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A novel fibroblast growth factor-1 ligand with reduced heparin binding protects the heart against ischemia-reperfusion injury in the presence of heparin co-administration

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Aims Fibroblast growth factor 1 (FGF1), a heparin/heparan sulfate-binding growth factor, is a potent cardioprotective agent against myocardial infarction (MI). The impact of heparin, the standard of care for MI patients entering the emergency room, on cardioprotective effects of FGF1 is unknown, however.

Methods and results To address this, a rat model of MI was employed to compare cardioprotective potentials (lower infarct size and improve post-ischemic function) of native FGF1 and an engineered FGF1 ($FGF1^{\Delta HBS}$) with reduced heparin-binding affinity when given at the onset of reperfusion in the absence or presence of heparin. FGF1 and $FGF1^{\Delta HBS}$ did not alter heparin's anticoagulant properties. Treatment with heparin alone or native FGF1 significantly reduced infarct size compared to saline ($P < 0.05$). Surprisingly, treatment with $FGF1^{\Delta HBS}$ markedly lowered infarct size compared to FGF1 ($P < 0.05$). Both native and modified FGF1 restored contractile and relaxation function ($P < 0.05$ versus saline or heparin). Furthermore, $FGF1^{\Delta HBS}$ had greater improvement in cardiac function compared to FGF1 ($P < 0.05$). Heparin negatively impacted the cardioprotective effects (infarct size, post-ischemic recovery of function) of FGF1 ($P < 0.05$) but not of $FGF1^{\Delta HBS}$. Heparin also reduced the biodistribution of FGF1, but not $FGF1^{\Delta HBS}$, to the left ventricle. FGF1 and $FGF1^{\Delta HBS}$ bound and triggered FGFR1-induced downstream activation of ERK1/2 ($P < 0.05$); yet, heparin co-treatment decreased FGF1-produced ERK1/2 activation, but not that activated by $FGF1^{\Delta HBS}$.

Conclusion These findings demonstrate that modification of the heparin-binding region of FGF1 significantly improves the cardioprotective efficacy, even in the presence of heparin, identifying a novel FGF ligand available for therapeutic use in ischemic heart disease.

Keywords Cardioprotection • Structural biology • Growth factor • Heparin • Protein engineering

1. Introduction

Myocardial infarction (MI), resulting from coronary artery disease, is a devastating event that is still one of the leading causes of morbidity and mortality.[1] Although there have been advances in clinical therapies for coronary artery disease, more than eight million individuals suffer a myocardial infarction every year.[1] Myocardial ischemia/reperfusion (I/R) injury can result in reversible functional myocardial deterioration (i.e.,

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stunning) and in irreversible tissue damage (i.e., infarction and fibrosis). Research on potential postconditioning agents and mechanisms for the clinical application to protect the myocardium from cellular damage after cardiac I/R injury could lead to novel therapies to reduce acute myocardial infarction-associated mortality and morbidity. Therefore, *in vivo* studies to evaluate the cardioprotective efficacy of molecules, such as fibroblast growth factor (FGF) in reperfusion injury are warranted before it can gain therapeutic utility in clinical scenarios of coronary artery syndromes and acute myocardial infarction.

Fibroblast growth factors (FGFs) are a family of 18 ligands (FGF1-10, FGF16–23) with the mouse FGF15 being ortholog of human FGF19.[2–6] All FGF members are structurally and functionally related and are either involved in embryonic development and/or postnatal metabolism and disease.[2–4,6] For example, a number of pharmacological and *in vitro* studies have suggested that FGF1 maintains the integrity/function of the myocardium by acting directly on cardiomyocytes or indirectly via its angiogenic properties.[7–15] However, even though a wealth of data from pre-clinical studies demonstrate that FGF1 is a promising therapeutic strategy to improve myocardial survival and cardiac function, there exist several issues that complicate the clinical application of FGFs for acute myocardial infarction. One of the issues may be its interaction with heparin; exogenous heparin treatment, either with unfractionated or low molecular weight (enoxaparin), is standard medical practice for patients with acute MI.[16–22]

Heparin is a type of heparan sulfate (HS) made exclusively by mast cells that has the highest amount of iduronic acid and of N- and O-sulfate residues. Generally, it is acknowledged that FGF1 executes its pleiotropic actions by promoting FGFR dimerization and activation in a HS-dependent fashion.[2,23–26] FGF1 binding to exogenous heparin or endogenous heparan sulfate proteoglycan (HSPG) protects it from proteases,[24] alters its bioavailability and biodistribution,[27] and aids in FGFR signaling.[23,26,28] Previous reports showed that simultaneous intramyocardial injection of enoxaparin (low molecular weight heparin) combined with FGF1 promoted capillary growth and regional myocardial blood flow at one week after infarction;[29] however, Hondermarck and colleagues[30] demonstrated that increasing doses of heparin co-administered with FGF1 or FGF2 weakened their binding to blood vessels in a heparin dose-dependent manner. Additionally, Newman and group showed that, in rat AT2 cells, low to moderate concentrations of heparin enhanced FGF1-mediated signals compared to FGF1 treatment alone, while a high concentration of heparin inhibited FGF1 activity.[31] In agreement with Newman and colleagues, Fannon and investigators found that, in Balb/c3T3 fibroblasts, a low concentration of heparin enhanced FGF2 receptor binding, while a high concentration of heparin inhibited binding.[32] Up to now, however, there are no *in vivo* studies looking into the role of heparin in the cardioprotective effect of FGFs, in particular FGF1, in MI. Although FGF1 has been previously investigated in I/R injury as a preconditioning or postconditioning agent in small and large animal models,[7–15,33] this research project is novel and innovative because, for the first time, the cardioprotective nature of this heparin-binding growth factor will be evaluated in the presence of heparin, a standard of care for acute coronary syndromes.[16–18]

In the present study, we initially set to modify interaction of FGF1 with heparin by mutating the FGF1 heparin-binding sites ($FGF1^{\Delta HBS}$) and then evaluated the cardioprotective efficacy properties of native and mutant FGF1 ($FGF1^{\Delta HBS}$) in the presence of heparin. This study reveals that heparin reduced the cardioprotective effect of native FGF1 against I/R injury; the impaired effect of FGF1 is partially due to its target redistribution by heparin away from the heart. Interestingly, reducing the heparin binding of FGF1 results in a marked decrease in infarct size and improvement in cardiac function, even in the presence of heparin co-therapy.

2. Methods

2.1 Pharmacological agents

Heparin sodium (1000 U/mL) was purchased from Sigma-Aldrich (MO, USA). Recombinant human hexa-histidine-tagged FGF1 (His-FGF1) were obtained from Dr. Moosa Mohammadi's laboratory. Non-tagged FGF1 was obtained from R&D system (USA). The $FGF1^{\Delta HBS}$ mutant, which carries the K127D/K128Q/K133V triple mutation in its HS-binding region (*Figure 1A*) was expressed in E.coli and purified to homogeneity using anion exchange chromatography followed by size exclusion chromatography (*Figure 1B*) in Dr. M. Mohammadi's lab. Heparin and FGFs were diluted in phosphate-buffered saline immediately before use.

2.2 Animals

Male Sprague Dawley rats weighing 240–300 g supplied by Harlan Laboratories were housed and handled according to the standards and guidelines set by *the Care and Use of Laboratory Animals* published by the US NIH (NIH Publication No. 85–23, revised 2011). All animal experimental protocols were approved by the University of Cincinnati Institutional Animal Care and Use Committee. All animals were acclimatized for at least 48 h before experimental use.

2.3 Experimental protocol and exclusion criteria

Rats were randomly divided into four sets of studies. Two sets were to assess the pharmacodynamics effects cardioprotective efficacy of native FGF1 and $FGF1^{\Delta HBS}$ and FGF1 signaling and the other two sets were to evaluate the pharmacokinetic properties of native FGF1 and modified FGF1 ($FGF1^{\Delta HBS}$). The first set of experiments was for the ischemia/reperfusion study in which animals were divided into six groups as follows: (1) saline group: saline treatment given intravenously for 10 min immediately upon reperfusion, (2) heparin group: heparin, 60 U/kg intravenous (i.v.) bolus, given immediately at reperfusion + 12 U/kg/h i.v. infusion for 120 min of reperfusion, (3) FGF1 group: FGF1, 10 μg/kg i.v. infusion, given for 10 min immediately at reperfusion, (4) FGF1 + heparin: heparin, 60 U/kg bolus, given immediately upon reperfusion followed by FGF1, 10 μg/kg i.v. infusion, given for 10 min and 12 U/kg/h i.v. infusion given for 110 min of reperfusion,[5] modified FGF1 ($FGF1^{\Delta HBS}$) group: $FGF1^{\Delta HBS}$, 10 μg/kg i.v. infusion, given for 10 min, or (6) $FGF1^{\Delta HBS}$ + heparin: heparin, 60 U/kg bolus, given immediately at reperfusion followed by $FGF1^{\Delta HBS}$, 10 μg/kg i.v. infusion, given for 10 min and 12 U/kg/h i.v. infusion for 110 min of reperfusion. All rats were subjected to 30 min of regional ischemia and 120 min of reperfusion. Drugs were administered at the onset of reperfusion via the jugular vein (*Figure 2A*). The final dose (10 μg/kg, i.v.) of native and modified FGF1 was determined by performing preliminary dose response studies (1 μg/kg, 10 μg/kg, and 100 μg/kg; data not shown); the dose range was derived from published experiments (2.6 μg/kg, i.v. and 10 μg/kg, i.v.) that have previously been shown to be effective in protecting the heart against ischemia-reperfusion injury in rodent models.[7,10–12,33,34] A total of ninety-six rats was completed for the I/R study. Sample size for each group ranged from 9 to 16 depending on the power analysis. A total of thirty-five rats were excluded from the study based on the lack of

