Involvement of FrzA/sFRP-1 and the Wnt/Frizzled Pathway in Ischemic Preconditioning

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Abstract—Phosphorylation and subsequent inactivation of glycogen synthase kinase (GSK)-3β via the Akt/PI3-Kinase pathway during ischemic preconditioning (PC) has been shown to be cardioprotective. As FrzA/sFRP-1, a secreted antagonist of the Wnt/Frizzled pathway, is expressed in the heart and is able to decrease the phosphorylation of GSK-3β in vitro on vascular cells, we examined its effect during PC using transgenic mouse overexpressing FrzA in cardiomyocytes (α-MHC promoter) under a conditional transgene expression approach (tet-off system). Overexpression of FrzA inhibited the increase in GSK-3β phosphorylation as well as protein kinase C (PKC) epsilon activation in transgenic mice after PC as compared with littermates. Phospho-Akt/P-Akt, phospho-JNK, or the cytoplasmic β-catenin levels were not modified, phospho-p38 (P-p38) was slightly increased in transgenic mice after PC as compared with littermates. FrzA transgenic mice displayed a larger infarct size and a greater worsening of cardiac function compared with littermates. All these differences were reversed by the addition of doxycycline. This study demonstrates for the first time that disruption of a β-catenin independent Wnt/Frizzled pathway induces the activation of GSK-3β and reverses the benefit of preconditioning. (Circ Res. 2005;96:1299-1306.)

Key Words: animal models of human disease ■ cell signaling/signal transduction ■ genetically-altered mice ■ ischemia ■ heart

Brief episodes of ischemia/reperfusion, termed ischemic preconditioning (PC), protect the myocardium from the damage induced by subsequent and more prolonged ischemia.1 PC has proved crucial in the protection of surviving damaged myocardium. There is also evidence that it might be operative in man.2,3 Postischemic contractile dysfunction in all species tested so far. There is also evidence that it might be operative in man.2,3 The different signaling pathways involved in PC result in important roles in the protection and enhanced cell survival afforded by PC in the heart.7 Multiple pathways other than the PI3-kinase–PKB/Akt-dependent pathway regulate the activity of GSK-3β, eg, the Wnt signaling pathway. Wnt can inactivate GSK-3β through 2 distinct pathways.8,9 In the Wnt canonical pathway, phosphorylation of GSK-3β (at a residue different from Ser9) results in increased β-catenin levels and its translocation to the nucleus.10 Additionally, PKC could be involved in Wnt-induced GSK-3β inhibition by a phosphorylation at Ser9.11–15 Recent reports show that in some conditions, Ser9 phosphorylation of GSK3β potentiates Wnt canonical signaling.10,16

We are currently working on a secreted Frizzled-related protein, FrzA, which is an antagonist of the Wnt/Frizzled pathway.17–18 Bovine FrzA is related to human SARP2 (or human sFRP) and to mouse sFRP-1.19–21 We have demonstrated that high-FrzA levels may be detected during cardiac maturation and in cardiomyocytes in adulthood.17,22 We and others have shown that FrzA/sFRP-1 binds Wnt to prevent it from accessing its cell-surface receptor Frizzled, and that it blocks the canonical Wnt pathway as measured by cytosolic accumulation of β-catenin in endothelial cells (ECs).22–24 In parallel, we have recently shown that FrzA/sFRP-1 induces a constant and significant decrease in the phosphorylation of GSK-3β at Ser9 in cultured ECs, independent of the PI3K/Akt signaling pathway.24,25

Because FrzA/sFRP-1 is physiologically expressed in the heart and could activate GSK-3β through different pathways, and because GSK-3β has been demonstrated to be involved in cardioprotection during PC, we hypothesized that FrzA might play a major role in myocardial protection after PC. To test this hypothesis, we developed transgenic mice overex-

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pressing FrzA in cardiomyocytes (α-MHC promoter) using a conditional transgene expression approach (tet-off system). A mouse model of ischemia reperfusion with PC was set up. The results show that FrzA overexpression inhibits the induction of Ser9 phosphorylation of GSK-3β in cardiomyocytes independent of the Akt pathway, and compromises the protection in the heart afforded by PC.

