Liu B, Kurisaki A, Matsuzaki T, Sugino H. Synergistic activity of activin A and basic fibroblast growth factor on tyrosine hydroxylase expression through Smad3 and ERK1/ERK2 MAPK signaling pathways. *J Endocrinol* 2005;184:493–504. [PubMed: 15749808]
26. Heffron DS, Mandell JW. Opposing roles of ERK and p38 MAP kinases in FGF2-induced astroglial process extension. *Mol Cell Neurosci* 2005;28:779–90. [PubMed: 15797724]
27. Matsuoka M, Igisu H, Nakagawa K, Katada T, Nishina H. Requirement of MKK4 and MKK7 for CdCl₂- or HgCl₂-induced activation of c-Jun NH₂-terminal kinase in mouse embryonic stem cells. *Toxicol Lett* 2004;152:175–81. [PubMed: 15302099]
28. Hortala M, Estival A, Pradayrol L, Susini C, Clemente F. Identification of c-Jun as a critical mediator for the intracrine 24 kDa FGF-2 isoform-induced cell proliferation. *Int J Cancer* 2005;114:863–9. [PubMed: 15609298]
29. Xiao L, Liu P, Sobue T, Lichtler A, Coffin JD, Hurley MM. Effect of overexpressing fibroblast growth factor 2 protein isoforms in osteoblastic ROS 17/2.8 cells. *J Cell Biochem* 2003;89:1291–301. [PubMed: 12898525]
30. Pasumarthi KB, Kardami E, Cattini PA. High and low molecular weight fibroblast growth factor-2 increase proliferation of neonatal rat cardiac myocytes but have differential effects on binucleation and nuclear morphology. Evidence for both paracrine and intracrine actions of fibroblast growth factor-2. *Circ Res* 1996;78:126–36. [PubMed: 8603495]
31. Klagsbrun M, Baird A. A dual receptor system is required for basic fibroblast growth factor activity. *Cell* 1991;67:229–31. [PubMed: 1655276]
32. Prats H, Kaghad M, Prats AC, Klagsbrun M, Lelias JM, Liauzun P, Chalon P, Tauber JP, Amalric F, Smith JA, et al. High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. *Proc Natl Acad Sci U S A* 1989;86:1836–40. [PubMed: 2538817]

