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## Fibroblast growth factor-2-induced cardioprotection against myocardial infarction occurs via the interplay between nitric oxide, protein kinase signaling, and ATP-sensitive potassium channels

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### Abstract

Fibroblast growth factor-2 (FGF2) protects the heart from ischemia–reperfusion (I-R) injury via a vast network of protein kinases. In the heart, downstream effectors of these FGF2-triggered signals have not yet been identified. It is hypothesized that nitric oxide (NO) signaling and ATP-sensitive potassium ( $K_{ATP}$ ) channel activity are key effectors of protein kinases activated by FGF2-mediated cardioprotection. Hearts with a cardiac-specific overexpression of *FGF2* (*FGF2* Tg) were subjected to I-R injury in the absence or the presence of selective inhibitors of NO synthase (NOS) isoforms or sarcolemmal (sarc $K_{ATP}$ ) and mitochondrial (mito $K_{ATP}$ )  $K_{ATP}$  channels. Multiple NOS isoforms are necessary for FGF2-mediated cardioprotection, and nitrite levels are significantly reduced in *FGF2* Tg hearts upon inhibition of protein kinase C or mitogen-activated protein kinases. Likewise, sarc $K_{ATP}$  and mito $K_{ATP}$  channels are important for cardioprotection elicited by endogenous FGF2. These findings suggest that FGF2-induced cardioprotection occurs via protein kinase-NOS pathways as well as  $K_{ATP}$  channel activity.

### Keywords

Growth factor; nitric oxide synthase; extracellular signal regulated kinase (ERK); p38; PKC; transgenic mouse; ischemia–reperfusion

### Introduction

Since it was established that brief periods of ischemia are capable of protecting the heart against subsequent injury (Murry et al. 1986), a potential therapeutic intervention for

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patients with coronary (ischemic) heart disease that can mimic this phenomenon has been the subject of many investigations (Kharbanda 2010). Among these potential therapies is fibroblast growth factor-2 (FGF2).

FGF2 has been implicated in multiple protective pathways related to ischemia–reperfusion (I-R) injury including cardioprotection, the inflammatory response, angiogenesis, and vascular remodeling (Detillieux et al. 2003; Liao 2008). A number of pharmacological and *in vitro* studies have suggested that FGF2 can act directly on cardiomyocytes (Padua et al. 1995; Jiang et al. 2002; Jiang et al. 2004) to maintain the integrity and function of the myocardium during I-R injury. Our laboratory has demonstrated in an *ex vivo* mouse model of I-R injury that cardiac overexpression of human FGF2 decreases ischemic damage, as measured by the recovery of post-ischemic ventricular function and myocardial infarct size (House et al. 2003). These cardioprotective effects of FGF2 have been described by findings in other laboratories as well (Hampton et al. 2000; Sheikh et al. 2001; Jiang et al. 2002). Additionally, previous findings from our laboratory (House et al. 2003, 2005, 2007) and others (Jiang et al. 2002) have shown involvement of both the mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) pathways in FGF2-induced cardioprotection. These kinase pathways activated by FGF2 are also involved in other modes of cardioprotection (Barancik et al. 2000; Strohm et al. 2000; Lips et al. 2004; Budas et al. 2007). However, additional pathways, which may be independent of or interact with kinase signaling, may also play a role in the mediation of the protective effect of FGF2.

Certain biological actions, in particular vascular and angiogenic functions, of FGFs have been shown to occur through nitric oxide (NO) signaling (Huang et al. 1997; Tiefenbacher and Chilian 1997; Cuevas et al. 1999; Hampton et al. 2000; Parenti et al. 2001) or ATP-sensitive potassium ( $K_{ATP}$ ) channel activity (Cuevas et al. 1991; Boussairi and Sassard 1994; Tiefenbacher and Chilian 1997; Kajita et al. 2001). Hampton and colleagues determined that exogenously FGF2 applied prior to ischemia was able to protect the heart from post-ischemic dysfunction in hearts in an inducible nitric oxide synthase (iNOS)-dependent manner (Hampton et al. 2000). However, it remains unclear whether the reduction in infarct size and improvement in cardiac function mediated by endogenously expressed FGF2 in ischemic cardiomyocytes relies on the activity of any species of NOS.

Vascular studies have implicated  $K_{ATP}$  channel activation in the signal transduction and biological activity of FGF1 and FGF2 in angiogenesis and vasodilation (Cuevas et al. 1991; Boussairi and Sassard 1994; Tiefenbacher and Chilian 1997; Kajita et al. 2001). However, there is no evidence, to date, as to the involvement of  $K_{ATP}$  channels (whether mitochondrial or sarcolemmal) in the cardioprotective effect mediated by FGF2.

In this study, mouse hearts with a cardiac-specific overexpression of human *FGF2* were subjected to ischemia and reperfusion in the presence and the absence of NOS inhibitors selective against iNOS and neuronal (nNOS) as well as inhibitors selective for sarcolemmal (sarc $K_{ATP}$ ) or mitochondrial (mito $K_{ATP}$ )  $K_{ATP}$  channels, and cardiac function, infarct size, and NO release were measured to test the hypothesis that cardioprotection induced by overexpression of FGF2 is linked to NOS-associated pathways and sarc $K_{ATP}$  and mito $K_{ATP}$  channels during I-R injury.

## Methods

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. Non-transgenic (NTg) mice and mice with a cardiac-specific overexpression of all FGF2 isoforms (*FGF2*Tg MHC20 and *FGF2*Tg MHC 25), on a FVB/N background, were randomly assigned to the studies. A total of 17 mice were excluded from the I-R injury or pharmacological inhibition studies. For each group, 11 NTg mice and 15 *FGF2*Tg mice completed the I-R studies or the I-R +pharmacological NOS isoform inhibition studies, six to eleven mice of each genotype completed the I-R +pharmacological protein kinase inhibition studies or the I-R +pharmacological  $K_{ATP}$  channel inhibition studies. Exclusion from any I-R study was based on the signs of aortic or pulmonary vein leak in the working heart preparation. Aortic leak was represented as an aortic pressure <60 mmHg on Langendorff, retrograde perfusion mode. Pulmonary vein leak was demonstrated as an aortic flow <2.0 ml/min, low (<4 mmHg) atrial pressure, and a perfusate gas  $pO_2$  >380 mmHg or a visible leak (i.e. hole in ventricle or atrium) in the heart.

### Model of low-flow global ischemia in isolated work-performing heart preparation

After a 30 min equilibration period at a basal cardiac workload of 250 (ml/min)\*mmHg, the venous return was quickly (<30 s) reduced by 1 ml increments to a flow of 1 ml/min for 60 min to elicit a low-flow, global ischemic insult, leading to irreversible injury (i.e. cardiac dysfunction and myocardial infarction). Coronary flow was reduced by approximately 90% of its original rate (from 2.3 to 0.2 ml/min) during this global ischemia, mimicking a severe coronary artery stenosis. To maintain the work demand on the heart during ischemia, the heart was paced 10–15 beats per minute above its intrinsic heart rate. After 60 min of low-flow ischemia, venous return was quickly (<30 s) increased by 1 ml increments to a flow of 5 ml/min, and reperfusion occurred for 120 min. Functional data, perfusate gases, and coronary effluent were obtained at designated time points of baseline, low-flow ischemia, and reperfusion. Indices of diastolic and systolic function (e.g. end-diastolic pressure, half relaxation time, and time to peak pressure), percent recovery of function, and left ventricular developed pressure as well as coronary flow changes and myocardial oxygen consumption were assessed at baseline, ischemia, and reperfusion.

### Pharmacological assessment of NO signaling

$N^G$ -nitro-L-arginine methyl ester hydrochloride (L-NAME), a non-selective NOS inhibitor (Huang et al. 1997), N6-(1-iminoethyl)-L-lysine hydrochloride (L-NIL), a NOS inhibitor selective for the inducible NOS isoform with minimal effects on other NOS isoforms (iNOS) (Moore et al. 1994), 2-(1-(3-Dimethylaminopropyl)-1H-indol-3-yl)-3-(1H-indol-3-yl)maleimide-HCl (bisindolylmaleimide also known as GF109203x or GFX) (GFX), a nonspecific inhibitor of multiple PKC isoforms at their catalytic ATP-binding sites (Bit et al. 1993), or SB203580 hydrochloride (4-(4-fluorophenyl)-2-(4-methyl-sulfinylphenyl)-5-(4-pyridyl)-1H-imidazole), a p38 inhibitor (Cuenda et al. 1995) were dissolved in Milli-Q water. 1-(2-Trifluoromethyl-phenyl) imidazole (TRIM), a NOS inhibitor selective for the

nNOS isoform with some selectivity for iNOS and no activity for endothelial NOS (eNOS) (Handy and Moore 1997) was dissolved in ethanol. U0126 (1,4-diamino-2,3-dicyano-1, 4-bis[2-aminophenylthio]butadiene), an inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK)1/2, upstream kinases of ERK activation (Favata et al. 1998) was dissolved in dimethyl sulfoxide (DMSO). All drugs were diluted in Krebs's solution to obtain final concentrations of L-NAME, 400  $\mu$ M; L-NIL, 400  $\mu$ M; TRIM, 100  $\mu$ M; GFX, 1  $\mu$ M; U0126, 2.5  $\mu$ M; SB203580, 2  $\mu$ M.