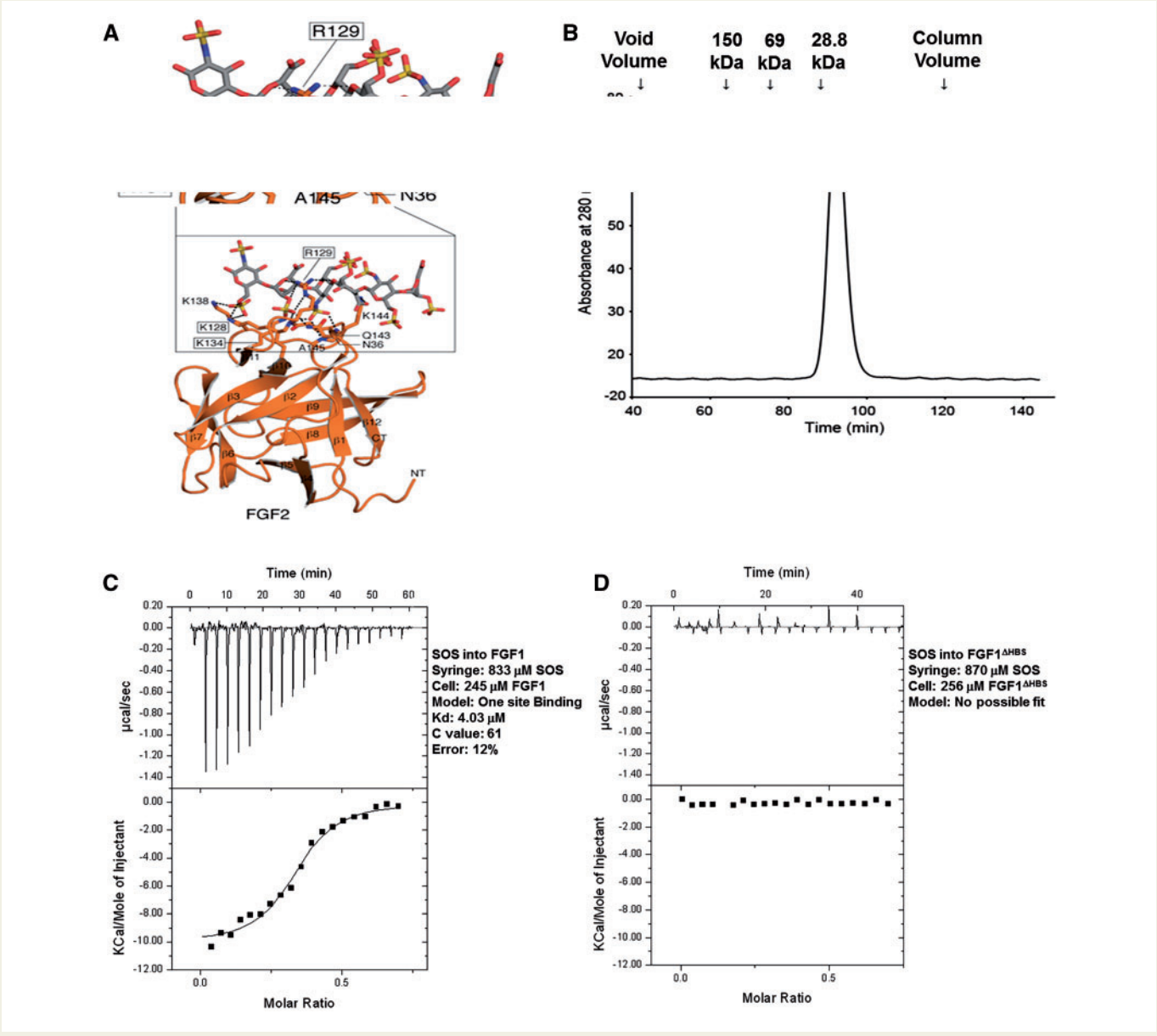

Figure 1 Structure-guided design of the FGF1$^{\Delta HBS}$ construct. (*A*) Expanded view of the hydrogen bonding interactions (dashed lines) between the HS-binding site of FGF2 and heparin hexasaccharide as observed in the crystal structure. FGF2 is shown as an orange ribbon diagram with HS-interacting residues rendered in sticks. The three FGF2 residues namely, K128, R129 and K134 that make important hydrogen bonding with HS are boxed. The corresponding three residues in FGF1 are K127, K128 and K133 which were mutated to aspartic acid, glutamine and valine, respectively, to engineer the FGF1$^{\Delta HBS}$ construct. (*B*) FGF1$^{\Delta HBS}$ elutes as a single symmetric peak at its predicted molecular weight from a Superdex 200 sizing column. Retention times of protein standards are given above the chromatogram. (*C* and *D*) Analysis of the interactions of wildtype FGF1 and FGF1$^{\Delta HBS}$ with SOS. Indicated solutions of SOS were injected into solutions of wildtype FGF1 or FGF1$^{\Delta HBS}$ in the cell. Wildtype FGF1 (panel C) binds SOS with a K_d of 4 µM; whereas, FGF1$^{\Delta HBS}$ (panel D) fails to bind SOS.

cyanosis of the heart or typical elevation of the ST segment in the electrocardiogram during ischemia, and death from malignant arrhythmia.

For the biodistribution or FGF1 signaling study, rats were randomly divided into six groups as described above (*Figure 2B*). Following drug treatment (immediately following 10 min of treatment or 110 min post-treatment at the point which mimics the length of time of reperfusion), solid organs and blood (see sections "Tissue collection for exogenous FGF1 biodistribution" and "Cardiac preparation to detect the activation of FGF1 downstream molecules") were collected. A total of 33 rats were used for biodistribution and FGF1 signaling studies, and 3 rats were excluded due to anesthetic overdose. Also, a total of 8 plasma samples were excluded because of hemolysis.

For elimination half-life of native FGF1 and FGF1$^{\Delta HBS}$, 12 rats were randomly divided into two groups and treated intraperitoneally with either FGF1 form as described in the section "Pharmacokinetic evaluation of exogenous FGF1 or FGF1$^{\Delta HBS}$".

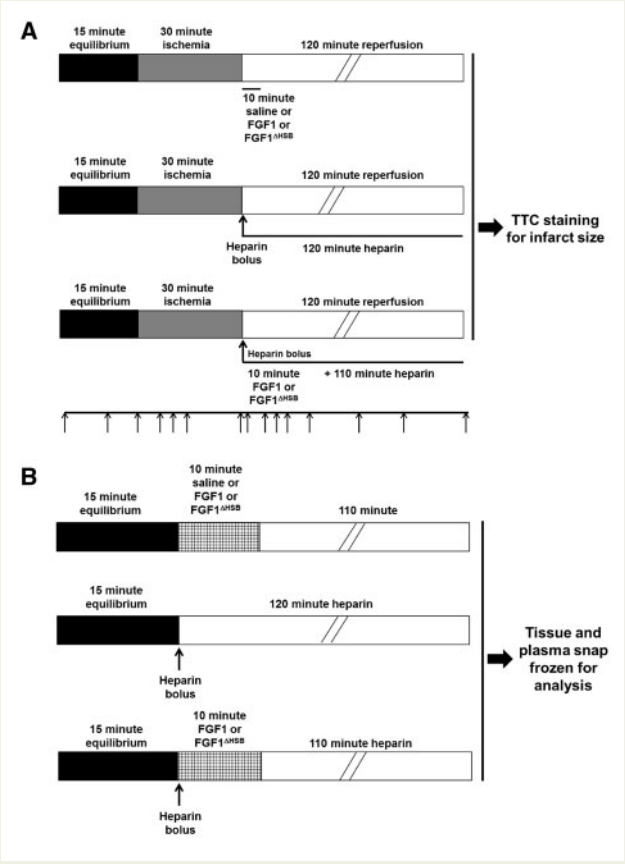

Figure 2 Experimental design and protocols. (*A*) *In vivo* ischemia/reperfusion protocol for assessment of heparin on the cardioprotective effect of FGF1 and the novel FGF1 ligand with reduced heparin binding (FGF1$^{\Delta HBS}$). Sprague Dawley rats were subjected to 30 min of ischemia and 120 min of reperfusion. Hemodynamic parameters were measured at baseline, ischemia, and reperfusion (black arrows). (*B*) Schematic of FGF1 administration protocol for analysis of the tissue distribution or signaling of FGF1 or FGF1$^{\Delta HBS}$ in the absence or presence of heparin. Solid organs and blood were collected at the point which mimics the full length of time of reperfusion for ELISA assay. The left ventricle was collected immediately post-treatment or the point which mimics the full length of time of reperfusion for FGF1 signaling.

2.4 Assessment of blood clotting time

In sets of experiment, blood-clotting time was detected at baseline and 15 min after drug treatment. In brief, a nick 1–2-mm depth was made at 5 cm and 3 cm of the proximal tail for baseline and post-drug blood clotting time, respectively; the tail nick was blotted with 4 × 4 gauze every 15 s until bleeding totally stopped, and blood clotting time was recorded from the onset of bleeding until it stopped.

2.5 *In vivo* I/R model

The *in vivo* I/R model was established by ligating and loosening the left anterior descending artery (LAD) previously described by our laboratory.[35–37] In brief, rats were anesthetized with thiobutabarbital sodium salt hydrate, Inactin® hydrate (100 mg/kg, i.p., Sigma-Aldrich), and intubated and artificially ventilated via a rodent respirator (respiratory rate: 75 strokes/min; tidal volume: 3 mL; Model 683, Harvard, USA). The heart was exposed through a left thoracotomy and pericardiotomy, and a 6–0 silk suture was placed around the left anterior descending (LAD) coronary artery. After equilibration for 15 min, hearts were subjected to 30 min of ischemia by ligating the LAD via a snare occluder and reperfusion occurred for 120 min via loosening the snare occluder. The appearance of cyanosis of the heart and significant ST-segment elevation in the electrocardiogram were used to verify successful ligation.

2.6 Hemodynamic analysis

Hemodynamic measurement was assessed via a Millar MIKRO-TIP transducer (SPR-320, Houston, TX, USA) placed into the left ventricle via the right carotid artery. Left ventricular systolic (LVSP), end-diastolic pressure (LVEDP), heart rate (HR), rate of contraction (+dP/dt) as well as rate of relaxation (-dP/dt) were collected (DigiMed data analysis) during specific timepoints of baseline, ischemia and reperfusion (*Figure 2A*).

2.7 Infarct size measurement

At the end of the ischemia-reperfusion injury study, the LAD was re-occluded with the suture used previously for the establishment of ischemia. 5% Evans blue dye solution was given via the jugular vein to define the area-at-risk (AAR). Then, the heart was arrested in diastole with 1 mL of 15% potassium chloride, and the animal was euthanized with an anesthetic overdose followed by a bilateral pneumothorax for removal of the heart. The heart was excised, washed with saline, frozen in -80 °C for 5–6 min, sliced transversely into 2–3 mm thick sections, incubated in 1% 2, 3, 5-triphenyl tetrazolium chloride (TTC, pH 7.4) for 30 min at 37 °C in the dark followed by fixing in 4% paraformaldehyde and digitally photographed. Infarct size (IS), depicted as the percentage of the area-at-risk, was determined by Image J software (NIH, 1.61 version).

2.8 Pharmacokinetic evaluation of exogenous FGF1 or FGF1$^{\Delta HBS}$

The *in vivo* half-life of FGF1 or FGF1$^{\Delta HBS}$ (given as a single, intraperitoneal dose of 0.5 mg/kg body weight) was assessed, using the human FGF1 immunoassay ELISA kit (R&D System, MN), from blood (i.e., serum) collected at varying times as described previously.[38] The pharmacokinetic parameters of FGF1 or FGF1$^{\Delta HBS}$ were analyzed using the Drug and Statistics Software (DAS, v 2.0; Mathematical Pharmacology Professional Committee of China).

2.9 Tissue collection for exogenous FGF1 biodistribution

The rat was anesthetized with Inactin® hydrate (100 mg/kg, i.p.), and the external jugular vein was isolated for saline, FGF1, FGF1$^{\Delta HBS}$ (mutant FGF1) and/or heparin administration. At the end of the experiment (based on half-life of FGF1[39,40]), the animal was euthanized with an anesthetic overdose followed by excision and collection of the heart (including atria, left ventricle, and right ventricle), liver, lungs, kidneys, spleen, skeletal muscle, and brain (negative control as i.v. administered FGFs do not cross the blood-brain barrier[41]). Blood from the jugular vein was collected into the tubes containing 0.5 M EDTA and complete mini protease inhibitor cocktail (Roche), centrifuged at 2, 000 g for 15 min at 4°C, and the separated plasma was saved. Both tissue and plasma were snap-frozen in liquid nitrogen and stored at -80 °C for evaluation of exogenous FGF1 biodistribution.