**Materials and Methods**

**Generation of Transgenic Mice**

Two transgenic mouse lines were used. The first type, the α-MHC-tTA mouse, expressed a tetracycline-controlled transactivator (tTA) under the control of a mouse α-MHC promoter previously described20 (Jackson Laboratory, Bar Harbor, Me). The animals of generation >6 aged 2 to 3 months were used for this study. The second line, TRE-FrzA, was produced by microinjection of a construct containing the bidirectional tet-responsive promoter, which allows simultaneous expression of FrzA and LacZ. The FrzA transgene fused in the C terminus with a myc-his epitope in pCDNA3 expression vector (Invitrogen) was excised from the plasmid backbone17 with NotI and Pme I restriction enzymes and cloned in the Sall/blunt-NotI sites located between the tetO promoter and the simian virus 40 polyadenylation signal of the bidirectional tet-inducible pBl-G vector allowing coexpression of the LacZ reporter gene and FrzA mRNA. Five founder transgenic mice were obtained by pronuclear microinjections of an 8-kb Asel–Asel fragment into fertilized eggs in the Center for Transgene Technology (Bordeaux, France). Two independent-line mouse strains (lines 11 and 56) were used for phenotypic analysis. Double-transgenic mice (called α-MHC-tTA/TRE-FrzA) were obtained by crossing the TRE-FrzA mice with the α-MHC-tTA transactivator mouse strain. TRE-FrzA and α-MHC-tTA monoclonal transgenic litters were no different from wild-type mice (not shown), and TRE-FrzA monoclonal transgenic mice were used as controls and called littersmates. Only males were used for experiments.

For genotyping, genomic DNA was isolated from mouse-tail biopsies using the NucleoSpin Tissue kit according to the manufacturer’s protocol (Macherey-Nagel) and was analyzed by PCR. FrzA and tTA transgenes were routinely detected with the primers for FrzA sense 5’-TgT gTC CTC CAT gTg ACA ACG AGC-3’ and antisense 5’-TgA gAT gAg TTT TTg TTC ggg C-3’, for tTA sense 5’-gCT GTA CgC GGA CCC ACT TT 3, and antisense 5’-CTC ACT TTT gCC CTT Tag AA-3’, respectively.

**Documentation of Transgene Expression**

To assess whether FrzA expression was confined to cardiomyocytes, experiments were performed on different normal cardiac adult tissue sections on α-MHC-tTA/TRE-FrzA mice and TRE-FrzA litters. The FrzA transgene has a c-myc cassette. Immunohistochemistry was performed essentially as previously described using polyclonal anti c-myc antibody (Santa Cruz, sc-789) on paraffin-embedded sections.22 β-galactosidase expression was evaluated by histochemical staining with the chromogenic substrate X-Gal, or was evaluated by immunohistochemical staining using polyclonal antibody anti β-galactosidase (1/1000, Chemicon) as previously described.23

**Regulated Expression of Transgene**

To repress tTA-dependent transactivation and FrzA expression, doxycycline was given at 200 μg/mL in drinking water bottles containing 2.5% sucrose and wrapped with aluminum foil. The water containing doxycycline was changed every 2 days to avoid precipitation. Doxycycline was started 1 week before surgery and was maintained until euthanized. We found no adverse effects of doxycycline on the macroscopic tissue morphology when administered to mice for as long as 2 months.

**Experimental Study**

This study was conducted in accordance with both institutional guidelines and those in force in the European community for experimental animal use (L358 – 86/609/EEC). Transgenic α-MHC-tTA/TRE-FrzA and littermates males weighing 26 to 28 g were anesthetized with an intraperitoneal injection of averten (TriBromo-Ethanol, 12 μL/g of 2.5% solution).24 Mice were intubated orally with a cathelin 22 G (Johnson and Johnson) and ventilated with a rodent ventilator (Harvard apparatus) supplemented with oxygen (95%). Body temperature was maintained at 37°C by using a rectal thermometer and infrared heating lamp. Mice were installed in a supine position with paws taped to an ECG. A thoracotomy was performed in the fourth intercostal space. After retraction of the ribs, the heart was exposed and the pericardium was opened. Using a 1-mm piece of PE 10 tubing and a surgical tie (a 7/0 silk suture), the proximal left anterior descending coronary artery was occluded then left ventricular blanching and electrocardiographic ST segment elevation was used to assess myocardial ischemia.