Concentrations for NOS or PKC or MAPK inhibitors were chosen because these drug concentrations blocked NOS or protein kinase activation, respectively, as previously published (Martiny-Baron et al. 1993; Cuenda et al. 1995; Handy and Moore 1997; Favata et al. 1998; Hampton et al. 2000; Zhao et al. 2000) without any adverse effects (i.e. cardiac arrhythmias or increased left atrial pressure) which were observed at higher drug levels (unpublished observations with 10  $\mu$ M GFX, 5  $\mu$ M U0126, 5  $\mu$ M SB203580, and 400  $\mu$ M TRIM). These pharmacological agents were administered for the last 15 min of baseline and the first 15 min of ischemia and again for the last 15 min of ischemia and the first 15 min of reperfusion (Figure 1). Vehicle controls were also performed. Previous work by Kardami and colleagues (Jiang et al. 2004) demonstrated that FGF2 protects the heart against myocardial cell injury/death when it is given prior to or following ischemia; therefore, the timing of inhibitor treatment was for the purpose of blocking FGF2 signaling at key time points of the I-R protocol.

### **Inhibition of cardiac $K_{ATP}$ channels**

HMR1098 (10  $\mu$ M, a kind gift from Sonofi-Aventis, Frankfurt, Germany) was used to inhibit sarcolemmal (sarc)  $K_{ATP}$  channels. 5-Hydroxydecanoic acid (5-HD, 100  $\mu$ M) was used to inhibit mitochondrial (mito)  $K_{ATP}$  channels. Concentrations for  $K_{ATP}$  channel inhibitors were chosen because these drug concentrations blocked sarc $K_{ATP}$  or mito $K_{ATP}$  channels as previously published (Grover and Garlid 2000; Sato et al. 2000) without any adverse effects, which were observed at higher drug levels (unpublished observations with 100  $\mu$ M HMR1098 and 300  $\mu$ M 5-HD). To distinguish possible roles for cardiac  $K_{ATP}$  channels as early triggers or late effectors of cardioprotection initiated by FGF2, these pharmacologic agents were administered at one of the two time points during the I-R protocol: for the last 15 min of baseline and the first 15 min of ischemia, or for the last 15 min of ischemia and the first 15 min of reperfusion (Figure 1). Vehicle controls were also performed.

### **Measurement of myocardial infarct size**

Infarct size was determined by the histochemical stain, 2,3,5-triphenyltetrazolium chloride (TTC), which delineates viable vs. necrotic tissue as described previously by our laboratory (House et al. 2003). After the I-R study, NTg and FGF2 Tg hearts were perfused with warmed 1% TTC stain (pH 7.4) via the aortic cannula. The hearts were frozen, sliced transversely, digitally photographed, and weighed. The area at risk (whole heart) and the infarct zone were determined by computer morphometry (NIH imaging software, 1.61 version). Infarct size was depicted as a percent of the entire heart.

### Measurement of NO release into coronary effluent

To assess the amount of NO released in response to cardiac-specific overexpression of FGF2 in the context of I-R injury, coronary effluent was collected from NTg and *FGF2* Tg hearts subjected to I-R injury in the absence or the presence of NOS inhibitors. Additionally, to determine the interaction between protein kinases (e.g. PKC and MAPK) and NOS pathways in FGF2-induced cardioprotection, coronary effluent samples were collected at designated time points from NTg and *FGF2* Tg hearts subjected to I-R injury in the absence or the presence of protein kinase inhibitors. Time points collected included: every 2 min for the last 10 min of baseline, for the first 30 min and last 15 min of ischemia, during the increase in flow (from 2 to 4 ml/min), and every 2 min for the for the first 14 min and last 10 min of reperfusion. Quantitative determination of NO released at various time points of baseline, ischemia, and reperfusion was determined through the use of a Fluorometric Nitric Oxide Assay Kit (Calbiochem, Darmstadt, Germany). The basis for this reaction is the addition of 2,3-diaminonaphthalene (DAN) and then sodium hydroxide which converts nitrite to a fluorescent compound, 1(H)-naphthotriazole. Measurement of the fluorescence of 1(H)-naphthotriazole provides an accurate assessment of nitrite ( $\text{NO}_2^-$ ) concentration in a sample.

Two hundred microliters of each coronary effluent sample were aliquoted into wells on a 96-well plate. Ten microliters of DAN were added to each well and incubated for 10 min and 20  $\mu\text{l}$  of NaOH was then added to each well, and the plate was read in a GENios fluorometer (Tecan, Durham, North Carolina, USA) using an excitation wavelength of 365 nm and an emission wavelength of 450 nm. A standard curve was calculated using standards of 0.078 to 5  $\mu\text{M}$  of nitrite (Calbiochem). The concentration of the nitrite in coronary effluent samples was calculated using Prism 3.0 software and was normalized to coronary flow and heart weight.

### Western blot analysis of NOS isoform expression

Non-ischemic NTg and *FGF2* Tg hearts were subjected to Western immunoblot analysis to determine the expression of the level of eNOS, nNOS, and iNOS isoforms in response to cardiac-specific overexpression of FGF2. Non-ischemic NTg and *FGF2* Tg hearts were snap frozen in liquid nitrogen and homogenized as previously described by our laboratory (House et al. 2003, 2005, 2007). One hundred and fifty micrograms of each heart homogenate were subjected to Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), and Western blot analysis was performed using antibodies specific for eNOS (1:500 dilution, Santa Cruz Biotechnologies, Santa Cruz, California, USA), nNOS (1:500 dilution, BD Transduction Labs, San Jose, California, USA) or iNOS isoforms (1:500 dilution, BD Transduction Labs, San Jose, California, USA). Equal protein loading was assessed via Ponceau S (Sigma Aldrich, St. Louis, Missouri, USA) staining of total protein content. Densitometry of protein bands was performed using a Fluorchem 8800 gel imager (Alpha Innotech, San Leandro, California, USA).

## Statistical analysis

All values are expressed as mean  $\pm$  Standard Error of the Mean (SEM). Percent functional recovery and myocardial infarct size were compared using a oneway ANOVA followed by *post-hoc* analysis using the Student's *t*-test. Differences between nitrite release at various time points were compared using two-way ANOVA for time and treatment with repeated measures using the *post-hoc* test of Fisher's least significant difference. Western blot data was compared using a Student's *t*-test. Statistical differences were considered significant when  $p < 0.05$ .

## Results

### Effect of cardiac-specific overexpression of FGF2 on NOS isoform expression

The protein expression level of eNOS, iNOS, and nNOS was determined in non-ischemic NTg and *FGF2* Tg hearts to determine if overexpression of FGF2 primes the heart for ischemia-reperfusion insult through upregulation of NOSs, as these have been implicated in a variety of models of cardioprotection (Jones and Bolli 2006; Otani 2009). *FGF2* Tg hearts showed significantly increased expression of iNOS compared to NTg hearts ( $p < 0.05$ , Figure 2). However, there was no significant difference in the protein expression level of eNOS or nNOS in non-ischemic *FGF2* Tg or NTg hearts.

### Inducible NOS and activation mediates FGF2-induced cardioprotection from myocardial infarction but not post-ischemic contractile dysfunction

The importance of NOS activity in FGF2-induced cardioprotection was examined in the presence of pharmacological agents that inhibit NOS activity, including L-NAME (400  $\mu$ M), L-NIL (400  $\mu$ M), and TRIM (100  $\mu$ M). *FGF2* Tg and NTg hearts were subjected to global, low-flow ischemia for 60 min, followed by reperfusion for 120 min and treated with NOS inhibitor or vehicle (Figure 1). In the vehicle-treated hearts, significantly higher recovery of cardiac function and lower myocardial infarct volume were observed in *FGF2* Tg hearts when compared to NTg hearts ( $p < 0.05$ , Figure 3A,B) as previously demonstrated by our laboratory (House et al. 2003). Upon NOS inhibition with L-NAME, L-NIL, or TRIM, recovery of post-ischemic cardiac function in *FGF2* Tg hearts was not different from vehicle-treated cohort hearts (Figure 3A). However, NOS inhibition with either L-NAME, L-NIL, or TRIM significantly abrogated the reduction of infarct size in *FGF2* Tg hearts vs. vehicle treatment ( $p < 0.05$ , Figure 3B).