2.10 Enzyme-linked immunosorbent assays (ELISA) of exogenous FGF1

Snap-frozen tissue samples were powdered and homogenized in protein extraction buffer (20 mM Tris, 2 mM EDTA, 2 M NaCl, 1% NP40 with Roche complete mini EDTA-free protease inhibitor cocktail (1 tablet/10 mL) and 1 mM PMSF added right before use) and protein was extracted as previously described.[42] Exogenous levels of tissue and plasma FGF1 and FGF1$^{\Delta HBS}$ were determined by ELISA per the manufacturer's instructions (FGF1 immunoassay kit, R&D Systems). In brief, 80 μg protein was incubated with assay diluent for 2 h at room temperature, shaking in a 96-well plate coated with a monoclonal antibody against FGF1. After 4 washes, conjugate buffer was added followed by incubation for 2 h at ambient temperature. All samples were incubated for 30 min in the dark with substrate solution, followed by stop solution. The optical density (O.D) was measured at 450 nm with correction wavelength of 570 nm in GENios Microplate Reader (Tecan, USA). The FGF1 standard in the kit was diluted with assay diluent to produce a dilution series of 2000, 1000, 500, 250, 125, 62.5, and 31.2 pg/mL. The assay diluent alone served as the zero standard (0 pg/mL). Standard and unknown samples were assayed in duplicate. Standard curve was created by using Excel via plotting the log of the average FGF1 concentrations versus the log of the O.D. The concentration of exogenous FGF1 (native or mutant) in tissue and plasma samples was calculated from the standard curve.

2.11 Cardiac preparation to detect the activation of FGF1 downstream molecules

Hearts from rats treated with saline, native FGF1 or FGF1$^{\Delta HBS}$ in the presence or absence of heparin were collected immediately after administration and at 120-min post-treatment for evaluation of downstream signaling. One-half of each snap-frozen heart was powdered and homogenized in protein extraction buffer as previously described by our laboratory.[43–45] For FGFR1, 150 μg of total protein were loaded onto a 8% SDS-PAGE gel, for ERK1/2 activation, 20 μg of whole cell protein homogenate was loaded onto a 15% SDS-PAGE gel, and for PKCα and PKCδ, 100 μg of whole cell protein homogenate was loaded onto a 10% SDS-PAGE gel and then transferred to nitrocellulose membrane. 0.1% Ponceau S in 5% acetic acid was used to examine the transfer efficiency and loading equality as described previously.[43] Membranes were blocked with 5% dry milk and then incubated with primary antibody against phospho-FGFR1 (Y-654, 1: 500, Cell Signaling), phospho-ERK1/2 (1: 1000, Cell Signaling) or phospho-PKCα (1: 500, Santa Cruz) or phospho-PKCδ (1: 500, Santa Cruz) followed by incubation with HRP-conjugated secondary goat anti-rabbit antibody (1: 3000, Santa Cruz Biotechnology). Membranes were stripped and reprobed with antibodies against total FGFR1 (1: 500, Cell Signaling), total ERK (1: 1000, BD Transduction Labs) or total PKCα (1: 500, Santa Cruz) or PKCδ (1: 500, Santa Cruz). Bound antibodies were visualized by ECL system. Activation is indicated as a ratio of phosphorylated protein/total protein. β-actin (1: 1000, Cell Signaling) was also used as a loading control.

To detect levels of phospho-Akt, Akt, phospho-STAT3, STAT3, and GAPDH, an automated capillary Western blot was performed according to the WES user guide by ProteinSimple and all Wes reagents were purchased with the Wes Size Separation Master Kit (ProteinSimple, San Jose, CA). The cardiac samples (collected immediately post-treatment or 110-min post-treatment) were mixed together with 5 × Master Mix (DTT, fluorescence labeled maker, SDS) in a ratio of 5: 1 and then incubated at 70 °C for 5 min. The cardiac samples (0.4 mg/mL) and the biotin-labeled protein ladder (12 kDa, 40 kDa, 66 kDa, 116 kDa, 180 kDa and 230 kDa) were loaded into individual wells of the sample plate. Primary antibody against phospho-Akt (1: 50, Cell Signaling), Akt (1: 50, Cell Signaling), phospho-STAT3 (1: 50, Santa Cruz), STAT3 (1: 50, Millipore), and GAPDH (1: 50, Cell Signaling) were diluted with antibody diluent buffer. Loading conditions were determined by GAPDH. The experiment was then performed in Wes instrument as described by Wang and colleagues.[46]

2.12 Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Differences between groups were assessed by one-way or two-way ANOVA with Student-Newman-Keul post-hoc test or Kruskal-Wallace non-parametric test with Tukey's post-hoc test, depending on the end-point evaluated. $P < 0.05$ was considered statistically significant.

3. Results

3.1 FGF1 given immediately at reperfusion protects the heart against ischemia-reperfusion injury

Previous studies reported that pre-treatment with FGF1 prior to an ischemic insult improved cardiac function and enhanced cardiomyocyte survival.[7,9,14,15] As these studies indicate that this growth factor intervention protects the heart only when given prior to an ischemic event, its utility for use in the clinical setting of acute MI is limited. Therefore, elucidation of (non-angiogenic) cardioprotective activity of FGF1 when administered following an acute ischemic (MI) event is a necessary step to develop FGF1 as a potential therapeutic agent. Consistent with Cuevas and investigators,[8,10–12,47] our results demonstrate that human recombinant FGF1 given immediately at the onset of reperfusion rescued cardiomyocyte survival and cardiac function as represented by a reduction in infarct size and an improvement in post-ischemic contractility and relaxation (±dP/dt), respectively ($P < 0.05$, *Figures 3* and *4*, *Table 1*).

3.2 Heparin co-therapy reduces the cardioprotective effect of FGF1

Heparin treatment, either as unfractionated or low molecular weight (enoxaparin), is a standard medical practice for patients with acute MI.[16–22] Since FGF1 is a heparin-binding protein,[2,4] issues of heparin co-medication (per the 2010 NHLBI Workshop recommendations[48]) must be taken into consideration when developing FGF1 for acute MI therapy. To determine the effect of heparin on the cardioprotective activity of FGF1, infarct size and cardiac function was evaluated in the absence and presence of heparin co-administration (*Figure 2A*). Compared to saline treatment (58% infarct), heparin alone, at reperfusion, reduced infarct size (46% infarct), but not to the degree of FGF1 treatment (35% infarct, *Figure 3B*, $P < 0.05$); the heparin-induced reduction of myocardial infarct was similar to that first reported by Dr. Lucchesi's group in the 1990s.[49–52] Notably, heparin co-administration significantly inhibited FGF1-induced cardioprotection against MI (42% infarct, *Figure 3B*, $P < 0.05$). In addition, rats treated with FGF1 recovered to 69% of its baseline contractility compared to saline treatment (44%, *Figure 4A* and *E*, *Table 1*, $P < 0.05$). After 2 h of reperfusion, there was a significant difference in post-ischemic contractility between FGF1 treatment (69%) versus FGF1 + heparin (57%) co-therapy (*Figure 4A* and *E*, *Table 1*, $P < 0.05$), suggesting that heparin attenuates the FGF1-induced protection against post-ischemic cardiac dysfunction. There was no significant

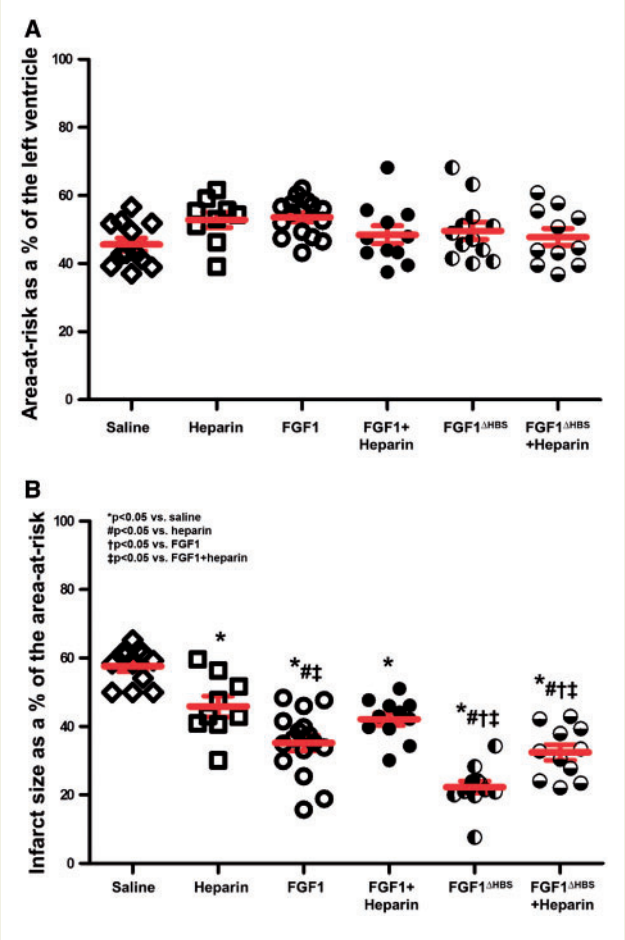

Figure 3 The role of heparin in the cardioprotective effect of FGF1 on preservation of cardiomyocytes after MI. (*A*) Percent (%) of area-at-risk normalized to the area of the left ventricle. (*B*) Percent (%) of infarct size normalized to the area-at-risk. $n = 9–16$. $^{*}P < 0.05$ vs. saline cohort, $^{\#}P < 0.05$ vs. heparin, $^{\dagger}P < 0.05$ vs. FGF1, $^{\ddagger}P < 0.05$ vs. FGF1 + heparin. The statistical test performed was 1-way ANOVA with Student-Newman-Keul post-hoc test.

difference in post-ischemic contractility between the saline and heparin group (*Figure 4A* and *E*, *Table 1*, $P < 0.05$). Other LV measures are depicted in *Table 1* (and *Figure 4B* and *F*) showing that rat hearts treated with FGF1 also have improved relaxation compared to saline treatment. Taken together, these data demonstrate that heparin reduces the cardioprotective effect of FGF1 against myocardial infarction and post-ischemic recovery of cardiac function.

3.3 Triple mutation in FGF1 heparin binding site (FGF1 mutant) is cardioprotective, leading to reduced infarct size and improved cardiac function even in the presence of heparin co-treatment compared to native FGF1

Based on the above data demonstrating that heparin inhibits the cardioprotective effect of FGF1 (*Figures 3* and *4*, *Table 1*), the question remains as to whether altering the HS-binding region of FGF1 to affect its binding affinity for heparin may change the cardioprotective efficacy of FGF1. From the crystallographic data, three basic residues at the HS binding site of FGF1, namely, K127, K128, and K133V, mediate the majority of hydrogen bonding with HS (*Figure 1A*). Accordingly, we reasoned that mutations of these three HS-binding residues should cause a major reduction in the HS-binding affinity of FGF1. The mutated FGF1 should retain some ability to promote HS-mediated FGFR dimerization and activation. A FGF1 mutant, termed FGF1$^{\Delta HBS}$, which carries the triple mutation in its HS-binding site, was designed. FGF1$^{\Delta HBS}$ was expressed in *E. coli* and purified to homogeneity using anion exchange chromatography followed by size exclusion chromatography. FGF1$^{\Delta HBS}$ elutes as a monodisperse peak at the predicted retention time, indicating that the mutations do not harm the tertiary folding of the ligand (*Figure 1B*). In fact, this was expected as K127, K128, and K133 are surface-exposed and do not play any role in ligand folding. To test the impact of the mutations on HS-binding ability of FGF1, isothermal titration calorimetry (ITC) was used to compare the interactions of native FGF1 and FGF1$^{\Delta HBS}$ with Sucrose Octasulfate (SOS), a known surrogate for HS. As shown in *Figure 1C* and *D*, native FGF1 bound SOS with a K_d of 4 μM; whereas, binding of FGF1$^{\Delta HBS}$ to SOS was negligible, demonstrating that the FGF1$^{\Delta HBS}$ mutant sustains a substantial loss in HS binding.