The standard ischemia-reperfusion (IR) protocol included a 20-minute ligation before reperfusion was induced by cutting the ligature. Restoration of blood flow was attested by left ventricular hyperemia. The IR with PC protocol included 4 cycles of 5 minutes of ischemia and 5 minutes of reflow, followed by the standard IR protocol (Figure 1). The chest was closed and the pneumothorax exsufflated. Mice were allowed to recover in a temperature-controlled area.

A sham group was done for α-MHC-tTA/TRE-FrzA mice and TRE-FrzA littersmates and called nonischemic. These animals were anesthetized and then a thoracotomy was performed and a 7/0 silk suture was fixed around the proximal left anterior descending coronary artery but the artery was not occluded. No ischemia was performed. The chest was closed and sham mice were allowed to recover.

In this experimental protocol, 8 groups were studied (Figure 1). Mice in group I (α-MHC-tTA/TRE-FrzA, n=18) and group II (TRE-FrzA littersmates, n=18) were subjected to the standard ischemia-reperfusion protocol (IR). Mice in group III (α-MHC-tTA/TRE-FrzA, n=18), group IV (TRE-FrzA littersmates, n=18), group V (α-MHC-tTA/TRE-FrzA + Doxycycline, n=18), and group VI (TRE-FrzA littersmates + Doxycycline, n=18) were subjected to the PC protocol. Mice in group VII (α-MHC-tTA/TRE-FrzA, n=18) and group VIII (TRE-FrzA littersmates, n=18) were the sham groups, thoracotomized but nonischemic.

**Necropsy Examination**

**Tissue Preparation**

Animals were euthanized 2 hours after the onset of reperfusion to study signaling pathways or 24 hours later to study cardiac function and infarct size. For immunohistochemistry, hearts were fixed in methanol or in 4% paraformaldehyde and embedded in paraffin. For protein or RNA extraction, tissue samples were rinsed in PBS to remove excess blood, flash-frozen in liquid nitrogen, and stored at -80°C until used.

**Immunohistochemistry**

Seven-micron, paraffin-embedded sections cut transversely from the mouse heart were used for immunohistochemistry as previously described.25

**Western Blot Analysis**

After 2 hours of reperfusion, mice hearts were excised (n=10 in each group). The ischemic zone was dissected and kept frozen. The fragments were homogenized in ice-cold lysis buffer and subjected to SDS polyacrylamide gel electrophoresis as previously described.26 The membranes were probed with the following antibodies: anti–c-myc (Santa Cruz) was used to study transgene expression; anti–phospho-GSK-3β (Cell Signaling), anti–total-GSK-3β (Cell Signaling), anti–β-catenin (Sigma), anti–phospho-β-catenin (Cell Signaling), and anti–phospho-JNK (Cell Signaling) antibodies were used to analyze the Wnt/Frizzled pathway; anti–phospho-Akt (Cell Signaling).
The particulate (maximum and minimum dP/dt) in a closed chest preparation.

end diastolic pressure (LVEDP), and first derivative of LV pressure

heart rate (HR), left ventricle systolic pressure (LVSP), left ventricle previously reported.29 The following parameters were recorded:

the common carotid artery was isolated, and a Millar 1.4 F catheter was placed in the aorta. After midline cervical skin incision, the right

were homogenized with a Polytron in a buffer containing Tris

/ H11005

infarct size (n=8 in each group) was determined as previously

Assessment of Area at Risk and Infarct Size

Infarct size (n=8 in each group) was determined as previously described.29 run for Western blot, and normalized with α-tubulin.

Subcellular fractionation for PKCe translocation: the frozen hearts were homogenized with a Polytron in a buffer containing Tris 20 mmol/L, pH 7.4, EDTA 2 mmol/L, EGTA 2 mmol/L, Mercaptoethanol 6 mmol/L, and protease inhibitors, then centrifuged at 1000g for 10 minutes. The supernatants were centrifuged at 100 000g for 60 minutes. The resulting supernatant and pellet were the cytosolic and particulate fractions, respectively.4 The particulate fraction was resuspended in lysis buffer with 1% triton. We used an anti-PKCe antibody from Transduction Laboratory.