### NO release into coronary effluent during I-R injury

Since iNOS expression is significantly increased in *FGF2* Tg hearts (Figure 2), production of NO may be increased in this group. To assess the level of NO released by *FGF2* Tg and NTg hearts during I-R injury, coronary effluent samples were collected during designated time points of equilibration, ischemia, and reperfusion. Levels of nitrite ( $\text{NO}_2^-$ ), a product of NOS activity *in vivo* and a reflection of NO release by the heart were measured in these samples. Nitrite concentration was significantly higher in *FGF2* Tg hearts compared to NTg hearts both prior to and following I-R injury ( $p < 0.05$ , Figure 4), indicating a higher NO release coupled with FGF2 overexpression. A significant ( $p < 0.05$ ) reduction in nitrite

release was observed in *FGF2*Tg hearts subjected to NOS inhibition compared to vehicle-treated hearts, and this decrease in nitrite release was similar for L-NAME (Figure 4A), L-NIL (Figure 4B) or TRIM treatment (Figure 4C), demonstrating that both inducible isoform (iNOS) and/or the neuronal isoform (nNOS) were necessary for the increase in NO production seen in *FGF2* overexpressing hearts.

### Protein kinases involved in NOS activation and FGF2-induced cardioprotection

Our laboratory has previously demonstrated that PKC and ERK activation is necessary for FGF2-induced cardioprotection against both cardiac dysfunction and myocardial infarction following I-R injury (House et al. 2005, 2007), and in this study, the possibility of crosstalk between these kinases and NOS activity to evoke the cardioprotective response of FGF2 is explored. There are two major forms of post-translational regulation which alter NOS isoform activity: subcellular targeting and phosphorylation (Massion and Balligand 2003). Modulation of NOS isoform activity can be mediated by a number of kinases, including PKCs and MAPKs (Forstermann et al. 1995; Forstermann 2010). Since FGF2 activates both these pathways to protect the heart from I-R injury (Jiang et al. 2002; House et al. 2005, 2007), it follows that NO may evoke the cardioprotective response of FGF2 by activation of MAPKs or PKC. Coronary effluent samples from hearts subjected to PKC or MAPK inhibition were evaluated for levels of nitrite, a product of NOS activity, released during I-R injury to determine whether PKC or MAPK signaling stimulates NOS activity, leading to FGF2-induced cardioprotection. A significant reduction in nitrite release was observed in *FGF2*Tg hearts receiving bisindolylmaleimide (GFX, 1  $\mu$ M), a non-selective PKC inhibitor ( $p < 0.05$ , Figure 5A), suggesting that PKC is upstream of NOS in FGF2-induced cardioprotection. Nitrite levels were also assessed in coronary effluent samples from hearts subjected to MAPK (MEK/ERK or p38) inhibition to determine whether MAPK signaling stimulates NOS activity, leading to FGF2-induced cardioprotection. A significant reduction in nitrite release was observed in *FGF2*Tg hearts receiving U0126 (2.5  $\mu$ M), the MEK/ERK inhibitor or SB203580 (2  $\mu$ M), the p38 inhibitor, ( $p < 0.05$ , Figure 5B,C), suggesting that both ERK and p38 are upstream of iNOS and/or nNOS in FGF2-induced cardioprotection against myocardial infarction.

### Sarcolemmal and mitochondrial $K_{ATP}$ channel involvement in FGF2-induced cardioprotection against myocardial dysfunction and infarction

Vascular studies have demonstrated that the vasoactive action of FGF2 is mediated in part by  $K_{ATP}$  channels (Cuevas et al. 1991; Boussairi and Sassard 1994; Tiefenbacher and Chilian 1997; Kajita et al. 2001). Both cardiac sarc $K_{ATP}$  and mito $K_{ATP}$  channels have been implicated in I-R injury and to be important effectors of cardioprotection, including ischemic preconditioning (Schultz et al. 1997; Gross and Fryer 1999; Grover and Garland 2000; O'Rourke 2000). Yet, the role of  $K_{ATP}$  channel in FGF2-mediated cardioprotection is not known. The loss of sarc $K_{ATP}$  channel function caused by 10  $\mu$ M HMR1098 treatment during the last 15 min of baseline and the first 15 min of ischemia eliminated the protective effect of FGF2 overexpression in the heart on post-ischemic recovery of function ( $p < 0.05$ , Figure 6A) and prevention of myocardial infarction ( $p < 0.05$ , Figure 6B). HMR1098 given during the last 15 min of ischemia and the first 15 min of reperfusion affected neither final

post-ischemic functional recovery (Figure 6C) nor infarct size (Figure 6D). This suggests that the sarcK<sub>ATP</sub> channel is a trigger for FGF2-induced cardioprotection.

Administration of 100 μM 5-HD had a deleterious effect on post-ischemic recovery of cardiac function and increased myocardial infarction in both NTg and *FGF2* Tg hearts when given during the last 15 min of baseline and first 15 min of ischemia ( $p < 0.05$ , Figures 7A,B). This result suggests that mitoK<sub>ATP</sub> channel opening during the onset of ischemia is essential for the ability of the heart to survive an irreversible ischemic insult. Additionally, 5-HD administration during the last 15 min of ischemia and the first 15 min of reperfusion resulted in diminished functional recovery in *FGF2* Tg hearts ( $p < 0.05$ , Figure 7C) and significantly increased infarct size (Figure 7D). These results also suggest that mitoK<sub>ATP</sub> is an end-effector of FGF2-induced cardioprotection.

## Discussion and conclusions

These data suggest that FGF2 overexpression modulates both NOS expression and NOS activity in the heart, and more importantly, that FGF2-induced cardioprotection against myocardial infarction is mediated via a NOS-dependent pathway. These findings indicate that NO signaling is a potential therapeutic target of endogenously expressed FGF2-induced cardioprotection against myocardial infarction. In addition, the application of pharmacologic blockers of K<sub>ATP</sub> channel function reveals that operation of these channels early in ischemia are crucial to the ability of the *FGF2* Tg heart to enhance recovery of function and minimize infarct size as well as in reperfusion to reduce myocardial infarction. This is the first demonstration that K<sub>ATP</sub> channels are involved in the cardioprotective effect of FGF2.

ATP-sensitive potassium (K<sub>ATP</sub>) channels are present in the mammalian heart, and experimental and clinical studies have identified the sarcolemmal (sarcK<sub>ATP</sub>) and/or mitochondrial (mitoK<sub>ATP</sub>) channel as an end-effector in the cardioprotective pathway of ischemic and pharmacological preconditioning (Gross and Fryer 1999; Grover and Garlid 2000; O'Rourke 2000). Yet, based on pharmacological studies using selective channel openers (such as diazoxide) and genetic knockout studies (involving ablation of the Kir6.2 subunit), there is disagreement in the field on the relative importance of sarcK<sub>ATP</sub> vs. mitoK<sub>ATP</sub> in cardioprotection (Grover and Garlid 2000; O'Rourke 2000). Some studies suggest that, although sarcK<sub>ATP</sub> is more important during preconditioning, mitoK<sub>ATP</sub> is more important for protection during long I-R injury (Wang et al. 2001; Suzuki et al. 2002; Marinovic et al. 2006). It appears, by way of consensus, that while neither sarcK<sub>ATP</sub> nor mitoK<sub>ATP</sub> is sufficient for the full cardioprotective phenotype, at least one of the two channels is necessary. Our present work has demonstrated that the sarcK<sub>ATP</sub> channel is an important trigger in the cardioprotective effect of FGF2 in the heart as inhibition of this channel in *FGF2* Tg resulted in poorer post-ischemic contractile dysfunction and larger infarct compared to vehicle-treated *FGF2* Tg (Figure 6A,B). The role of sarcK<sub>ATP</sub> channels in modulating cardiac function in diseased hearts (i.e. I-R injury or heart failure) is controversial such that similar observations were made in isolated perfused rat hearts subjected to low-flow ischemia by Gögelein and colleagues (2001); whereas no significant cardiodepressant effects were observed in anesthetized rats subjected to ischemia-reperfusion. Furthermore, evidence from mice deficient in (Suzuki et al. 2002) or with a



mutated form (Rajashree et al. 2002) of the inward-rectifying K<sup>+</sup> channel subunit (Kir6.2), a component of the cardiac sarcK<sub>ATP</sub> channel, revealed that this particular K<sub>ATP</sub> channel was important for normal myocardial function, and perturbations of this channel led to a poor recovery of cardiac function following ischemic injury. On the other hand, Pasdois et al. (2007) reported that 10 μM HMR1098 improved the recovery of cardiac function in Langendorff perfused rat hearts. Also, HMR1098 had no effect on cardiac function in *in vivo* failing canine hearts (Saavedra et al. 2002). The mitochondrial K<sub>ATP</sub> channel is essential for the ability of the heart to survive an irreversible ischemic insult as well as plays an accessory role as an end-effector of FGF2-induced cardioprotection against cardiac dysfunction and infarction (Figure 7). The importance of mitoK<sub>ATP</sub> channels in protecting the heart during I-R injury is well documented (reviewed in O'Rourke 2004), but this is the first data demonstrating its role in FGF2-mediated protection.