33. Williamson AJ, Dibling BC, Boyne JR, Selby P, Burchill SA. Basic fibroblast growth factor-induced cell death is effected through sustained activation of p38MAPK and up-regulation of the death receptor p75NTR. *J Biol Chem* 2004;279:47912–28. [PubMed: 15310753]
34. Gaubert F, Escaffit F, Bertrand C, Korc M, Pradayrol L, Clemente F, Estival A. Expression of the high molecular weight fibroblast growth factor-2 isoform of 210 amino acids is associated with modulation of protein kinases C delta and epsilon and ERK activation. *J Biol Chem* 2001;276:1545–54. [PubMed: 11031252]
35. Hagemann C, Blank JL. The ups and downs of MEK kinase interactions. *Cell Signal* 2001;13:863–75. [PubMed: 11728826]
36. Watson A, Eilers A, Lallemand D, Kyriakis J, Rubin LL, Ham J. Phosphorylation of c-Jun is necessary for apoptosis induced by survival signal withdrawal in cerebellar granule neurons. *J Neurosci* 1998;18:751–62. [PubMed: 9425017]
37. Le-Niculescu H, Bonfoco E, Kasuya Y, Claret FX, Green DR, Karin M. Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. *Mol Cell Biol* 1999;19:751–63. [PubMed: 9858598]
38. Guan QH, Pei DS, Zhang QG, Hao ZB, Xu TL, Zhang GY. The neuroprotective action of SP600125, a new inhibitor of JNK, on transient brain ischemia/reperfusion-induced neuronal death in rat hippocampal CA1 via nuclear and non-nuclear pathways. *Brain Res* 2005;1035:51–9. [PubMed: 15713276]
39. Uehara T, Bennett B, Sakata ST, Satoh Y, Bilter GK, Westwick JK, Brenner DA. JNK mediates hepatic ischemia reperfusion injury. *J Hepatol* 2005;42:850–9. [PubMed: 15885356]
40. Andreaka P, Nadhazi Z, Muzes G, Szantho G, Vandro L, Konya L, Turner MS, Tulassay Z, Bishopric NH. Possible therapeutic targets in cardiac myocyte apoptosis. *Curr Pharm Des* 2004;10:2445–61. [PubMed: 15320755]
41. Manning AM, Davis RJ. Targeting JNK for therapeutic benefit: from junk to gold? *Nat Rev Drug Discov* 2003;2:554–65. [PubMed: 12815381]
42. Burchill SA, Westwood G. Mechanism of basic fibroblast growth factor-induced cell death. *Apoptosis* 2002;7:5–12. [PubMed: 11773700]
43. Karsan A, Yee E, Poirier GG, Zhou P, Craig R, Harlan JM. Fibroblast growth factor-2 inhibits endothelial cell apoptosis by Bcl-2-dependent and independent mechanisms. *Am J Pathol* 1997;151:1775–84. [PubMed: 9403728]
44. Fox JC, Shanley JR. Antisense inhibition of basic fibroblast growth factor induces apoptosis in vascular smooth muscle cells. *J Biol Chem* 1996;271:12578–84. [PubMed: 8647868]
45. Garcia-Maya M, Anderson AA, Kendal CE, Kenny AV, Edwards-Ingram LC, Holladay A, Saffell JL. Ligand concentration is a driver of divergent signaling and pleiotropic cellular responses to FGF. *J Cell Physiol* 2006;206:386–93. [PubMed: 16155940]
46. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science* 1998;281:1322–6. [PubMed: 9735050]
47. Liu J, Lin A. Role of JNK activation in apoptosis: a double-edged sword. *Cell Res* 2005;15:36–42. [PubMed: 15686625]

Figure 1.

(A) Schematic for the generation of FGF2 LMWKO mice. The Tag and Exchange procedure was used to generate FGF2 LMWKO mice in which the AUG start site for the LMW isoform was mutated to eliminate that isoform. (B) Representative Western blot of FGF2 isoform expression. The homogenates from non-ischemic Wt, *Fgf2* KO and FGF2 LMWKO hearts were bound to heparin sepharose beads and subjected to SDS-PAGE. The amount of FGF2 expression was measured by immunoblotting using FGF2 antibody (1:1000). Wt hearts expressed one FGF2 LMW isoform (18kDa) and 2 FGF2 HMW isoforms (21 and 22 kDa). No FGF2 isoforms were present in *Fgf2* KO mouse hearts and no FGF2 LMW isoform was expressed in FGF2 LMWKO mouse hearts. (C) Quantification of HMW isoforms in FGF2

LMWKO mouse hearts. There was no difference in either the 21 kDa or 22kDa FGF2 HMW isoform expression in FGF2 LMWKO hearts compared to Wt hearts. n=6 for Wt, FGF2 LMWKO and *Fgf2* KO groups.

Figure 2.

Schematic of low-flow ischemia-reperfusion injury and drug treatment protocol. DMSO and CEP11004 were given 15 minutes prior to and during the first 15 minutes of ischemia as well as during the last 15 minutes of ischemia and first 15 minutes of reperfusion. Functional parameters and perfusate gases were taken at designated time points of baseline, ischemia and reperfusion (Black arrow). Coronary effluent collection was also taken at designated time points of baseline, ischemia and reperfusion. CE: coronary effluent collection.

Figure 3.