Next, the cardioprotective potential of FGF1$^{\Delta HBS}$ mutant when administered immediately upon reperfusion was evaluated in an *in vivo* rat model of myocardial infarction. FGF1$^{\Delta HBS}$ was ~2-fold more efficacious in reducing infarct size than native FGF1 (62% versus 39% reduction, respectively; *Figure 3*, $P < 0.05$). Importantly, heparin co-therapy had minor, and no statistically significant, inhibition on the cardioprotective effect of FGF1$^{\Delta HBS}$; whereas, the cardioprotective activity of native FGF1 was markedly attenuated in the presence of heparin (*Figure 3*). Of note, FGF1$^{\Delta HBS}$ significantly reduced the infarct size to an extent that was lower than native FGF1 in the absence (22% vs 35%, respectively, $P < 0.05$, *Figure 3*) or presence of heparin (32% vs 42%, $P < 0.05$, *Figure 4*), which indicates that with the same dosage, FGF1$^{\Delta HBS}$ was more efficacious than the native counterpart in protecting the heart from I/R injury. Both contractile and relaxation parameters were significantly improved +dP/dt and -dP/dt were markedly restored in FGF1$^{\Delta HBS}$ and FGF1$^{\Delta HBS}$+heparin following ischemic injury (*Figure 4C–F*, *Table 1*, $P < 0.05$), demonstrating that the recovery of post-ischemic cardiac function is not compromised by the presence of heparin. Interestingly, the recovery of post-ischemic cardiac function of FGF1$^{\Delta HBS}$ or FGF1$^{\Delta HBS}$+heparin was significantly improved compared to the native FGF1 cohorts (*Figures 3* and *4*, *Table 1*, $P < 0.05$). There were no significant differences in heart rate with the FGF1$^{\Delta HBS}$ and FGF1$^{\Delta HBS}$+heparin groups compared to the saline group (*Table 1*).

3.4 FGF1 and FGF1$^{\Delta HBS}$ do not affect the anticoagulant activity of heparin

When developing novel agents to treat ischemic heart disease, consideration needs to be made that any new therapy does not modify and/or interfere with heparin and its anticoagulant function, which is used as the standard of care for cardiac patients,[16–22] and therefore, would make the new therapy not clinically applicable. Therefore, we evaluated the effect of native FGF1 and FGF1$^{\Delta HBS}$ mutant on the coagulation time. There was no significant difference in blood clotting time during baseline (pre-treatment) or following administration of saline, FGF1 or FGF1$^{\Delta HBS}$ (post-treatment). However, in the heparin, FGF1 + heparin and FGF1$^{\Delta HBS}$+heparin groups, the coagulation time was significantly longer post-treatment than at baseline or following administration of saline, FGF1 or FGF1$^{\Delta HBS}$ (*Table 2*). These data demonstrate that neither native nor HS-binding mutant FGF1

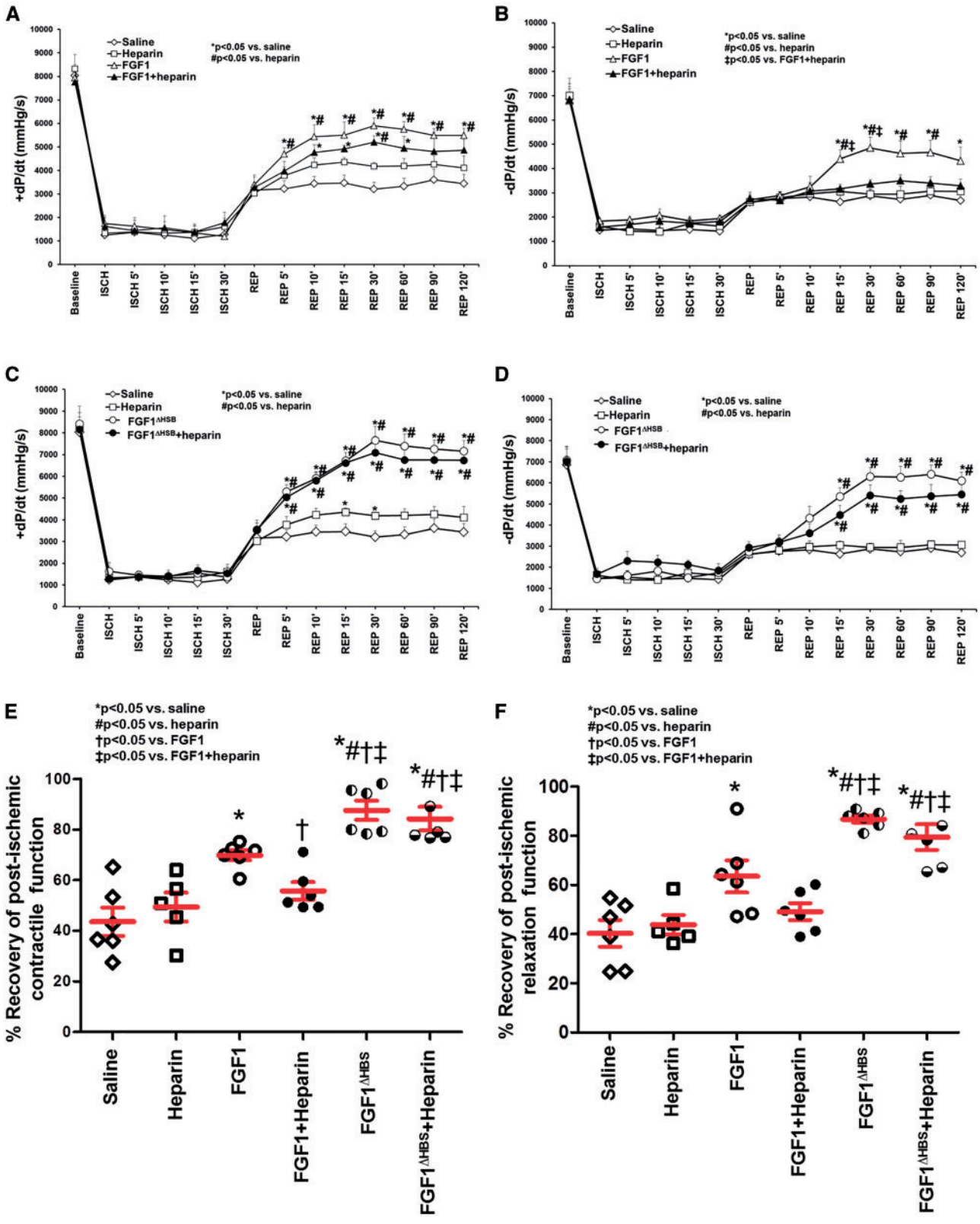

Figure 4 Post-ischemic recovery of contractile and relaxation function as measured by +dP/dt and –dP/dt, respectively. Contractile (*A*, *C*) and relaxation (*B*, *D*) function of FGF1 and modified FGF1 with reduced heparin binding ($FGF1^{\Delta HBS}$) in the absence or presence of heparin. Heparin reduced the post-ischemic improvement of cardiac function induced by FGF1 (panels A and B). $FGF1^{\Delta HBS}$ improved post-ischemic cardiac function, even in the presence of heparin (panels *C* and *D*). Percent (%) recovery of contractility (panel E) or relaxation (panel F) is +dP/dt or -dP/dt, respectively, at 120-min post-reperfusion normalized to baseline. $n = 5$ (for heparin). $n = 6$ (for saline, FGF1, FGF1 + heparin, $FGF1^{\Delta HBS}$, and $FGF1^{\Delta HBS}$ + heparin). $^{*}P < 0.05$ vs. saline cohort, $^{\#}P < 0.05$ vs. heparin, $^{\dagger}P < 0.05$ vs. FGF1, $^{\ddagger}P < 0.05$ vs. FGF1 + heparin. For panels A-D, the statistical test performed was a repeated measures 2-way ANOVA with Student-Newman-Keul post-hoc test. For panel E-F, the statistical test performed was 1-way ANOVA with Student-Newman-Keul post-hoc test.

Table 1 Cardiac parameters prior to and during I/R injury

	HR (beats/min)	LVSP (mmHg)	LVEDP (mmHg)	Tau (msec)	RT 1/2 (msec)
Baseline					
Saline	408 ± 20	148 ± 7	4 ± 1	17 ± 1	36 ± 3
Heparin	407 ± 17	139 ± 14	4 ± 1	17 ± 2	46 ± 9
FGF1	381 ± 23	142 ± 14	5 ± 1	16 ± 2	39 ± 6
FGF1+Heparin	396 ± 47	144 ± 8	5 ± 1	19 ± 3	49 ± 11
FGF1ΔHBS	405 ± 14	148 ± 6	5 ± 1	18 ± 1	45 ± 5
FGF1ΔHBS+Heparin	423 ± 14	142 ± 7	4 ± 1	17 ± 2	38 ± 5
5' Ischemia					
Saline	398 ± 20	63 ± 12	19 ± 3	33 ± 4	23 ± 3
Heparin	407 ± 38	54 ± 8	24 ± 2	32 ± 4	25 ± 4
FGF1	372 ± 13	59 ± 10	23 ± 4	32 ± 6	24 ± 5
FGF1+Heparin	408 ± 30	55 ± 8	22 ± 4	31 ± 4	27 ± 2
FGF1ΔHBS	396 ± 18	59 ± 4	22 ± 3	32 ± 5	25 ± 4
FGF1ΔHBS+Heparin	409 ± 18	56 ± 5	21 ± 4	34 ± 4	28 ± 3
30' Ischemia					
Saline	356 ± 32	55 ± 10	21 ± 3	31 ± 5	31 ± 2
Heparin	385 ± 9	52 ± 7	25 ± 3	34 ± 6	28 ± 2
FGF1	365 ± 14	61 ± 11	23 ± 4	32 ± 4	31 ± 4
FGF1+Heparin	385 ± 38	57 ± 8	25 ± 3	32 ± 6	27 ± 2
FGF1ΔHBS	382 ± 11	51 ± 8	21 ± 4	20 ± 3	26 ± 3
FGF1ΔHBS+Heparin	392 ± 15	54 ± 7	22 ± 4	33 ± 4	27 ± 5
5' Reperfusion					
Saline	382 ± 10	91 ± 8	16 ± 2	27 ± 2	31 ± 5
Heparin	395 ± 11	91 ± 6	16 ± 1	26 ± 2	31 ± 3
FGF1	366 ± 13	111 ± 7*	13 ± 3	23 ± 4	37 ± 4
FGF1+Heparin	373 ± 40	102 ± 9	17 ± 2	25 ± 5	38 ± 7
FGF1ΔHBS	380 ± 13	130 ± 5*#†‡	11 ± 1	21 ± 3	41 ± 9
FGF1ΔHBS+Heparin	395 ± 12	127 ± 6*#†‡	13 ± 3	22 ± 4	36 ± 6
30' Reperfusion					
Saline	378 ± 11	106 ± 4	12 ± 2	25 ± 4	30 ± 4
Heparin	384 ± 10	108 ± 7	10 ± 1	23 ± 3	36 ± 7
FGF1	332 ± 10	122 ± 4*	8 ± 1	21 ± 4	40 ± 4
FGF1+Heparin	374 ± 27	115 ± 6	10 ± 2	21 ± 2	38 ± 9
FGF1ΔHBS	367 ± 16	140 ± 3*#‡	5 ± 1#	17 ± 2	43 ± 8
FGF1ΔHBS+Heparin	377 ± 14	134 ± 6*#‡	7 ± 1	21 ± 2	34 ± 6
60' Reperfusion					
Saline	360 ± 21	101 ± 3	10 ± 1	26 ± 3	29 ± 3
Heparin	373 ± 22	111 ± 2*	10 ± 1	22 ± 2	33 ± 5
FGF1	345 ± 24	126 ± 7*	8 ± 1*	21 ± 4	36 ± 6
FGF1+Heparin	354 ± 16	113 ± 7	9 ± 1	22 ± 2	35 ± 3
FGF1ΔHBS	370 ± 12	139 ± 4*#‡	5 ± 1*#	18 ± 1	43 ± 6
FGF1ΔHBS+Heparin	363 ± 14	134 ± 3*#‡	7 ± 1*	22 ± 3	35 ± 5
90' Reperfusion					
Saline	353 ± 18	104 ± 3	12 ± 2	26 ± 4	28 ± 2
Heparin	366 ± 20	109 ± 7	11 ± 3	23 ± 3	34 ± 7
FGF1	349 ± 18	128 ± 6*	9 ± 1*	20 ± 5	34 ± 3
FGF1+Heparin	367 ± 14	118 ± 9	11 ± 1	23 ± 5	37 ± 5
FGF1ΔHBS	361 ± 16	139 ± 5*#	5 ± 2*	18 ± 2	43 ± 6
FGF1ΔHBS+Heparin	373 ± 10	135 ± 4*#	8 ± 2	20 ± 2	36 ± 3
120' Reperfusion					
Saline	353 ± 14	104 ± 2	12 ± 5	26 ± 1	34 ± 6
Heparin	377 ± 11	108 ± 1	11 ± 2	24 ± 2	33 ± 4
FGF1	332 ± 19	124 ± 8*	9 ± 2	19 ± 4	38 ± 6