All three NOS isoforms, eNOS, iNOS, and nNOS, have been detected in the myocardium (Forstermann et al. 1994). There is also a growing body of evidence that NOS isoforms play prominent roles in cardiomyocyte regulation in physiological and disease states. It has been well established that nNOS participates in the regulation of cardiac function (Sears et al. 2003; Zhang et al. 2008) and, after I-R, can impact cardiac function, infarct development, and remodeling (Dawson et al. 2005; Saraiva et al. 2005; Otani 2009; Burkard et al. 2010). Additionally, it is well documented that iNOS modulates the disease state of cardiac I-R injury and is necessary or instrumental in cardioprotection (Jones and Bolli 2006). Evidence has indicated that exogenously added FGF2 reduced myocardial stunning in the mouse heart via an iNOS-dependent mechanism (Hampton et al. 2000). Here, we examine if iNOS and/or nNOS are responsible for the protective effects of FGF2, since several biological actions of FGFs have been shown to occur through NO signaling (Huang et al. 1997; Cuevas et al. 1999).

This study supports a role for iNOS in mediating FGF2-induced cardioprotection against myocardial infarction (Figure 3B). Other laboratories have demonstrated upregulation of iNOS and eNOS in the cardiovascular system in response to FGF2 (Kostyk et al. 1995; Tiefenbacher and Chilian 1997; Hampton et al. 2000), and our investigations have confirmed that cardiac-specific overexpression of FGF2 resulted in an upregulation of iNOS (Figure 2), suggesting a priming effect of FGF2 on myocardial tissue through induction of iNOS expression prior to the onset of ischemia. While iNOS was the only isoform with upregulated expression in mice overexpressing FGF2 in the heart, FGF2 has previously been shown to increase the expression of eNOS in endothelial cells (Kostyk et al. 1995); however, there is no significant induction of eNOS in the heart by cardiac-specific overexpression of FGF2 (Figure 2).

Although cardiac-specific FGF2 overexpression does not change the levels of nNOS in the non-ischemic heart, the data presented here suggest that nNOS activity may be necessary for FGF2-mediated cardioprotection (Figure 4B). nNOS localizes to the sarcoplasmic reticulum in cardiomyocytes and alters SR Ca<sup>2+</sup> uptake (Xu et al. 1999) as well as has an impact on the survival of mice after I-R injury (Saraiva et al. 2005), which is associated with a decreased infarct size and production of reactive oxygen species in post-ischemic hearts (Burkard et al. 2010). Overall, these findings suggest that nNOS may adjust calcium cycling

in the heart to protect the heart from injury associated with  $\text{Ca}^{2+}$  overload. Our data give credit to this hypothesis, indicating that FGF2-mediated protection from cardiomyocyte injury/death may require nNOS activity.

This study shows a role for NO in FGF2-mediated protection from infarct development (Figure 3B), but not post-ischemic contractile dysfunction, as NOS-inhibitor-treated *FGF2* Tg hearts have the same functional recovery following I-R injury as *FGF2* Tg hearts without NOS inhibition (Figure 3). This uncoupling of irreversible tissue death (infarct development) and reversible acute functional injury (stunning) has been seen previously in other models of cardioprotection (Mitchell et al. 1993; Cohen et al. 1999) and is further evident that distinct biological pathways are responsible for each (Bolli 1990). There is a complex relationship between stunning and infarction, and while both can occur as a result of I-R, the pathways leading to each are biochemically distinct (reviewed in Buja and Entman 1998; Bolli and Marban 1999; Pommel et al. 2010). It should be noted that, while irreversible cardiac dysfunction can and may develop in response to tissue death in the long term (Bolli 1990), the function of the heart immediately after reperfusion, a measure of stunning, was not affected. The data presented here suggest that NOS isoforms activated by overexpression of FGF2 favors pathways that alter cell survival, instead of those that affect post-ischemic function. This has been documented in other models of NO-mediated cardioprotection, where inhibition of NO caused no difference in functional parameters of the isolated heart, but resulted in a change in infarct size (Cohen et al. 2010), although it should be noted that these findings are not unequivocal (Schulz et al. 2004).

Multiple potential mechanisms may mediate the protection from I-R injury provided by enhanced NO production in *FGF2* Tg hearts. Our laboratory has demonstrated that PKC and ERK activation acutely mediate FGF2-induced cardioprotection against both cardiac dysfunction and myocardial infarction following I-R injury (House et al. 2005, 2007), which may in turn modulate substrates that have relevance for myocardial function or protection from I-R injury, including NOS isoform (Forstermann et al. 1995; Forstermann 2010) and  $\text{K}_{\text{ATP}}$  channel (Light et al. 2000; Jaburek et al. 2006) expression and activity. While the effects of phosphorylation on nNOS are residue-specific, it should be noted that phosphorylation of nNOS at S847 (Rameau et al. 2004), as well as S1412 (Adak et al. 2001; Rameau et al. 2007) increases the activity of the enzyme. The findings presented here implicate PKC, ERK1/2 and p38 as regulators of NO release in the ischemic heart during I-R injury (Figure 5). Interestingly, FGF2 activation of PKC- $\alpha$  has been shown to result in an increase in the phosphorylation of eNOS at S1179, and a subsequent increase in NO production in endothelial tissue (Partovian et al. 2005); this is of particular importance, as this site is analogous to the positive nNOS regulator S1412 (Adak et al. 2001). There is also evidence demonstrating that the activation of PKC- $\epsilon$  associated with the cardioprotective effect of ischemic preconditioning is prevented by the inhibition of NO pathway (Ping et al. 1999). Furthermore, PKC-mediated post-translational modification is linked to the regulation of sarc $\text{K}_{\text{ATP}}$  and mito $\text{K}_{\text{ATP}}$  channels (Kwak et al. 1996; Light et al. 2000). There is evidence that PKC- $\delta$  and - $\epsilon$  are necessary for sarc $\text{K}_{\text{ATP}}$  or mito $\text{K}_{\text{ATP}}$  channel opening in cardioprotection and other cellular functions (Ohnuma et al. 2002; Harada et al. 2004). Mito $\text{K}_{\text{ATP}}$  opening and inhibition of the associated pro-apoptotic cardiac mitochondrial permeability transition pore via the PKC- $\epsilon$  pathway prevents cardiomyocyte apoptosis in a

model of cardioprotection (Baines et al. 2003). Previous studies in our laboratory demonstrated that PKC isoforms  $\alpha$ ,  $\delta$ , and  $\epsilon$  are localized to the sarcolemma or mitochondria in *FGF2* Tg hearts at either baseline or various time points of I-R injury (House et al. 2007). Since these pathways operate downstream of FGF2 (Jiang et al. 2002; House et al. 2005; Liao et al. 2007) and upstream of  $K_{ATP}$  channel opening (Ohnuma et al. 2002), it is likely that at least one kinase cascade, attributed to cardioprotective FGF2 signaling, terminates at the mito $K_{ATP}$  and/or sarc $K_{ATP}$  channel to influence its opening and induce physiological changes that protect the heart from I-R injury.

Subfamilies of the MAPK pathway have also been implicated in modulating NOS signaling in the heart (Singh et al. 1996), and recently it has been shown that MAPK inhibition abrogates iNOS activity in sildenafil-induced cardioprotection (Das et al. 2009). In addition, the ERK pathway has been implicated in FGF2-induced cytoprotection against iNOS-mediated apoptosis (Iwai-Kanai et al. 2002). These findings support the conclusions presented here that MAPK and PKC both regulate NOS function in the heart during I-R, in hearts with increased expression of FGF2. With the finding that NOS and NO signaling are downstream of kinase activation (Figure 5), resulting in decreased myocardial infarct size following I-R injury, but not post-ischemic cardiac dysfunction, these data suggest that the kinase-NOS pathway is involved in the reduction of infarct development. Therefore, the preserved cardiac function in the presence of NOS inhibition (Figure 3A), but not PKC or MAPK inhibition (see references House et al. 2005, 2007), is most likely due to kinase activation and downstream changes in activity of proteins involved in calcium handling and/or myofibril function (Bolli and Marban 1999) independent of NO signaling. Ongoing studies are being performed to identify these mechanisms involved in the improvement of post-ischemic cardiac function induced by FGF2 overexpression.