Statistical Analyses

Results were expressed as mean±SD. Comparisons of continuous variables between 2 groups were performed by a one-way ANOVA and subsequently, if statistical significance was observed, by a two-sided paired t test (Statview 5 to 1, Abacus). A value of P<0.05 was considered significant.

Results

Characterization of Transgenic Mice

We bred heterozygous TRE-FrzA with heterozygous α-MHC-tTA mice and observed the anticipated Mendelian inheritance pattern; ie, 24.1% of the offspring were binary transgenic α-MHC-tTA/TRE-FrzA. This result also indicated the lack of fetal toxicity related to the FrzA transgene. The size, body, and heart weight of α-MHC-tTA/TRE-FrzA mice were normal and there was no difference with littermates. This further indicated that FrzA expression did not outwardly impede fetal development or heart growth. No overt difference in tissue morphology of the different organs (including hearts) was apparent by light microscopy.

To determine that FrzA expression was selectively targeted to the heart in α-MHC-tTA/TRE-FrzA mice, different adult tissues were examined by RT-PCR, Western blot, and immunolabeling. FrzA-myc tag or β-galactosidase was never detected in tissues other than in the heart in α-MHC-tTA/TRE-FrzA mice. We verified that FrzA was not expressed in the heart of littermates and that FrzA overexpression was not indicated by light microscopy.

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This regulation was further confirmed by the lack of β-galactosidase positive cells in heart tissue 1 week after doxycycline treatment (Figure 2B). The tight suppression achieved by doxycycline on FrzA expression indicated that the chance of leakage of transgene expression is small. Finally, we tested that FrzA overexpression in α-MHC-tTA/TRE-FrzA mice was not impaired by IR or PC protocols (data not shown and Figure 2A).

**FrzA Overexpression in Cardiomyocytes Did Not Impair P-Akt Induction After PC but Increased P-p38**

Initially, we investigated whether Phosho-Akt (P-Akt) and Phospho-p38 (P-p38) were induced after PC to attest the validity of the model. P-Akt and P-p38 were detectable at very low levels in nonischemic myocardium extracts and were upregulated in tissue extracts after the PC protocol in double transgenic and littermate mice. No significant difference in P-Akt expression was observed between α-MHC-tTA/TRE-FrzA and littermate heart extracts after PC, suggesting that FrzA overexpression did not affect this pathway (Figure 3A). After PC, P-p38 level was slightly higher in α-MHC-tTA/TRE-FrzA mice than in littermates (Figure 3A). Doxycycline treatment did not impair P-Akt and P-p38 increase after PC in either α-MHC-tTA/TRE-FrzA or littermate groups. Interestingly, doxycycline treatment reversed the increase in P-p38 after PC in α-MHC-tTA/TRE-FrzA compared with littermates, demonstrating a specific effect of FrzA on the P-p38 pathway.
Frza Overexpression Blocked the PC-Induced Ser9 Phosphorylation of GSK-3β but Did Not Modify Cytosolic β-Catenin Levels

In this study, we aimed to identify the Wnt cascade as a novel signaling pathway involved in ischemic PC. As shown in Figure 3B, IR alone did not induce Ser9 phosphorylation of GSK-3β (Ser9 P-GSK-3β) in littermate compared with nonischemic littermate. However, after IR, phospho-GSK-3β appeared slightly diminished in α-MHC-tTA/TRE-FrzA mice compared with littermates. PC protocol led to a large increase in Ser9 P-GSK-3β in littermate heart extracts. In α-MHC-tTA/TRE-FrzA, overexpression of FrzA abolished the PC-induced phosphorylation of GSK-3β compared with littermate mice (Figure 3C). After PC, the Ser9 P-GSK-3β level was lower in FrzA-overexpressed heart extracts than in nonischemic heart extracts (Figure 3C). Doxycycline addition restored the level of Ser9 P-GSK-3β in α-MHC-tTA/TRE-FrzA mice similar to that in littermates (Figure 3C) after PC. These results indicate that FrzA can block P-GSK-3β increase after IR with PC.

There was an identical increase in cytosolic β-catenin levels after PC in heart extracts from α-MHC-tTA/TRE-FrzA and littermates (Figure 3C). Phosho-JNK was detectable in nonischemic heart extracts from littermate and α-MHC-tTA/TRE-FrzA mice. After PC, expression of phospho-JNK was downregulated equally in α-MHC-tTA/TRE-FrzA and littermates (Figure 3A).