Continued

Table 1 *Continued*

	HR (beats/min)	LVSP (mmHg)	LVEDP (mmHg)	Tau (msec)	RT 1/2 (msec)
FGF1+Heparin	364 ± 20	113 ± 10	11 ± 2	22 ± 2	35 ± 3
FGF1ΔHBS	357 ± 21	141 ± 5*#	6 ± 2#	18 ± 2	46 ± 6
FGF1ΔHBS+Heparin	372 ± 13	132 ± 4*#	7 ± 2	20 ± 2	39 ± 5

Data expressed as mean ± SEM. HR, heart rate. LVSP, left ventricular systolic pressure, LVEDP, left ventricular diastolic pressure, +dP/dt, rate of contraction, -dP/dt, rate of relaxation, $RT_{1/2}$, half relaxation time. *$P < 0.05$ vs. saline. #$P < 0.05$ vs. heparin. †$P < 0.05$ vs. FGF1. ‡$P < 0.05$ vs. FGF1 + heparin. $n = 5$ (for heparin). $n = 6$ (for saline, FGF1, FGF1 + heparin, FGF1ΔHBS, and FGF1ΔHBS + heparin).

co-administered with unfractionated heparin co-treatment affect the anti-coagulant property of heparin therapy alone.

3.5 FGF1 and modified FGF1 ($FGF1^{\Delta HBS}$) have similar elimination half-life

One possibility to account for the enhanced cardioprotective activity of $FGF1^{\Delta HBS}$ was that the elimination half-life was varied from native FGF1. Mean serum concentration-time profiles for FGF1 and $FGF1^{\Delta HBS}$ are shown in *Table 3*. The non-compartmental *in vivo* pharmacokinetic properties of FGF1 and $FGF1^{\Delta HBS}$ showed that the absorption phase half-life ($t_{1/2\alpha}$) and the elimination phase half-life ($t_{1/2z}$) of $FGF1^{\Delta HBS}$ were not significantly different from FGF1. The maximum serum concentration (C_{max}) and area-under-curve (AUC) for $FGF1^{\Delta HBS}$ were 4-fold and 2-fold greater than those for FGF1, respectively. However, the mean time to reach maximum serum concentration (T_{max}) of $FGF1^{\Delta HBS}$ was reached earlier than with FGF1. This result implies that the absorption rate of $FGF1^{\Delta HBS}$ after intraperitoneal administration was relatively fast, which is to be expected as the weak HS binding affinity of $FGF1^{\Delta HBS}$ should allow this ligand to avoid entrapment by HS in the pericellular/extracellular milieu.

3.6 Heparin co-treatment modifies the biodistribution of exogenous FGF1 to the heart

It is reported that heparan sulfates on the cell surface or extracellular matrix can concentrate or sequester FGFs which accounts for some of the modulatory activities of heparin on FGFs.[25,53–56] Therefore, the effect of heparin on exogenous FGF1 tissue localization was determined. Saline treatment is indicative of the endogenous level of FGF1 for each organ or in plasma. There was no difference in saline or heparin treatment with regard to FGF1 levels in plasma or organs evaluated. This suggests that heparin treatment does not lead to secretion of endogenous FGF1 from the organs. Compared to saline treatment, a significant amount of exogenous FGF1 was distributed to kidney, spleen, heart, liver, and plasma, while less went to lung and skeletal muscle (*Figure 5A* and *C*, $P < 0.05$). However, upon co-administration of FGF1 and heparin, exogenous FGF1 biodistribution was significantly decreased in kidney, spleen, heart, liver and plasma (*Figure 5A* and *D*, $P < 0.05$), suggesting heparin could change the biodistribution of exogenous FGF1 and influence the cardioprotective efficacy of FGF1. Most notably, when in the presence of heparin therapy, the greatest reduction in FGF1 biodistribution was at the heart (*Figure 5A* and *D*, $P < 0.05$). These findings are consistent with other observations that heparin modifies the distribution of FGF1 treatment.[57, 58]

Table 2 Blood clotting time of FGF1- and $FGF1^{\Delta HBS}$-treated rats in the presence or absence of heparin

Group	Baseline (sec)	Post-Treatment (sec)
Saline	178 ± 11	168 ± 10
Heparin	173 ± 8	415 ± 9*#
FGF1	163 ± 12	160 ± 12
FGF1+Heparin	154 ± 16	549 ± 53*#
FGF1ΔHBS	153 ± 9	160 ± 9
FGF1ΔHBS+Heparin	181 ± 7	440 ± 10*#

*$P < 0.05$ vs. saline post-treatment. #$P < 0.05$ vs. baseline cohort., $n =$ 10-18 per group.

Table 3 Pharmacokinetics of native FGF1 and $FGF1^{\Delta HBS}$ following intraperitoneally treatment

Parameters	FGF1 ($n = 6$)	$FGF1^{\Delta HBS}$ ($n = 6$)
$AUC_{(0\text{-}9h)}$ (μg·h/L)	267.70 ± 37.40	559.70 ± 104.50 **
$AUC_{(0\text{-}\infty)}$ (μg·h/L)	279.20 ± 37.80	565.60 ± 105.60**
R_AUC (t/∞)	95.80 ± 1.00	99.00 ± 0.40
$t_{1/2\alpha}$ (h)	0.13 ± 0.07	0.40 ± 0.18
$t_{1/2z}$ (h)	2.00 ± 0.20	1.99 ± 0.56
T_{max} (h)	1.25 ± 0.27	0.50 ± 0.00 **
V_z (L/kg)	5.24 ± 0.84	2.60 ± 0.70**
CL_z (L/h·kg)	1.82 ± 0.27	0.92 ± 0.22**
C_{max} (μg/L)	93.90 ± 28.70	367.00 ± 82.40**

AUC, area-under-the-curve. $t_{1/2\alpha}$, absorption phase half-life. $t_{1/2z}$, elimination phase half-life. T_{max}, mean time to reach maximum serum concentration. V_z, volume of distribution. CL_z, clearance. C_{max}, maximum serum concentration. **$P < 0.01$ vs. FGF1.

The next question is whether heparin alters the biodistribution of $FGF1^{\Delta HBS}$. As shown in *Figures 5B* and *C*, in the absence of heparin, $FGF1^{\Delta HBS}$ mainly went to the heart including atria, left ventricle, and right ventricle. In fact, there was a greater biodistribution of $FGF1^{\Delta HBS}$ to the left ventricle compared to native FGF1. In the presence of heparin, the amount of $FGF1^{\Delta HBS}$ to the heart was similar to that of $FGF1^{\Delta HBS}$ alone (*Figure 5B* and *D*). These findings indicate that modifying the heparin-binding region of FGF1 retains and, even, enhances its