This study, for the first time, demonstrates involvement of NO signaling and  $K_{ATP}$  channel activity in the cardioprotective effect elicited by endogenous FGF2. This is similar to signaling cascades activated by other tyrosine kinase receptor ligands such as epithelial growth factor (Namiki and Akatsuka 1990; Philipp et al. 2006) and insulin-like growth factor (Haylor et al. 1991; Tsukahara et al. 1994) in vascular function. In addition, our data confirm the direct or indirect interplay between NO and  $K_{ATP}$  channels in cardioprotection and I-R injury. For example, there is well-documented evidence that many pharmacological cardioprotective agents elicit their actions via NO and  $K_{ATP}$  channel function (Uchiyama et al. 2003; Xu et al. 2004; Cohen et al. 2006; Bai et al. 2011). Furthermore, evidence by Tsuura and colleagues (1994) and that of Kawano and colleagues (2009) demonstrate that NO, by modulation of the glycolytic pathway in pancreas or direct S-nitrosylation of channel in sensory neurons, respectively, can affect  $K_{ATP}$  channel activity and function.

### Limitations.

An important limitation of these studies is that the role of eNOS in FGF2-mediated protection remains to be elucidated. The effects of eNOS on the infarcted heart have been the subject of some controversy, and there have been conflicting reports regarding eNOS's role in cardioprotection (Sharp et al. 2002; Guo et al. 2008). This study does not rule out the possibility that eNOS may provide some of the cardioprotection induced by FGF2

overexpression in the heart. Regrettably, the tools for studying eNOS are currently limited, as there are no selective eNOS inhibitors, and two distinct strains of eNOS knockout mice have produced incongruous data in the hands of different groups (Bell and Yellon 2001; Sharp et al. 2002). While it has been demonstrated here that iNOS and/or nNOS are necessary for FGF2-mediated cardioprotection, the question of whether eNOS is also involved will be the subject of future studies as better tools are developed, and will be necessary to completely understand NO signaling in FGF2-induced cardioprotection. An additional limitation of this study is the relative selectivity of the nNOS inhibitor TRIM. It has been shown that TRIM may also inhibit iNOS at concentrations sufficient to inhibit nNOS (Handy et al. 1996), although TRIM has been used previously to distinguish nNOS from iNOS in ischemia studies in pigs (Adams et al. 2007). However, our studies examining the effect of protein kinase inhibition on nitrite levels suggest that a phosphorylatable NOS, in addition to iNOS, has a role in FGF2-induced cardioprotection. Further delineation of nNOS and eNOS in cardioprotection triggered by FGF2-protein kinase signaling is warranted. A limitation in the field of  $K_{ATP}$  channel function in I-R injury is the selectivity of the pharmacological inhibitors for sarc $K_{ATP}$  and mito $K_{ATP}$  channels; yet, the main tool for discriminating these two  $K^+$  channels is pharmacological manipulation. While HMR1098 is indeed highly selective for sarc $K_{ATP}$ , there is controversy regarding selectivity of 5-HD for mito $K_{ATP}$  vs. possible direct effects on succinate dehydrogenase activity (Hanley and Daut 2005).

In summary, the data suggest that activation of NOSs and the production of NO during I-R injury mediate FGF2-induced cardioprotection from post-ischemic myocardial cell death, but not post-ischemic cardiac dysfunction (Figure 3). This study shows that cardiac-specific overexpression of FGF2 results in a NOS-dependent increase in NO production, which is acutely regulated by PKC, ERK, and p38 (Figure 5). Furthermore, for the first time, these findings indicate a role of sarcolemmal and mitochondrial  $K_{ATP}$  channels in FGF2-induced cardioprotection against cardiac dysfunction and myocardial infarction (Figures 6 and 7). NO and  $K_{ATP}$  channels are part of a complex network of intracellular signals, which currently include PKC isoforms and MAPKs, activated by FGF2 to protect the heart against cardiac dysfunction and myocardial infarction during I-R injury (Figure 8).

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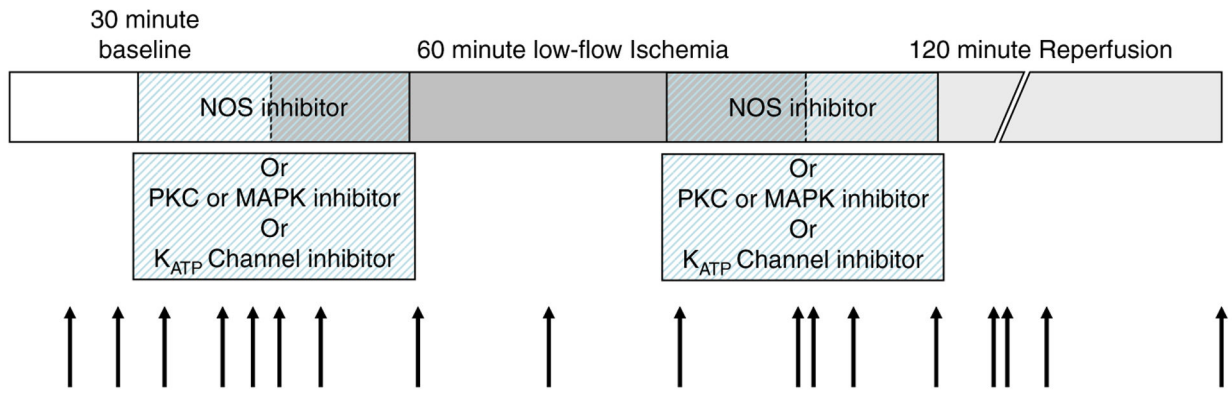
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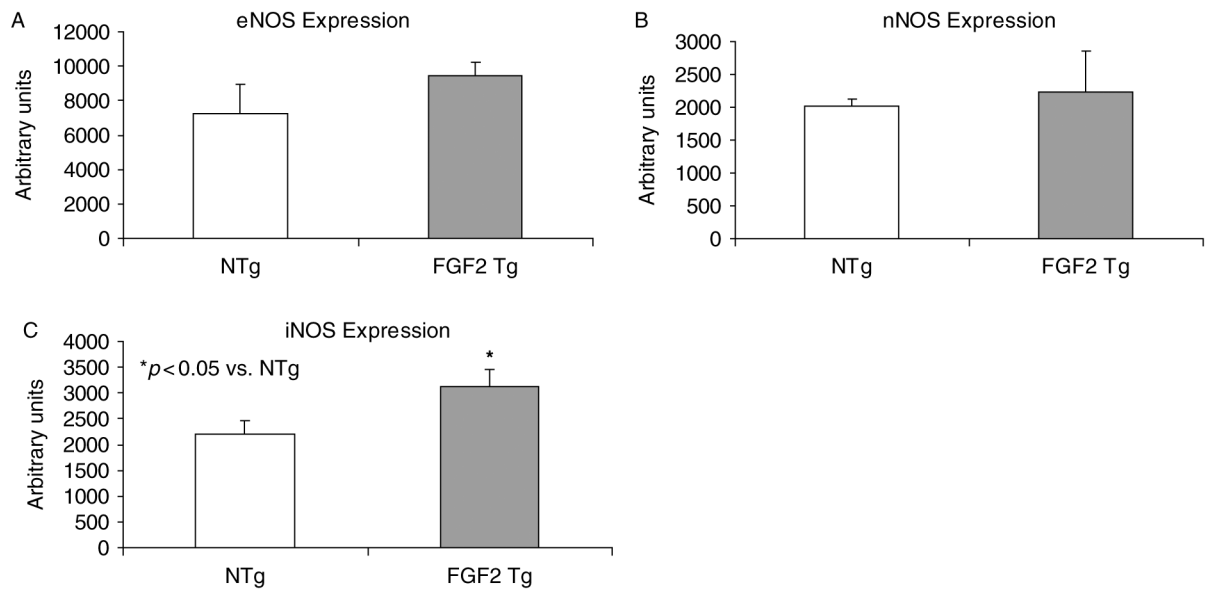
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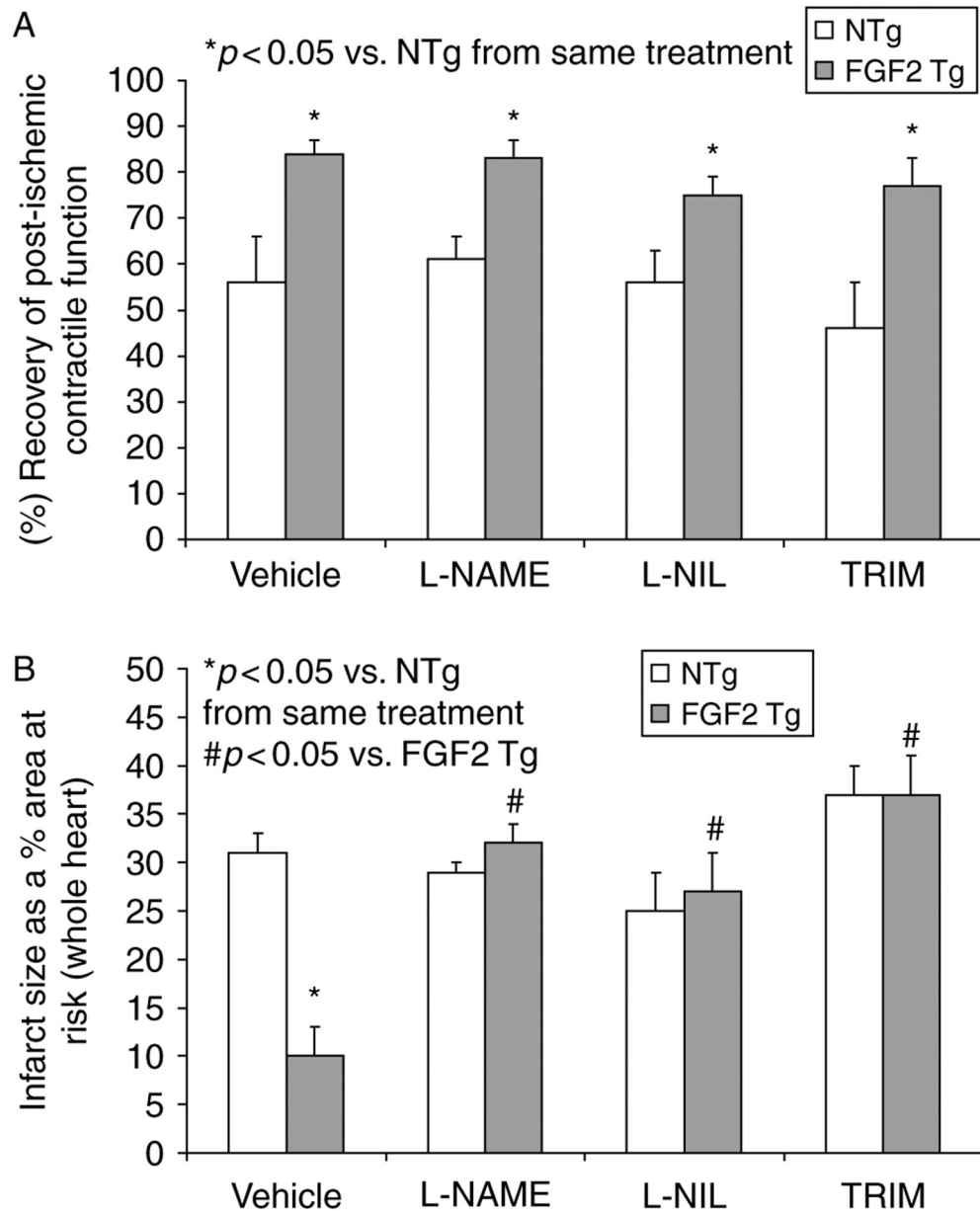