Frza Overexpression Blocked the PC-Induced Translocation of PKC

The translocation of epsilon-protein kinase C (PKCε) by ischemic preconditioning has been involved as a protective event in many studies. As shown in Figure 4, preconditioning led to PKCε translocation to particulate fraction in littermate mice. Overexpression of FrzA resulted in a decrease of PKCε translocation after PC as compared with littermates. Under doxycycline conditions, PKCε isozyme was equally present in the particular fraction of α-MHC-tTA/TRE-FrzA mice compared with littermates (Figure 3B).

PC Reduced Infarct Size in Littermates but Not in α-MHC-tTA/TRE-FrzA Mice

We studied the link between the reduction of GSK-3β phosphorylation by FrzA and the cardiac protection afforded by PC.
No significant difference was observed in the area at risk in the different groups including IR and PC (Figure 5A). As demonstrated in Figure 5B, the PC protocol significantly reduced infarct size in littersmates as compared with α-MHC-tTA/TRE-FrzA. PC did not result in a significant infarct size reduction in α-MHC-tTA/TRE-FrzA mice. Doxycycline addition restored the reduction in infarct size in α-MHC-tTA/TRE-FrzA mice as in littersmates in the PC protocol (Figure 5B).

PC Improved Cardiac Performance in Littermates but Not in α-MHC-Tta/TRE-FrzA Mice

No difference was observed in heart rate or in left ventricle systolic pressures in any group (Table). Left ventricle diastolic pressures were significantly increased in α-MHC-tTA/TRE-FrzA mice compared with littersmates after Ischemia-Reperfusion with PC (Table). Before ischemia, contractile index dp/dt min and max were not different in littersmate and α-MHC-tTA/TRE-FrzA groups (Figure 5C). However, after the IR protocol, dp/dt min and dp/dt max were significantly decreased and there were no differences between the 2 groups (Figure 5C). PC improved contractile parameters in littersmates but not in the α-MHC-tTA/TRE-FrzA group. Doxycycline restored contractile parameters to a similar degree in α-MHC-tTA/TRE-FrzA mice and littersmates (Figure 5C).

Discussion

FrzA, a secreted frizzled-related protein antagonist of the Wnt/Frizzled pathway, was originally isolated in an attempt to identify genes specifically involved in the induction or maintenance of the quiescent, differentiated vascular endothelial phenotype. Our laboratory has already demonstrated that FrzA is detected at high levels during mouse cardiovascular embryogenesis. In adulthood, FrzA is detected in cardiomyocytes and in the endothelium and media of most vessels. Recently, we demonstrated an upregulation of sFRP-1/FrzA and distinct Wnt and fz member expression in the heart after myocardial infarction. Using transgenic mice overexpressing FrzA under a CMV promoter, we showed that FrzA could control the healing process after myocardial infarction. Previously we confirmed that FrzA is capable of regulating vascular cell proliferation and can induce an angiogenic response. We and other groups have recently shown that FrzA and sFRP-1 affect the phosphorylation state of GSK-3β on Ser9 in different cell types, independently of the PI3K/Akt signaling pathway. In view of recent evidence pointing to the crucial role of phosphorylation (and inactivation) of GSK-3β in ischemic PC, we sought to investigate whether the activation of GSK-3β induced by FrzA is involved in myocardial recovery after PC.

To explore the effect of FrzA in PC, we generated an inducible transgene/FrzA expression in which we targeted conditional expression of FrzA to cardiomyocytes with the use of tTA driven by the α-MHC promoter, as recently described. Binary α-MHC-tTA/TRE-FrzA mice showed a strong basal expression of FrzA restricted to the cardiomyocytes, as demonstrated by Western blot and immunohistochemistry with the use of the tag c-myc and β-galactosidase as a reporter gene in this bigenic construction. Doxycycline was shown to switch the transgene expression off with no leakage and was used as a supplementary control. Our mouse model of IR with PC was validated by the increased expression of P-Akt, P-p38, and Ser9 P-GSK-3β and by the decreased infarct size and improved cardiac functions as compared with the IR without the PC group, as previously reported.