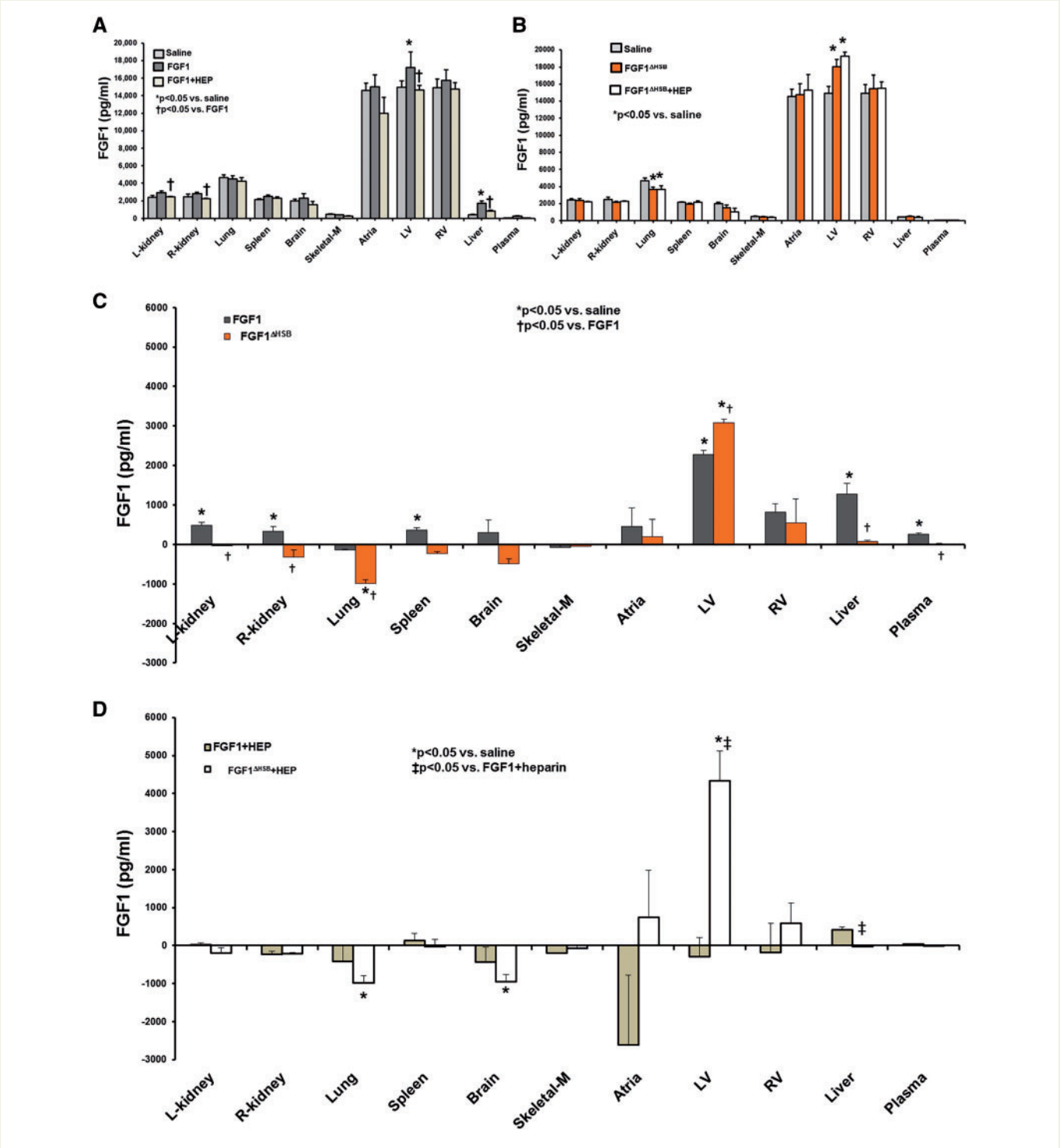

Figure 5 The tissue distribution of FGF1 and FGF1$^{\Delta HBS}$ in the absence or presence of heparin. (*A*) Total FGF1 levels (endogenous and exogenous) in saline treatment (endogenous FGF1, white bar), native FGF1 (includes endogenous + exogenous, gray bar), and native FGF1 + heparin (includes endogenous exogenous, light brown bar). (*B*) Total FGF1 levels (endogenous and exogenous) in saline treatment (endogenous FGF1, white bar), modified FGF1, FGF1$^{\Delta HBS}$, (includes endogenous + exogenous, gray bar), and FGF1$^{\Delta HBS}$ + heparin (includes endogenous + exogenous, light tan bar). (*C*) Exogenous native FGF1 (gray bar) and FGF1$^{\Delta HBS}$ (orange bar) were both significantly targeted to the heart; whereas, exogenous native FGF1 was targeted also to the kidney, spleen, liver and plasma. (*D*) Targeting to heart of exogenous native FGF1 + heparin (white bar) was markedly reduced compared to FGF1 alone (panel *C*) and FGF1$^{\Delta HBS}$+ heparin (light tan bar). Tissue accumulation of exogenous native FGF1 or FGF1$^{\Delta HBS}$; exogenous FGF1 of each tissue is described as a subtraction of FGF1 concentration in native FGF1- or FGF1$^{\Delta HBS}$-treated rats from that of saline treatment. L-kidney, left kidney; R-kidney, right kidney; Skeletal-M, skeletal muscle; LV, left ventricle; RV, right ventricle. $n = 5$ (for saline, heparin), $n = 7$ (for FGF1, FGF1 + heparin, FGF1$^{\Delta HBS}$ + heparin, FGF1$^{\Delta HBS}$). *$P < 0.05$ vs. saline. $^{\dagger}P < 0.05$ vs. FGF1, $^{\ddagger}P < 0.05$ vs. FGF1 + heparin. The statistical test performed was 2-way ANOVA with Student-Newman-Keul post-hoc test.

biodistribution/targeting to the heart even in the presence of heparin therapy, and demonstrates that heparin can no longer sequester FGF1 from its target site, the heart.

3.7 Heparin co-therapy does not alter the activation of FGF1 downstream signaling triggered by modified FGF1 (FGF1$^{\Delta HBS}$)

To ensure that the mutations in the heparin-binding site did not impair FGF binding to its receptor, ITC was used to compare the binding interactions of native FGF1 and FGF1$^{\Delta HBS}$ with the extracellular ligand-binding domain of FGFR3c, one of the cognate FGFRs of FGF1. Native FGF1 and FGF1$^{\Delta HBS}$ affinities to FGFR3c ecodomain show that the HS-binding site mutations do not impact FGFR binding ability of FGF1 (*Figures 6A* and *B*). Importantly, the ITC data showing that the FGF1$^{\Delta HBS}$ retains normal receptor binding affinity confirm that the HS mutations have no adverse effect on tertiary folding of the ligand. In support of this, FGFR1 activation (i.e., tyrosine phosphorylation) *in vivo* by FGF1 or FGF1$^{\Delta HBS}$ was similar (*Figure 6C*).

It is reported that a low concentration of heparin restored the activity of FGF1 in HS-deficient cells *in vitro*, while a high concentration of heparin completely inhibited its function.[31] To determine the influence of

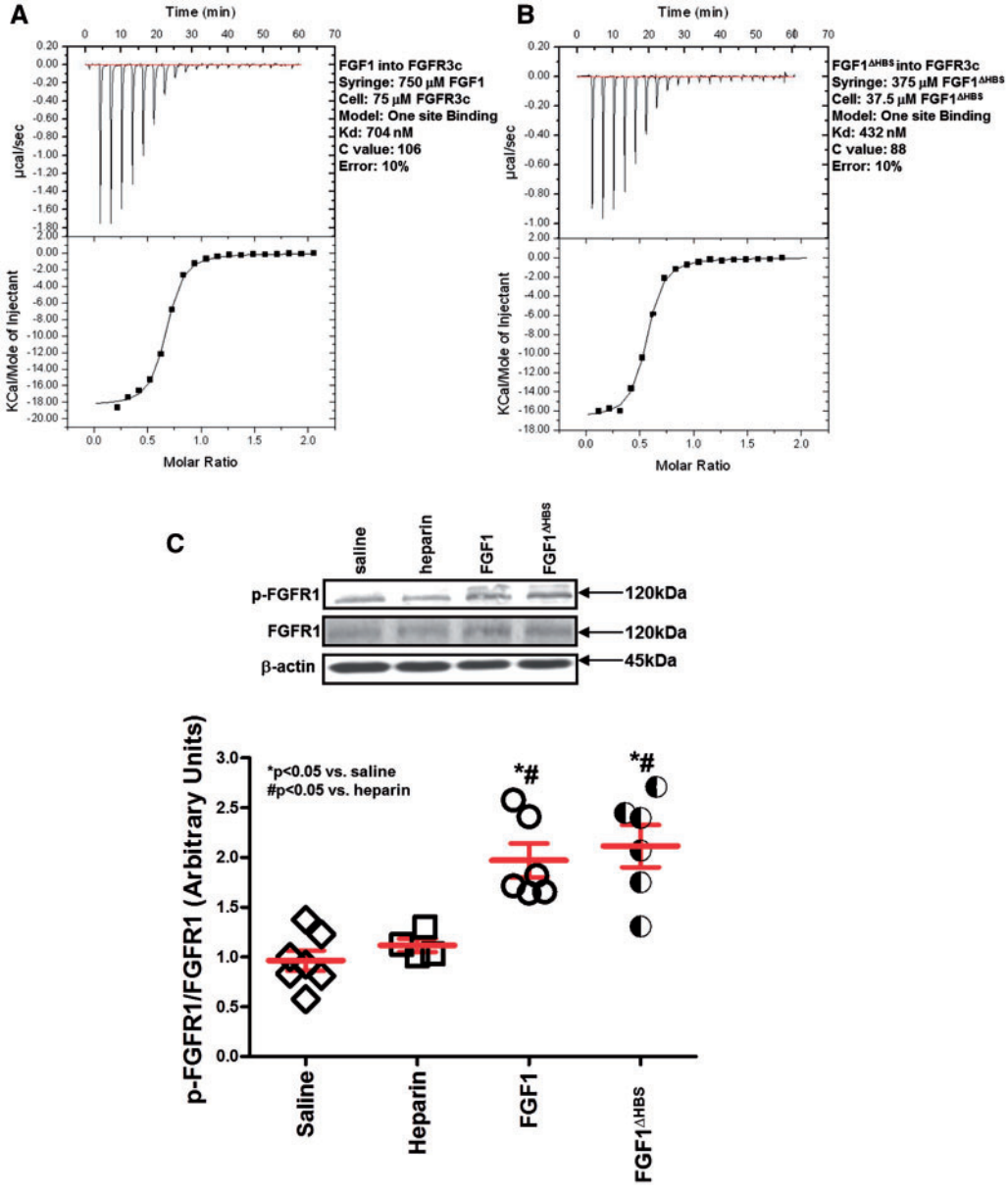

Figure 6 *In vitro* and *in vivo* characterization of the FGF1$^{\Delta HBS}$ construct in FGF receptor binding and activation. (*A* and *B*) Analysis of the interactions of native FGF1 and FGF1$^{\Delta HBS}$ with FGFR3c. Indicated solutions of native FGF1 or FGF1$^{\Delta HBS}$ were injected into solutions of FGFR3c ectodomain in the cell. Native FGF1 (panel *A*) and FGF1$^{\Delta HBS}$ (panel *B*) bind FGFR3c ectodomain with affinities of 704 nM and 432 nM, respectively. (*C*) *In vivo* activation (i.e., phosphorylation) of FGFR1 in the left ventricle is similar for native FGF1 and FGF1$^{\Delta HBS}$. $n = 5–7$. $^{*}P < 0.05$ vs. saline. $^{\#}P < 0.05$ vs. heparin. The statistical test performed for panel C was Kruskal-Wallis non-parametric test with Tukey's post-hoc test.

heparin on the activity of FGF1-FGFR1 signaling in the heart, ERK, PKC, Akt, and STAT3 activation, well-known pathways downstream of FGFR and RISK (reperfusion injury salvage kinase) and SAFE (survivor activating factor enhancement) mechanisms of cardioprotection, were assessed in left ventricle collected immediately after and 120-min post-drug administration. Immediately post-FGF1, FGF1 + heparin, FGF1$^{\Delta HBS}$, or FGF1$^{\Delta HBS}$+heparin treatment, phosphorylation of ERK1/2 was significantly increased compared to saline or heparin only treatment (*Figure 7A*, $P < 0.05$). However, heparin markedly reduced ERK1/2 activation stimulated by FGF1 (*Figure 7A*, $P < 0.05$). Surprisingly, there was a significantly higher level of phosphorylated ERK1/2 in the hearts collected from the FGF1$^{\Delta HBS}$-treated group compared to native FGF1 treatment whether in the absence or presence of heparin (*Figure 7A*, $P < 0.05$). By 2-h post-treatment, ERK activation returned to saline control levels (*Figure 8A*). Although heparin treatment had a minor cardioprotective effect (*Figure 4*), it was most likely not via ERK as its activation was not different than saline treatment (*Figure 7A*). Consistent with this finding, studies by Lucchesi's laboratory demonstrated that heparin-mediated cardioprotection occurred via inhibition of complement activation of the immune system.[49,52] Neither activation of PKCα or PKCδ nor activation of Akt of the RISK pathway and STAT3 of the SAFE pathway were different among the treatment groups or timepoints (*Figure 7B–E* and *Figure 8B–E*). These data suggest that both FGF1 and FGF1$^{\Delta HBS}$ activated FGFR1 signaling (i.e., ERK1/2); yet, heparin co-therapy reduced FGF1 signaling, but not that of FGF1$^{\Delta HBS}$.

4. Discussion

A number of key and clinically relevant findings were noted in the present study. First, heparin, the standard of care for MI patients entering the emergency room,[16–22] abolishes the cardioprotective action (infarct size and cardiac function recovery) of FGF1 when both were co-administered at the onset of reperfusion. Second, a novel, rationally designed FGF1 ligand with reduced heparin binding (FGF1$^{\Delta HBS}$) has been demonstrated, for the first time, when given immediately upon reperfusion, to elicit a markedly greater cardioprotective efficacy than native FGF1 in the absence as well as in the presence of heparin. Third, heparin treatment changes the tissue distribution of exogenous FGF1 and reduces the availability of FGF1 to the heart; however, tissue distribution of the modified FGF1$^{\Delta HBS}$ mutant to the heart still occurs even in the presence of heparin. Fourth, even in the presence of heparin, FGF1$^{\Delta HBS}$ interaction with FGFR1 on the heart has enhanced ERK signaling, an important component of the Reperfusion Injury Salvage Kinase (RISK) pathway. Overall, these findings suggest that the attenuation of the cardioprotective effect of FGF1 by heparin co-therapy is most likely due to heparin's ability to alter the availability of FGF1 to the heart, and that the novel modified FGF1 ligand (with reduced heparin binding, FGF1$^{\Delta HBS}$) is of great promise as a co-medication strategy with heparin for patients exhibiting MI.