**Figure 1.**

Schematic of low-flow I-R protocol. Functional measurements and perfusate gases were taken at baseline, low-flow ischemia, and reperfusion (black arrows). Inhibitors were administered 15 min prior to ischemia and/or during the first 15 min of ischemia as well as during the last 15 min of ischemia and first 15 min of reperfusion (hashed bars).



**Figure 2.** Level of NOS isoforms [eNOS (Panel A), nNOS (Panel B), and iNOS (Panel C)] in non-ischemic NTg and *FGF2* Tg hearts. Overexpression of FGF2 resulted in an increase in iNOS expression compared to NTg hearts, suggesting that overexpressing FGF2 in the heart modulates signaling pathways such as NOS and NO which are involved in cardioprotection.  $n = 4-6$  hearts per group. \* $p < 0.05$  vs. NTg.

**Figure 3.**

(A) Percent recovery of post-ischemic contractile function and (B) myocardial infarct size (as a % of the whole heart) in vehicle- and NOS inhibitor-treated NTg and *FGF2* Tg hearts. (A) *FGF2* Tg hearts had a significantly improved recovery of post-ischemic contractile function compared to NTg hearts. L-NAME, L-NIL, or TRIM treatment to *FGF2* Tg hearts did not affect the cardioprotective effect of *FGF2* overexpression on contractile dysfunction. Percent recovery of post-ischemic contractile function, + dP/dt following 60 min of ischemia and 120 min of reperfusion compared to baseline + dP/dt. (B) Overexpression of *FGF2* significantly reduced infarct size (>50% reduction) compared to NTg hearts. L-NAME, L-NIL, or TRIM treatment to *FGF2* Tg hearts abolished the cardioprotective effect of *FGF2*. *n*

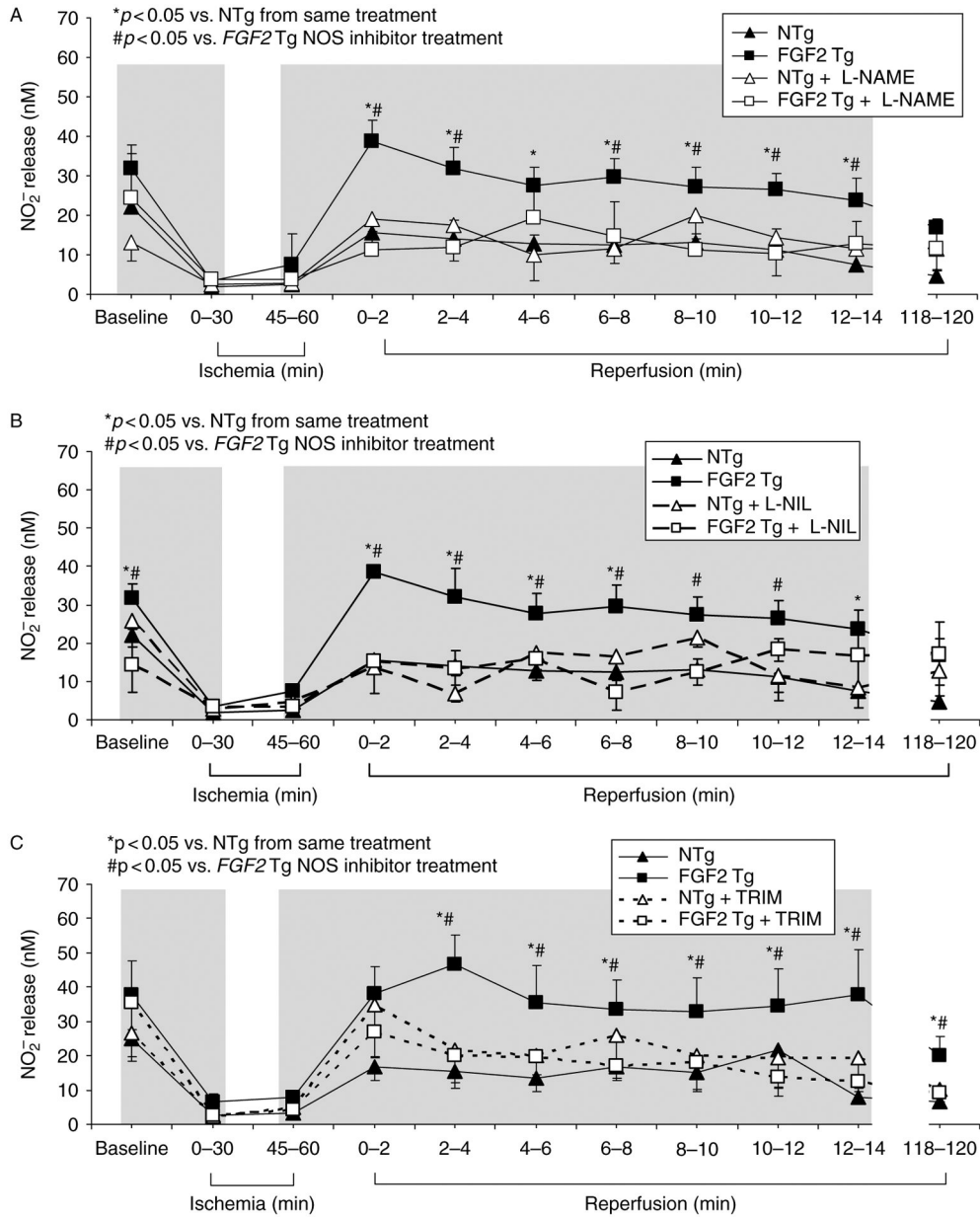
= 11–15 hearts per group. \* $p < 0.05$  vs. NTg from same treatment. # $p < 0.05$  vs. vehicle treatment.