After PC, we found that cardiac-specific overexpression of FrzA blocked the PC-induced phosphorylation of GSK-3β at Ser9, and that inhibition of FrzA overexpression by doxycycline treatment restored the phosphorylation of GSK-3β on Ser9 in binary mice. Results obtained by overexpressing FrzA were consistent with the hypothesis, ie, FrzA overexpression increased infarct size and altered cardiac function in bigenic mice compared with littersmates, while doxycycline treatment restored the cardiac protection afforded by PC. Indeed, Tong et al showed that ischemic preconditioning increases phosphorylation of GSK-3β and that this inhibition of GSK-3β is protective in classic PC. Pharmacological inhibition of GSK-3β was found by others to mimic the cardioprotective effect of PC, to improve recovery of post-ischemic function and to reduce infarct size. Moreover, dominant-negative GSK-3β transfected cells reduced apoptosis and enhanced cell survival, thus explaining why inhibition of GSK-3β may be cardioprotective. GSK-3β has already been described as playing key roles in various pathophysiological heart mechanisms such as hypertrophy or development. Because GSK-3β negatively regulates downstream signaling mechanisms, inactivation of GSK-3β (phosphorylation) removes the negative constraint due to GSK-3β and stimulates many cellular functions.

In previous studies, it has been established that FrzA may modulate GSK-3β activity by different pathways on different targets. Firstly, FrzA blocked the canonical pathway of Wnt signaling and led to activation of GSK-3β, as measured by the decrease in cytosolic accumulation of β-catenin in endo-
Thelial cells (EC) or in cardiomyocytes. Secondly, FrzA led to a constant and significant decrease in GSK-3β phosphorylation at Ser9 in EC, independently of the phosphorylation state of PKB/Akt. The demonstrated ability of FrzA or sFRP-1 to antagonize both the canonical pathway and a β-catenin independent pathway suggest that FrzA can elicit distinct cellular responses, depending on which receptors are present. In PC, we did not find any effect of FrzA overexpression on β-catenin level, suggesting that FrzA had only a modest effect on the canonical Wnt pathway in this model. Phosphorylation of GSK-3β at Ser9 residue was decreased after PC in binary mice, although we did not find any difference in Akt phosphorylation between binary and littermates after PC. These data suggest that FrzA acts on GSK-3β independently of the PI3-kinase/PKB/AKT pathway activation, as described by Tong et al. Recent reports suggest that the noncanonical Wnt pathway could lead to PKC activation and subsequent GSK-3β phosphorylation. The present experiment suggests that PKC could be a target of FrzA/sFRP-1.

Given that JNK and p38 are potential downstream targets after PC and are thought to play a role in cardioprotection and that JNK has been described as a mediator of noncanonical Wnt signaling, we analyzed JNK and p38 phosphorylation under FrzA overexpression. We found a similar decrease in JNK phosphorylation after PC in littermates and binary mice. JNK activation/phosphorylation during ischemia has been suggested by some studies but in one report, a reduction in JNK phosphorylation during ischemia was found. We found an increase in p38 phosphorylation after PC in littermates. Unexpectedly, however, the increase was significantly greater in binary animals and was reversed by doxycycline. While numerous reports point to p38 being an important signaling component in ischemic PC, further studies are required to understand how FrzA leads to p38 phosphorylation.

To avoid any concern with our previous results showing that FrzA reduce infarct size, we point out that in the previous study we used a nonreperfused model of myocardial infarction in transgenic mice overexpressing FrzA under a CMV promoter. We showed that general overexpression of FrzA interacted directly or indirectly with the different phases of infarct healing. In preliminary experiments on this nonreperfused model of myocardial infarction, we found that specific cardiomyocyte overexpression of FrzA (α-MHC-tTA/TRE-FrzA) did not result to a reduction of the infarct size. These differences could be explained by a diffuse versus a cardiomyocyte exclusive localization of FrzA expression.

We should mention that the limitations of the present study include any weaknesses associated with the use of transgenic animals and the murine model of myocardial IR.

In conclusion, our results confirm that GSK-3β plays a key role in cardioprotection after ischemic PC. They suggest for the first time that GSK-3β could be involved in PC independently of the Akt or JNK pathway. Finally, we demonstrate that the interruption of the Wnt/frizzled pathway by the secreted inhibitor FrzA plays an important role in limiting the cardioprotection afforded by PC.

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