A key factor to facilitate the translation of novel cardioprotective therapies into the clinical setting is the timing of administration of the cardioprotective agent. Given the clear benefit of establishing prompt reperfusion on myocardial salvage and clinical outcomes,[59–67] it is important to identify and develop cardioprotective agents that will be given at the time of reperfusion without delaying reperfusion, and that is the rationale for administering either FGF1 or FGF1$^{\Delta HBS}$ immediately upon reperfusion. A clinical study (J-WIND trial) demonstrated that the atrial natriuretic peptide analogue, carperitide, administered at the time of primary percutaneous coronary intervention (PPCI) lowered creatinine kinase, a clinical measure of myocardial infarct size.[68] Similar to the J-WIND trial[68] and supportive and consistent with a handful of previously published *in vivo* studies from the late 1990 s by Cuevas and colleagues,[8,10–12,47] which identified FGF1 as a postconditioning agent, our current study demonstrates that FGF1 or FGF1$^{\Delta HBS}$ administered immediately upon reperfusion protected the heart from MI (*Figure 3*, $P < 0.05$) and LV dysfunction (*Figure 4*, $P < 0.05$). On the other hand, there are proof-of-concept clinical studies demonstrating that drug treatment (such as cyclosporine, metropolol, exenatide, adenosine) during late ischemia to early reperfusion, and prior to PPCI or thrombolytic therapy, reduces infarct size and improves cardiac function.[69–73] There also is evidence that the acute beneficial effects of FGF1 against the steps involved (i.e., oxidative stress, apoptosis, neutrophil infiltration) in myocardial infarction (and cardiac dysfunction) have been observed pre-clinically when FGF1 was given prior to an ischemic event.[7,9,14,15] Yet, it is unknown whether FGF1 and FGF1$^{\Delta HBS}$ can (or cannot) elicit cardioprotection when given during ischemia, thereby adding to it efficacy as a clinical therapeutic for ischemic disease, and this is a limitation of the current study.

The FGF-heparin/heparan sulfate interactions modulate the multiple biological outcomes of FGFs, including FGF1. Heparin/heparan sulfate (HS) is implicated to prevent FGF1 from degradation and facilitate the active formation of FGF1-FGFR complex.[2,6,23–26,28] There is also evidence that heparin alters the bioavailability,[27] inhibits FGF-mediated signaling[31, 32] as well as weakens FGF binding to tissue.[30] The current study demonstrates that, when given immediately upon reperfusion, heparin or FGF1 alone protected the heart against myocardial infarction as evidenced by reduced infarct size and improved cardiac contractile and diastolic function at 2-h post-ischemia (*Figures 3* and *4* and *Table 1*), which supports previous work that heparin[49–52] or FGF1[7–15] protects from ischemia-reperfusion injury. However, with heparin and FGF1 co-therapy, the cardioprotective effect of FGF1 was completely abolished (*Figures 3* and *4*). Further evidence that anticoagulant or antithrombotic agents used in coronary artery disease modulate the cardioprotective effect of other pharmacological agents is provided by Gross and colleagues.[74] These investigators showed that aspirin co-treatment, a prophylactic for ischemic heart disease, abrogates morphine's cardioprotective effect.

Heparin (unfractionated or low molecular weight form) is the standard of care for patients with MI per the guidelines of the American Heart Association and American College of Cardiology.[16–22] Therefore, new cardioprotective therapeutics need to demonstrate efficacy in the presence of heparin without altering heparin's anticoagulant activity. Although heparin inhibited FGF1-induced cardioprotection against myocardial infarction and dysfunction, FGF1 did not alter anticoagulant activity of heparin (*Table 2*).

Our observation that FGF1-induced cardioprotection is abrogated by heparin co-therapy may be, in part, attributed to exogenous heparin affecting the bioavailability of exogenous FGF1 to the heart (see *Figures 3–5*). This finding from our data is supported by a number of studies. Ligand bioavailability at the target site of action is a significant limitation for an intravenous FGF therapeutic in acute MI because of FGF's heparin-binding properties. Free heparin/HS can trap or sequester FGFs in the blood and other extracellular spaces and inhibit FGF activity.[75–77] Most intravenously administered FGF is expected to be cleared by heparan sulfate proteoglycans (HSPG) ubiquitously expressed in all tissues/

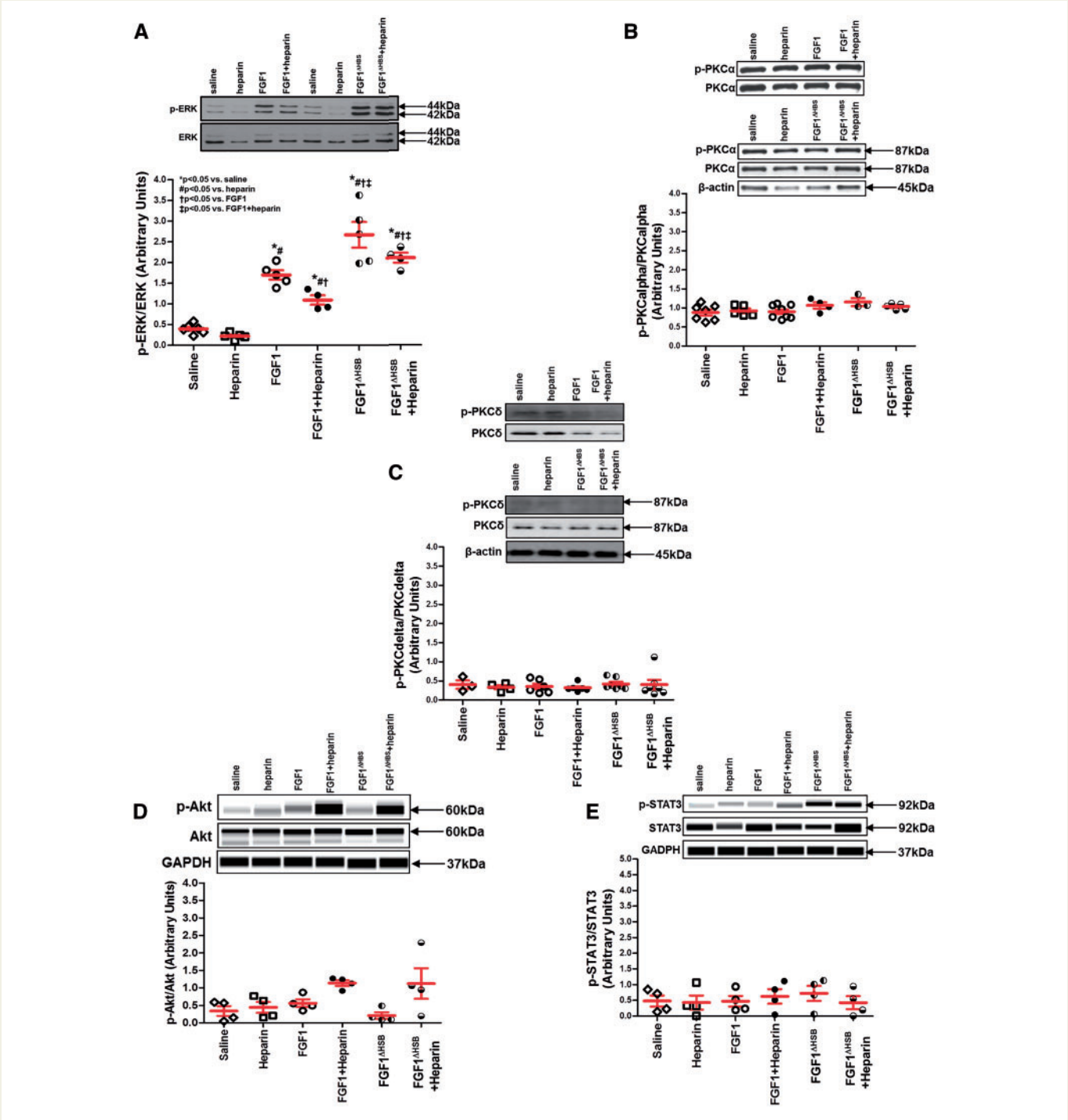

Figure 7 FGFR signaling involved in cardioprotection. The activation (i.e., phosphorylation) of cardioprotective kinases in the RISK and SAFE pathways measured in the left ventricle collected immediately post-FGF1 or post-modified FGF1 with reduced heparin binding ($FGF1^{\Delta HBS}$) administration in the presence or absence of heparin. (*A*) ERK activation. (*B*) PKCα activation. (*C*) PKCδ activation. (*D*) Akt activation. (*E*) STAT3 activation. ERK activation (panel *A*) was markedly increased in native FGF1 and $FGF1^{\Delta HBS}$, with a significantly higher level of phosphorylated ERK1/2 in the hearts from the $FGF1^{\Delta HBS}$-treated group compared to native FGF1 treatment whether in the absence or presence of heparin. PKCalpha or delta (Panel *B* and *C*) or Akt (panel *D*) or STAT3 (panel *E*) activation was not different among treatment groups evaluated. Akt and STAT3 activation (panel *D* and panel *E*, respectively) were determined via an automated capillary Western blot (WES sytem) by ProteinSimple. For ERK activation, $n = 4–6$. For PKCα activation, $n = 3–8$. For PKCδ activation, $n = 3–7$. For Akt or STAT3 activation, $n = 4$. $^*P < 0.05$. The statistical test performed for panels A-E was Kruskal-Wallis non-parametric test with Tukey's post-hoc test.