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**Figure 4.** Effect of NO release, as measured by nitrite ( $\text{NO}_2^-$ ) levels in vehicle- and NOS inhibitor-treated NTg and *FGF2*Tg hearts. Nitrite release was significantly elevated in *FGF2*Tg hearts compared to NTg hearts. L-NAME treatment resulted in a decreased level of nitrite release *FGF2*Tg hearts, indicating that NOS activity was inhibited (A), L-NIL treatment (B) or TRIM administration (C) resulted in a decreased level of nitrite release in *FGF2*Tg hearts, indicating that NOS activity was inhibited and that iNOS and/or nNOS activity was important for NO production leading to protection against I-R injury. NTg, + black triangle; NTg + L-NAME, open triangle; NTg + L-NIL, open triangle/dashed line; NTg + TRIM, open triangle/gray dashed line; *FGF2*Tg, black square; *FGF2*Tg + L-NAME, open square; *FGF2*Tg + L-NIL, open square/dashed line; and *FGF2*Tg + TRIM, open square/gray

dashed line. Gray region, time points of inhibitor treatment (15 min prior to through first 15 min of ischemia and last 15 min of ischemia through first 15 min of reperfusion).  $n = 11-15$  hearts per group. \* $p < 0.05$  vs. NTg from same treatment. # $p < 0.05$  vs. FGF2 Tg NOS inhibitor treatment.

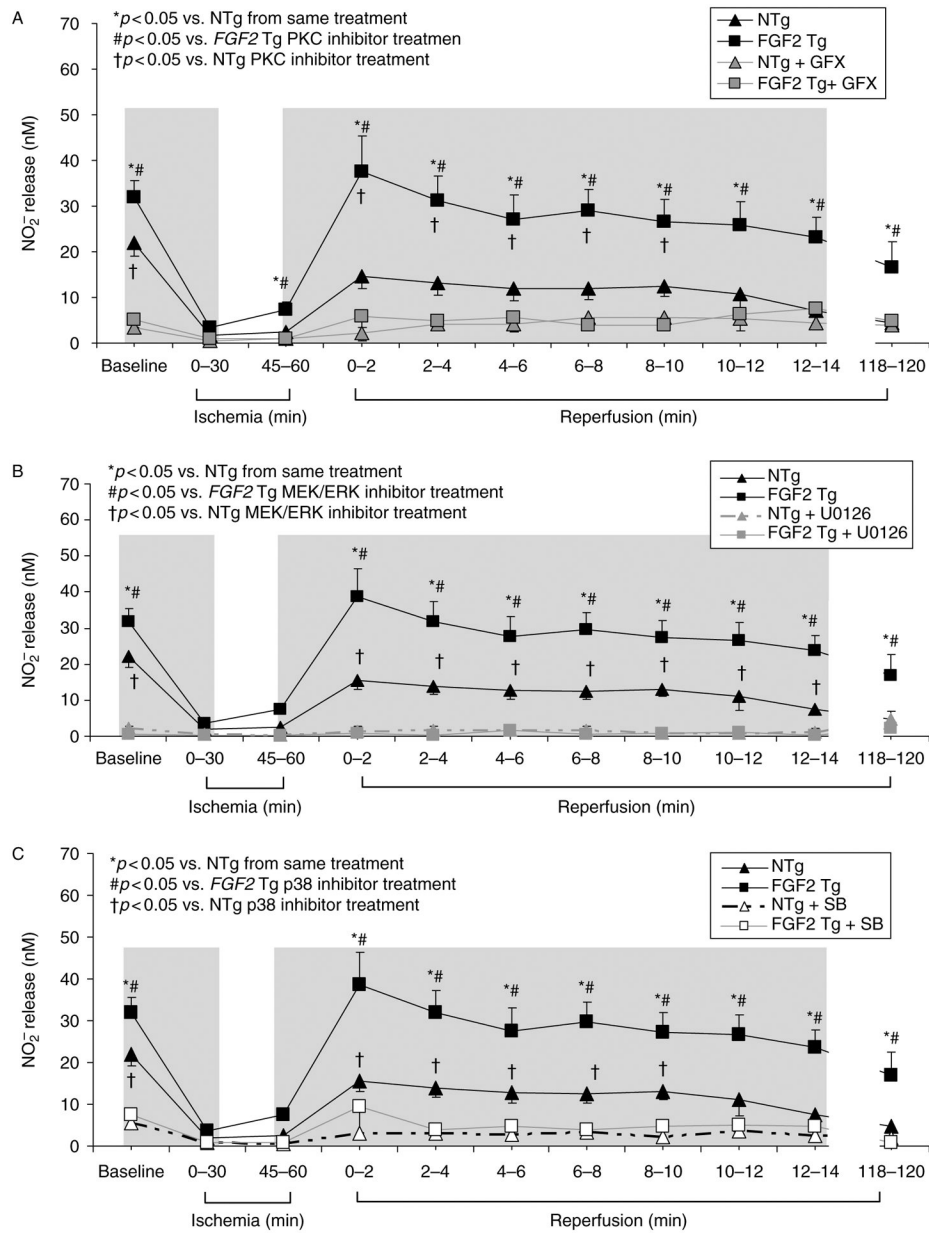
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**Figure 5.**

Effect of NO release, as measured by nitrite ( $\text{NO}_2^-$ ) levels in vehicle- and protein kinase (PKC, ERK or p38) inhibitor-treated NTg and *FGF2* Tg hearts. Nitrite release was significantly elevated in *FGF2* Tg hearts compared to NTg hearts. PKC inhibition (GFX treatment, 1  $\mu\text{M}$ ) resulted in a decreased level of nitrite release in both *FGF2* Tg and NTg hearts, indicating that NOS activity was regulated by PKC signaling (A). MEK/ERK inhibition (U0126, 2.5  $\mu\text{M}$ ) (B) or p38 inhibition (SB203580, 2  $\mu\text{M}$ ) (C) resulted in a decreased level of nitrite release in both *FGF2* Tg and NTg hearts, indicating that NOS activity was inhibited and that protein kinases regulate NOS activity and NO production leading to protection against I-R injury. NTg, black triangle; NTg + GFX, gray triangle; NTg + U0126, gray triangle/dashed gray line; NTg + SB, open triangle/dashed line; *FGF2* Tg,

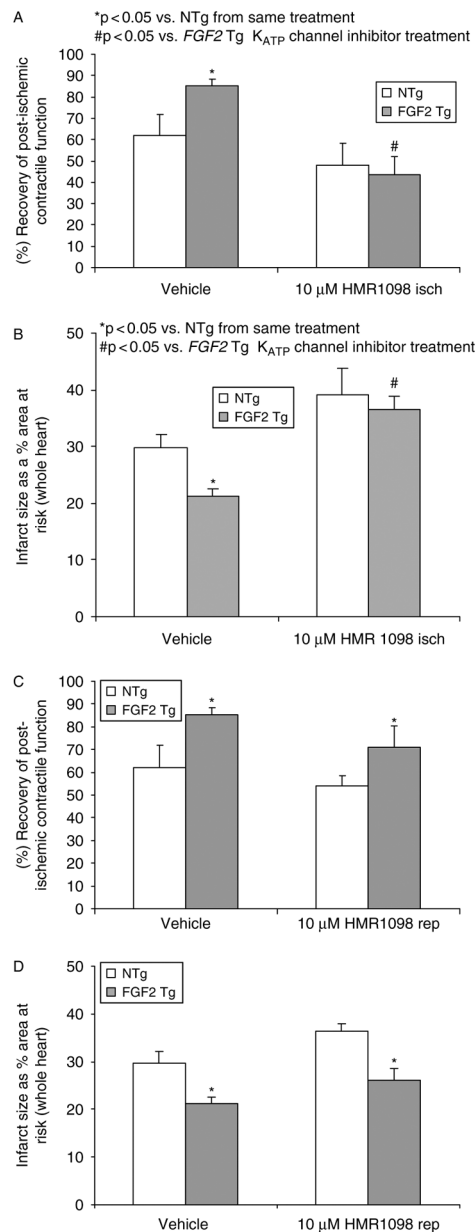
black square; *FGF2*Tg + GFX, gray square; *FGF2*Tg + U0126, gray square/dashed gray line; and *FGF2*Tg + SB, open square/gray line. Gray region, time points of inhibitor treatment (15 min prior to through first 15 minutes of ischemia and last 15 minutes of ischemia through first 15 min of reperfusion).  $n = 6-11$  hearts per group. \* $p < 0.05$  vs. NTg from same treatment. # $p < 0.05$  vs. *FGF2*Tg protein kinase inhibitor treatment. † $p < 0.05$  vs. NTg protein kinase inhibitor treatment.