MAPK kinase activation as indicated by phosphorylation level in non-ischemic Wt, *Fgf2* KO and FGF2 LMWKO hearts. (A) Western blot depicting the phosphorylation state of ERK, p38 MAPK and JNK in non-ischemic Wt, *Fgf2* KO and FGF2 LMWKO hearts. 100 μ g of homogenate from non-ischemic Wt, *Fgf2* KO and FGF2 LMWKO hearts was immunoblotting using phospho-ERK, p38 MAPK, JNK and total ERK, p38 MAPK and JNK antibodies (1:1000). There was no significant difference in ERK activation (B); however, there was a significant decrease in p38 MAPK activation (C) and a significant increase in JNK activation (D) in non-ischemic *Fgf2* KO and FGF2 LMWKO hearts compared to Wt hearts. y axis: Ratio

of phospho-ERK, p38 MAPK and JNK vs total ERK, p38 MAPK and JNK. n=8/
groups.*p<0.05 vs. Wt.

Figure 4.

Activation as measured by phosphorylation level of JNK pathway at ischemia-reperfusion injury in Wt (diamond), *Fgf2* KO (square) and FGF2 LMWKO (triangle) hearts. (A) Western blot depicting activation of JNK pathway during different time points of ischemia-reperfusion injury in Wt, *Fgf2* KO and FGF2 LMWKO hearts. Homogenates of Wt, *Fgf2* KO and FGF2 LMWKO hearts subjected to different lengths of ischemia-reperfusion injury were evaluated via immunoblotting with phospho-JNK, MKK4, MKK7 and total JNK, MKK4 and MKK7 antibodies (1:1000). (B) There was a significant activation of JNK prior to ischemia, early ischemia and early reperfusion in *Fgf2* KO and FGF2 LMWKO vs. Wt hearts. (C) There was a significant activation of MKK7 in early reperfusion in *Fgf2* KO and FGF2 LMWKO vs. Wt

hearts. (D) There was a significant activation of MKK4 in early reperfusion in *Fgf2* KO and FGF2 LMWKO vs. Wt hearts. y axis: Ratio of phospho-JNK, MKK7 and MKK4 vs. total JNK, MKK7 and MKK4. n=6/groups. *p<0.05 vs. sham cohort. #p<0.05 vs. Wt.

Figure 5.

Percent recovery of post-ischemic contractile function in Wt (black bar), *Fgf2* KO (gray bar) and FGF2 LMWKO (white bar) hearts following DMSO or CEP11004 treatment. Wt, *Fgf2* KO and FGF2 LMWKO hearts were subjected to 60 minutes ischemia and 120 minutes reperfusion treated either by DMSO or CEP11004. Percent recovery was measured as the ratio of +dP/dt at 60 minutes ischemia and 120 minutes reperfusion vs. +dP/dt at baseline. There was a poorer recovery of post-ischemic contractile function in *Fgf2* KO and FGF2 LMWKO DMSO-treated vs. Wt DMSO-treated groups. Inhibition of the JNK pathway significantly restored the post-ischemic recovery of contractile function in all three groups. y axis: Percent of recovery measured by the ratio of +dP/dt at 60 minutes ischemia and 120 minutes reperfusion vs. +dP/dt at baseline. n=6 for DMSO- and CEP11004- treated Wt, *Fgf2* KO and FGF2 LMWKO hearts. *p<0.05 vs. DMSO-treated cohort. †p<0.05 vs. CEP11004-treated Wt hearts. #p<0.05 vs. DMSO-treated Wt hearts.

Figure 6.

MAPK phosphorylation (i.e., activation) following 60 minutes ischemia and 120 minutes reperfusion in DMSO- and CEP11004-treated Wt (black bar), *Fgf2* KO (gray bar) and FGF2 LMWKO (white bar) hearts. (A) Western blot depicting MAPK activation in DMSO- and CEP11004- treated Wt, *Fgf2* KO and FGF2 LMWKO hearts after 60 minutes ischemia and 120 minutes reperfusion. Homogenates of Wt, *Fgf2* KO and FGF2 LMWKO hearts subjected to ischemia-reperfusion injury were analyzed by immunoblotting using phospho-ERK, p38 MAPK, JNK and total ERK, p38 MAPK and JNK antibodies (1:1000). (B) JNK phosphorylation was significantly increased in DMSO-treated *Fgf2* KO and FGF2 LMWKO vs. Wt hearts. There was a significant decrease in JNK phosphorylation in CEP11004-treated

groups compared to DMSO-treated groups. There was no significant difference in p38 MAPK (C) and ERK (D) activation between DMSO- and CEP11004-treated groups. y axis: Ratio of phospho-ERK, p38 MAPK and JNK vs total-ERK, p38 MAPK and JNK. n=6/group. *p<0.05 vs. DMSO-treated groups. #p<0.05 vs. Wt cohort.