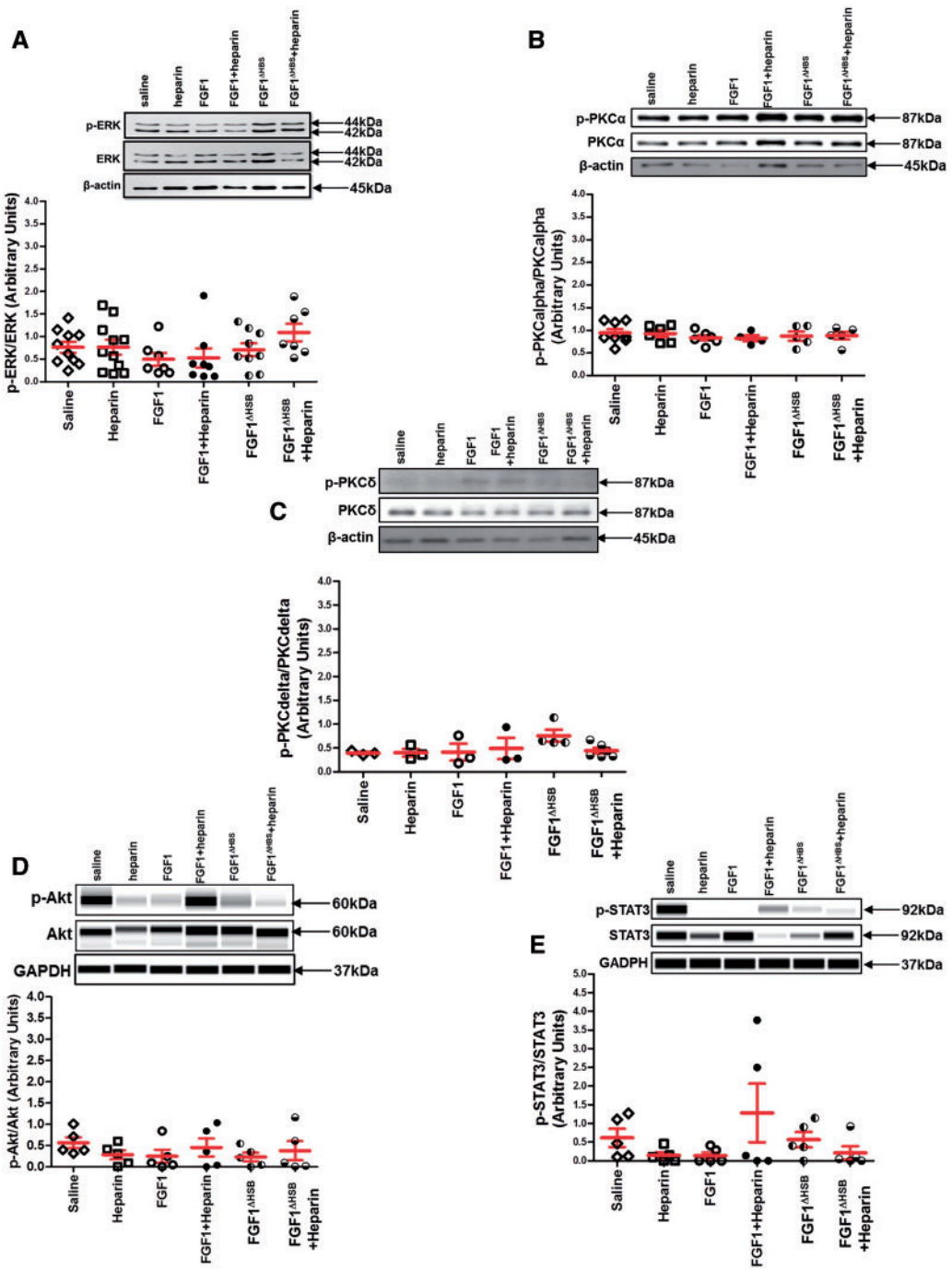

Figure 8 FGFR signaling involved in cardioprotection. The activation (i.e., phosphorylation) of cardioprotective kinases in the RISK and SAFE pathways measured in the left ventricle collected 110-min post-FGF1 or post-modified FGF1 with reduced heparin binding ($FGF1^{\Delta HBS}$) administration in the presence or absence of heparin. (*A*) ERK activation. (*B*) PKCα activation. (*C*) PKCδ activation. (*D*) Akt activation. (*E*) STAT3 activation. ERK activation (panel A), PKCalpha or delta (Panel *B* and *C*) or Akt (panel *D*) or STAT3 (panel *E*) activation was not different among treatment groups evaluated. Akt and STAT3 activation (panel *D* and panel *E*, respectively) were determined via an automated capillary Western blot (WES sytem) by ProteinSimple. For ERK activation, $n = 7–11$. For PKCα activation, $n = 4–8$. For PKCδ activation, $n = 3–6$. For Akt or STAT3 activation, $n = 5$. The statistical test performed for panels A–E was Kruskal-Wallis non-parametric test with Tukey's post-hoc test.

organs, thus limiting FGF bioavailability at cardiac tissue sites.[27] For example, the biodistribution of i.v. administered rhFGF2 in rat to liver, kidneys, and spleen and to a lesser extent, heart and lungs is a reflection of FGF-HS proteoglycan interactions.[78] Xia and investigators evaluated pharmacokinetic parameters of modified FGF1 ligands with altered protein stability or heparin-binding and demonstrated that the distribution and redistribution profiles were determined by HSPG affinity and that heparin competes with HS for binding to FGF1.[79] In fact, Hondermarck

and colleagues[30] demonstrated that increasing doses of heparin co-administered with FGF weakened the binding of FGF to blood vessels in a heparin dose-dependent manner. Furthermore, Xue and colleagues reported that exogenous heparin treatment resulted in a redistribution of the heparin–FGF1 complex from the cell surface to the medium, thus leading to reduced effectiveness of FGF1.[77] Similarly, *in vivo* evaluation of ^{99}mTc-labeled FGF1 tissue biodistribution showed that heparin prevented its binding to liver, but not kidney as well as increased FGF1 excretion.[57] Our results support some of these effects of heparin on FGF1 tissue biodistribution such that left ventricular or plasma levels of FGF1 were less in the presence of heparin, suggesting a redistribution of exogenous FGF1 from target organs and/or an increase in FGF1 excretion (although not measured in the current study), respectively, with heparin co-therapy. However, there is evidence that FGF-heparan sulfate/heparin interactions are also of benefit. HSPGs aid with the proper presentation of FGFs to FGFRs and formation of stable FGF/FGFR complexes.[25,75,80] HSPGs of the ECM can act as reservoirs for FGFs, prevent proteolytic degradation and increase local gradients of FGF during stimulation of endothelial cells.[25,75,80] For example, heparin regulated the *in vitro* activity of FGF1 on neurite outgrowth by altering its proteolytic degradation, thereby increasing its biological half-life from 7 to 39 h.[40] Since FGF1 biodistribution is not simply restricted to the organs studied, other tissue including eye, adrenal glands, and bone marrow are also potential "target organs."[78]

Site-directed mutagenesis is an important technique for altering cytokine function and affecting its efficacy and/or potency. Recently, FGFs have been modified to eliminate undesirable properties, but still keep or potentiate the beneficial actions. For example, native FGF1 was manipulated to increase its thermostability and half-life.[79,81–85] In addition, a truncated form of FGF1 has been created to remove its mitogenic activity and protect cardiomyocytes *in vitro* and *in vivo* without tumorigenesis.[7] Furthermore, protease resistant variants of FGF1 have been developed to prolong its biological activity.[79,81–85] Encouraged by these achievements, we employed a novel, modified FGF1 with reduced heparin-binding affinity (FGF1$^{\Delta HBS}$) (*Figures 1C* and *D*), which is still compatible with FGFR binding (*Figure 6*) and signaling (*Figures 7* and *8*), in the treatment of MI. In the current study, heparin had little inhibitory effect on FGF1$^{\Delta HBS}$ compared to native FGF1 as measured by the preservation of cardiac survival and muscle function (*Figures 3* and *4*, respectively). Furthermore, similar to native FGF1, FGF1$^{\Delta HBS}$ targeted largely to the heart (2282 ± 97 pg/mL vs. 3073 ± 101 pg/mL, respectively) compared to other tissue types (*Figure 5C*); however, unlike native FGF1 which in the presence of heparin co-treatment led to a re-distribution away from the heart, FGF1$^{\Delta HBS}$ even with heparin administration still directed mostly to the heart as FGF1$^{\Delta HBS}$ alone (4336 ± 775 pg/mL vs. 3073 ± 101 pg/mL, respectively) compared to other organs evaluated (see *Figure 5C* and *D*). Although FGF1$^{\Delta HBS}$ has reduced heparin/heparan sulfate-binding, it still accumulates significantly to the heart like its native form; this may most likely be due to the rat heart being highly sulfated, composed of 60% heparan sulfate.[86] This increased FGF1$^{\Delta HBS}$ sequestration to the heart most likely resulted in the enhanced ERK signaling observed (*Figure 7A*). These findings demonstrate that FGF1 ligand with reduced heparin binding (FGF1$^{\Delta HBS}$) may be a promising strategy for acute treatment of MI; yet, future studies are still needed to address its long-term effects post-MI. This is in light of previously published observations that FGF1 plus enoxaparin has been reported to promote capillary growth and increased regional myocardial blood flow one week after infarction; the reasons may be due to that heparin protects FGF1 from degradation, increases the expression of FGF2 and enhance angiogenic potential of FGF1 in the left ventricle.[29,87–89] Overall, these studies suggest that heparin plays biphasic roles in ischemia; it may reduce the acute protective effect of FGF1 in MI, but long-term, the combination of heparin + FGF1 may be of great promise for MI treatment by increasing angiogenesis activity.

The heparin-binding site of FGF is distinct from the FGF receptor (FGFR) binding site.[90, 91] The ITC data (*Figure 6A* and *B*) show that the HS-binding site mutations do not impact FGFR binding ability of FGF1$^{\Delta HBS}$ (i.e., retains normal receptor binding affinity), and confirm that the HS mutations have no adverse effect on tertiary folding of the ligand. Moreover, the *in vivo* data (*Figures 6C* and *7A*) demonstrate that the mutant FGF1 (FGF1$^{\Delta HBS}$) activates (as measured by phosphorylation status) downstream FGF signaling (e.g., ERK), providing further evidence that normal receptor binding activity is preserved *in vivo* with FGF1$^{\Delta HBS}$. Although it is well-documented that binding to heparan sulfate aids with the proper presentation of FGFs to FGFRs and formation of stable FGF/FGFR complexes and signaling,[24–26,75,80,91–94] this appears to not be the case for the cardioprotective activity of FGF1$^{\Delta HBS}$. Even in the absence of heparin binding, FGF1$^{\Delta HBS}$ activated FGFR1 and ERK signaling in the heart, possibly due to the elevated concentration of FGF1$^{\Delta HBS}$ to the heart. Supporting our finding, several studies have reported that FGF1 can interact with FGFR and trigger downstream signaling pathways even in the absence of heparan sulfate binding.[81,95–99] This enhanced affinity to the receptor, and higher ERK activation of FGF1$^{\Delta HBS}$ suggests that in conjunction with the re-distribution of FGF1$^{\Delta HBS}$ to the heart, elevated ERK signaling may be the mechanism by which FGF1$^{\Delta HBS}$ elicits a greater protection against ischemia-reperfusion injury. Surprisingly, there was no difference in activation of the other cardioprotective kinases studied (*Figures 7B–E* and *8B–E*), including PKC, Akt or STAT3 of which the latter two kinases are involved in RISK and SAFE pathways of cardioprotection and post-conditioning.[100–103] Normally, FGF1 activates FGFR which is coupled to intracellular signaling pathways including the RAS-MAPK, PI3K-Akt, PLCγ-PKC, and STAT pathways.[104–107] The stimulated receptor then recruits and activates several docking proteins containing src homology (SH-2) domains, e.g., Phospholipase C (PLC) γ and Shb, or phosphotyrosine binding domains, e.g., SHC and FRS2 (FGFR substrate 2).[108–111] The phosphorylation of the phosphotyrosine site, Y-654, is elevated in the presence of FGF1 or FGF1$^{\Delta HBS}$ (*Figure 6C*), which leads a binding complex of SHC-FRS2-GRB2-SOS-RAS-Raf-1 and activation of MAPK signaling.[112–116] In our study, FGF1$^{\Delta HBS}$ triggers increased ERK activation, but not PKC, Akt or STAT3 signaling. It is currently unknown whether this selective activation of ERK is indicative of biased agonism[117–123] or biases in the formation of heterodimer versus homodimer which can occur receptor tyrosine kinases[124, 125] and significant further study would need to occur to demonstrate this.

Taken together, the findings in this study, for the first time, demonstrate that: 1) intravenous administration of FGF1 at the onset of reperfusion protects the heart against cardiac ischemia injury, while heparin reduces the protective effect of FGF1; 2) heparin reduces the availability of FGF1 to the heart, which may be potential mechanism(s) of why heparin lessens the cardioprotective effect of FGF1, 3) novel FGF1 ligand with reduced heparin binding (FGF1$^{\Delta HBS}$) lowers infarct size and improves cardiac function in the presence of heparin, although it has a similar elimination half-life profile of native FGF1, and 4) FGF1$^{\Delta HBS}$ enhances the cardioprotective signaling (e.g., ERK activation) even in the presence of heparin to a greater extent than native FGF1, suggesting another potential mechanism of how this novel FGF1 ligand may be a promising therapeutic strategy for the treatment of myocardial infarction.

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Conflict of interest: J. Schultz and M. Mohammadi have an University of Cincinnati invention disclosure filed on "Novel FGF therapeutics for use in ischemic diseases" – 113-084 (May 2013).

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