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**Figure 6.** Effects of sarcolemmal  $K_{ATP}$  channels in endogenous FGF2-induced cardioprotection on post-ischemic recovery of contractile function (A and C) and myocardial infarct size (B and D) during ischemia and reperfusion. (Panels A and B) Mouse hearts overexpressing FGF2 (*FGF2* Tg) and their wildtype cohort (NTg) were treated with vehicle or the sarcolemmal  $K_{ATP}$  channel inhibitor HMR1098 (10  $\mu$ M) during the last 15 min of baseline equilibration and the first 15 min of ischemia and assessed for the ability to recover contractile function or reduce infarct size following 60 min of low-flow ischemia and 120 min of reperfusion. Inhibition of the sarc $K_{ATP}$  channel at ischemia in *FGF2* Tg hearts lose cardioprotection against cardiac dysfunction and infarction. (Panels C and D) *FGF2* Tg and NTg hearts were treated with vehicle or the sarcolemmal  $K_{ATP}$  channel inhibitor HMR1098 (10  $\mu$ M) during

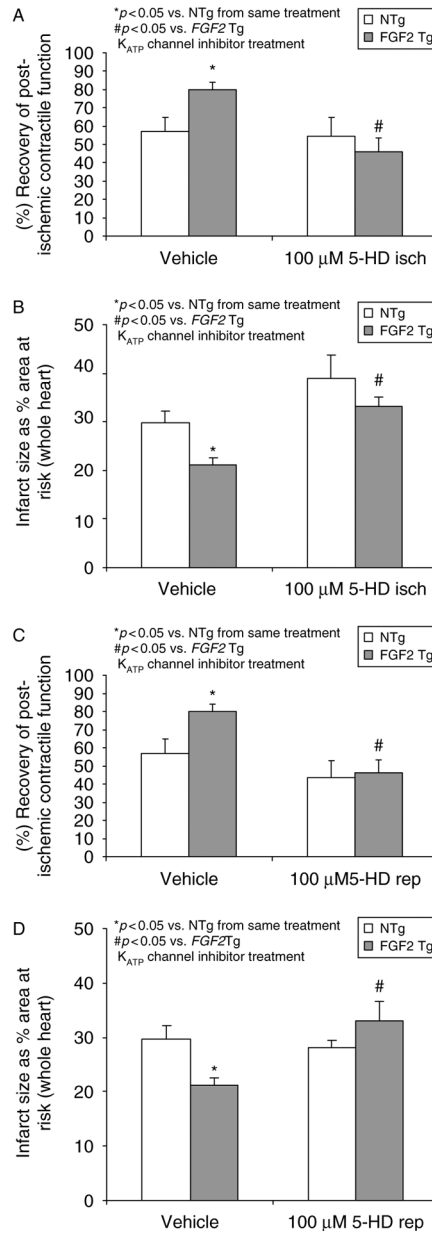
the last 15 min of ischemia and the first 15 min of reperfusion. Inhibition of the sarcK<sub>ATP</sub> channel at reperfusion had no effect on FGF2-induced cardioprotection suggesting that this channel is most likely not an end-effector in this protective phenotype.  $n = 6-11$  hearts per group. \* $p < 0.05$  vs. NTg from same treatment. # $p < 0.05$  vs. FGF2Tg K<sub>ATP</sub> channel inhibitor treatment.

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**Figure 7.**

Effects of mitochondrial  $K_{ATP}$  channels in endogenous *FGF2*-induced cardioprotection on post-ischemic recovery of contractile function (A and C) and myocardial infarct size (B and D) during ischemia and reperfusion. (Panels A and B) Mouse hearts overexpressing *FGF2* (*FGF2* Tg) and their wildtype cohort (NTg) were treated with vehicle or the mitochondrial  $K_{ATP}$  channel inhibitor 5-HD (100  $\mu$ M) during the last 15 min of baseline equilibration and the first 15 min of ischemia and assessed for the ability to recover contractile function or reduce infarct size following 60 min of low-flow ischemia and 120 min of reperfusion. Inhibition of the mito $K_{ATP}$  channel at ischemia in *FGF2* Tg hearts lose cardioprotection against cardiac dysfunction and infarction. (Panels C and D) *FGF2* Tg and NTg hearts were treated with vehicle or the mitochondrial  $K_{ATP}$  channel inhibitor 5-HD (100  $\mu$ M) during the

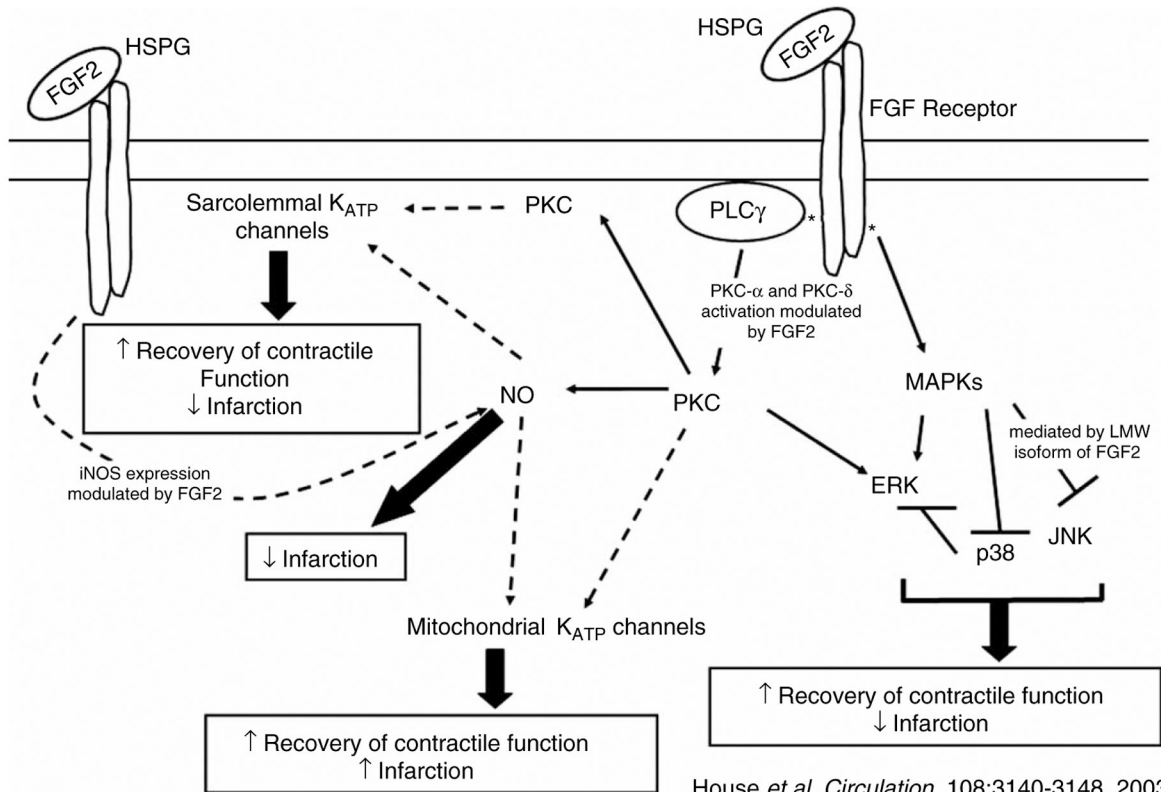
last 15 min of ischemia and the first 15 min of reperfusion. Similarly, inhibition of the mitoK<sub>ATP</sub> channel at reperfusion blocked FGF2-induced cardioprotection suggesting that this channel is most likely a trigger and an end-effector in this protective phenotype.  $n = 6-11$  hearts per group.  $*p < 0.05$  vs. NTg from same treatment.  $\#p < 0.05$  vs. *FGF2*Tg K<sub>ATP</sub> channel inhibitor treatment.

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**Figure 8.**

Schematic depicting the intracellular signaling network of endogenous FGF2-induced cardioprotection against cardiac dysfunction and myocardial infarction. Our laboratory previously demonstrated that endogenously expressed FGF2 in heart resulted in cardioprotection (House *et al.* 2003; Liao *et al.* 2007, 2010) via PKC and ERK activation (House *et al.* 2005) and p38 (House *et al.* 2005) as well as JNK inhibition (Liao *et al.* 2007). This protein kinase activation or inhibition was modulated by cross-talk between PKC and MAPKs (House *et al.* 2005; House *et al.* 2007). Our recent data demonstrate an involvement of NO and K<sub>ATP</sub> channels, directly or indirectly, to enhance post-ischemic recovery of cardiac function and/or reduce infarct size elicited by endogenous FGF2. Solid line indicates documented involvement of signaling cascade. Dashed line indicates potential pathway of protection. Upward arrow indicates improvement in recovery of post-ischemic contractile function and downward arrow indicates a reduction in infarct size.