Figure 7.

Activation of upstream (MKK4/7) and downstream (c-Jun) signals in the JNK pathway following 60 minutes ischemia and 120 minutes reperfusion in DMSO- and CEP11004-treated Wt (black bar), *Fgf2* KO (gray bar) and FGF2 LMWKO (white bar) hearts. (A) Western blot depicting MKK7, MKK4, and c-Jun activation from DMSO- and CEP11004-treated Wt, *Fgf2* KO and FGF2 LMWKO hearts after 60 minutes ischemia and 120 minutes reperfusion. Homogenates of Wt, *Fgf2* KO and FGF2 LMWKO hearts subjected to 60 minutes ischemia and 120 minutes reperfusion were analyzed by SDS-PAGE and immunoblotting using phospho-MKK7, MKK4, c-Jun and total MKK7, MKK4, c-Jun antibodies (1:1000). (B) MKK7 activation was significantly increased in DMSO-treated *Fgf2* KO and FGF2 LMWKO

vs. DMSO-treated Wt hearts. Moreover, there was a significant decrease in MKK7 activation in CEP11004-treated groups compared to DMSO-treated groups. (C) There was a significant decrease in MKK4 activation in CEP11004-treated groups compared to DMSO-treated groups. (D) c-Jun activation was significantly increased in DMSO-treated *Fgf2* KO and FGF2 LMWKO vs. DMSO-treated Wt hearts. Furthermore, there was a significant decrease in c-Jun activation in CEP11004-treated groups compared to DMSO-treated groups. y axis: Ratio of phospho-MKK7, MKK4 and c-Jun vs. total MKK7, MKK4 and c-Jun. n=6/group. *p<0.05 vs. DMSO-treated groups. #p<0.05 vs. DMSO-treated Wt group.

Figure 8.

Effect of JNK pathway inhibition on apoptosis signaling following 60 minutes ischemia and 120 minutes reperfusion in DMSO- and CEP11004-treated Wt (black bar), *Fgf2* KO (gray bar) and FGF2 LMWKO (white bar) groups. (A) Caspase 3 cleavage was significantly increased in DMSO-treated *Fgf2* KO and FGF2 LMWKO vs. DMSO-treated Wt hearts. Furthermore, there was a significant decrease in caspase 3 cleavage in CEP11004-treated vs. DMSO-treated groups. Inset: There was no significant difference in caspase 3 activation in non-ischemic *Fgf2* KO (gray bar) and FGF2 LMWKO (white bar) vs. Wt hearts (black bar). Homogenates from non-ischemic and ischemic-reperfused DMSO- and CEP11004-treated Wt, *Fgf2* KO and FGF2 LMWKO hearts were subjected to SDS PAGE and immunoblotting using cleaved

caspase 3 and total caspase 3 antibodies (1:500). (B) Representative heart sections depicting TUNEL-positive nuclei from DMSO- and CEP11004-treated Wt, *Fgf2* KO and FGF2 LMWKO groups following 60 minutes ischemia and 120 minutes reperfusion. (C) Quantification of TUNEL-positive nuclei following 60 minutes ischemia and 120 minutes reperfusion. Heart sections from non-ischemic and ischemic-reperfused DMSO- and CEP11004-treated Wt, *Fgf2* KO and FGF2 LMWKO groups were stained with TUNEL and DAPI. TUNEL-positive nuclei were significantly increased in DMSO-treated *Fgf2* KO and FGF2 LMWKO vs. DMSO-treated Wt hearts. There was a significant decrease in TUNEL-positive nuclei in CEP11004-treated vs. DMSO-treated groups. Inset: There was a significant increase in TUNEL-positive nuclei in non-ischemic *Fgf2* KO and FGF2 LMWKO vs. non-ischemic Wt hearts. Caspase 3 activation – y axis: activation of caspase 3 as measured by cleaved caspase 3 vs. total caspase 3, n=6/group. TUNEL staining – y axis: % TUNEL-positive nuclei as a % of total nuclei, n=4/groups and 96 fields/groups. *p<0.05 vs. DMSO-treated groups. #p<0.05 vs. DMSO-treated Wt group.

Table 1

Cardiac function in DMSO- and CEP11004-treated Wt, *Fgf2* KO and FGF2 LMWKO hearts.

	Baseline					Cardiac function at 60'ischemia/120'reperfusion						
	Wt (DMSO)	Fg2 KO (DMSO)	FGF2 LMWKO (DMSO)	Wt (CEP11004)	Fg2 KO (CEP11004)	FGF2 LMWKO (CEP11004)	Wt (DMSO)	Fg2 KO (DMSO)	FGF2 LMWKO (DMSO)	Wt (CEP11004)	Fg2 KO (CEP11004)	FGF2 LMWKO (CEP11004)
	100±2	100±1	101±2	101±2	96±2	105±2	67±7 [‡]	51±4 ^{#‡}	54±5 ^{#‡}	84±3 ^{*‡}	79±4 ^{*‡}	78±3 ^{*‡}
LVSP	7±1	7±1	7±1	7±1	7±1	7±1	21±4 [‡]	25±3 [‡]	18±2 [‡]	18±2 ^{*‡}	15±3 ^{*‡}	23±4 [‡]
LVEDP	4266±114	3985±66	4359±98	4268±69	3994±85	4463±77	2519±319 [‡]	1548 ±186 ^{‡#}	2102±261 [‡]	3409±173 ^{*‡}	3357±241 [*]	2948±254 ^{*‡}
+dP/dt	-3618 ±121	-3322±67	-3508±145	-3560±101	-3240±100	-3739±106	-1817 ±189 [‡]	-1158 ±98 ^{‡#}	-1568 ±165 ^{‡#}	-2259±127 ^{*‡}	-2122±139 ^{*‡}	-1999±146 ^{*‡}

All values were presented as mean±SEM of *n* independent experiments. LVSP: left ventricle systolic pressure (mmHg).

LVEDP: left ventricle end diastolic pressure (mmHg), ±dP/dt: derivative of change in contractile and relaxation pressure over time(mmHg/sec). N=12/groups.

* p<0.05 vs DMSO-treated cohort.

p<0.05 vs DMSO-treated Wt.

‡ p<0.05 vs Baseline cohort.

Table 2
Creatine kinase release from DMSO- and CEP11004-treated *Wt*, *Fgf2* KO and FGF2 LMWKO hearts.

		Baseline	Ischemia	Early Reperfusion	Late Reperfusion
<i>Wt</i>	DMSO	0.041±0.020	0.025±0.019 [†]	0.100±0.031 [†]	0.057±0.036
	CEP 11004	0.015±0.004 [*]	0.007±0.002 ^{†*}	0.046±0.013 ^{†*}	0.015±0.006 [*]
<i>Fgf2</i> KO	DMSO	0.043±0.015	0.015±0.007 [†]	0.114±0.056 [†]	0.019±0.005 [†]
	CEP 11004	0.031±0.023 [#]	0.001±0.001 [†]	0.060 ±0.028	0.021±0.010 [#]
FGF2 LMWKO	DMSO	0.034±0.012	0.011±0.002 [†]	0.129±0.034 [†]	0.017±0.005
	CEP 11004	0.027±0.012 [#]	0.005±0.005 ^{†*}	0.070 ±0.024 ^{#**†}	0.029±0.010 [#]

All values depicted as mean±SEM of *n* independent experiments. Early reperfusion: 0–14 minutes of reperfusion. Late reperfusion: 114–120 minutes of reperfusion. N=10/groups.

^{*} p<0.05 vs DMSO treated-cohort.

[#] p<0.05 vs *Wt* cohort.

[†] p<0.05 vs baseline.