Cardioprotective proteins upregulated in the liver in response to experimental myocardial ischemia

Shu Q. Liu,1 Brandon J. Tefft,1 Derek T. Roberts,1 Li-Qun Zhang,2 Yupeng Ren,2 Yan Chun Li,3 Yong Huang,3 Di Zhang,1 Harry R. Phillips,4 and Yu H. Wu1

1Biomedical Engineering Department, Northwestern University, Evanston, Illinois; 2Rehabilitation Institute of Chicago, Chicago, Illinois; 3Department of Medicine, Division of Biological Sciences, The University of Chicago, Chicago, Illinois; and 4Division of Cardiology, Duke University Medical Center, Durham, North Carolina

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Liu SQ, Tefft BJ, Roberts DT, Zhang LQ, Ren Y, Li YC, Huang Y, Zhang D, Phillips HR, Wu YH. Cardioprotective proteins upregulated in the liver in response to experimental myocardial ischemia. Am J Physiol Heart Circ Physiol 303: H1446–H1458, 2012. First published October 12, 2012; doi:10.1152/ajpheart.00362.2012.—Myocardial ischemia (MI) activates innate cardioprotective mechanisms, enhancing cardiomyocyte tolerance to ischemia. Here, we report a MI-activated liver-dependent mechanism for myocardial protection. In response to MI in the mouse, hepatocytes exhibited 6- to 19-fold upregulation of genes encoding secretory proteins, including α-1-acid glycoprotein (AGP)2, bone morphogenetic protein-binding endothelial regulator (BMPER), chemokine (C-X-C motif) ligand 13, fibroblast growth factor (FGF)21, neuregulin (NRG)4, proteoglycan 4, and trefoil factor (TFF)3. Five of these proteins, including AGP2, BMPER, FGF21, NRG4, and TFF3, were identified as cardioprotective proteins since suppression of a cardioprotective protein by small interfering (si)RNA-mediated gene silencing resulted in a significant increase in the fraction of myocardial infarcts (37 ± 9%, 34 ± 7%, 32 ± 8%, 39 ± 6%, and 31 ± 7%, respectively, vs. 48 ± 7% for PBS at 24 h post-MI). The serum level of the five proteins elevated significantly in association with protein upregulation in hepatocytes post-MI. Suppression of a cardioprotective protein by small interfering (si)RNA-mediated gene silencing resulted in a significant increase in the fraction of myocardial infarcts, and suppression of all five cardioprotective proteins with siRNAs further intensified myocardial infarction. While administration of a single cardioprotective protein mitigated myocardial infarction, administration of all five proteins furthered the beneficial effect, reducing myocardial infarct fractions from PBS control values from 46 ± 6% (5 days), 41 ± 5% (10 days), and 34 ± 4% (30 days) to 35 ± 5%, 28 ± 5%, and 24 ± 4%, respectively. These observations suggest that the liver contributes to cardioprotection in MI by upregulating and releasing secretory protective proteins. These proteins may be used for the development of cardioprotective agents.

myocardial infarction; myocardial protection; hepatocytes; secretory proteins; cardiohepatic interaction

MYOCARDIAL ISCHEMIA, while causing cardiomyocyte death and impairment of cardiac function, can activate innate protective mechanisms that mitigate myocardial infarction and promote myocardial repair. These mechanisms include, but are not limited to, the upregulation and release of secretory paracrine factors to stimulate cell survival and angiogenesis (4, 10, 27, 29, 34, 42, 45, 47, 53, 63–65), activation of cardiac resident stem cells to induce myocardial regeneration (32, 62), and mobilization of bone marrow cells to the ischemic myocardium to promote myocardial repair (15, 26, 31, 33, 50, 58). As cardiomyocyte death is the principal cause of myocardial functional deficits, a recognized treatment is to protect the ischemic myocardium from injury and minimize cardiomyocyte death (7, 20, 22, 23, 57, 68). Investigations on the naturally occurring mechanisms provide a foundation for development of cardioprotective strategies.

In a mammalian individual, an injury event in one organ may induce responses in other organs through environmental changes and hormone regulation. These systemic responses may be established to boost the protection of the injured organ, a mechanism critical to the survival of cell types with a limited capacity of protection, such as the cardiomyocyte. Recent observations showing that the liver responds to experimental myocardial ischemia and contributes to myocardial protection support interorgan protective mechanisms (35). Such protective mechanisms are also supported by the observations that bone marrow cells are mobilized to facilitate the repair of the ischemic myocardium (15, 26, 31, 50, 58). This interorgan protective phenotype may require coordinated actions of diverse mediators. In this investigation, we demonstrate that the liver responds to myocardial ischemia to upregulate and release secretory proteins, contributing to myocardial protection.

The present investigation stemmed from our recent discoveries that myocardial ischemia in the mouse induced hepatic cell mobilization into the circulatory system (35) and that a fraction of the mobilized hepatic cells engrafted to the lesion of myocardial infarction (36). We speculated that the mobilized hepatic cells might upregulate and release protective factors to the ischemic myocardium, mitigating cardiomyocyte injury. This speculation was supported by the observation that the administration of liver extract from donor mice with acute myocardial ischemia, but not with sham operation, rescued the injured myocardium, suggesting the presence of cardioprotective factors in the liver extract (37). To identify the cardioprotective proteins expressed by hepatic cells, we conducted a DNA microarray analysis and found that, in response to acute myocardial ischemia, hepatocytes displayed ~6- to 19-fold upregulation of genes encoding secretory proteins, including α-1-acid glycoprotein type (AGP)2, bone morphogenetic protein-binding endothelial regulator (BMPER), chemokine (C-X-C motif) ligand (CXCL)13, fibroblast growth factor (FGF)21, neuregulin (NRG)4, proteoglycan (PRG)4, and trefoil factor (TFF)3 (37). Given the observation that these proteins were upregulated in response to myocardial ischemia, they might include cardioprotective factors. The present investigation was designed to identify cardioprotective proteins from this protein population and evaluate the protective effect of the identified proteins on the ischemic myocardium in a
mouse model of coronary artery occlusion-induced myocardial ischemia.

METHODS

Coronary artery occlusion. Myocardial ischemia was induced in the mouse by occluding the left anterior descending coronary artery. C57BL/6 mice were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and ventilated via the trachea using a rodent respirator. Intercostal thoracotomy was carried out, and the left anterior descending coronary artery was occluded permanently by suture ligation immediately above the second diagonal branch (34, 37). Sex- and body weight-matched C57BL/6 mice were used as controls with sham operation using identical procedures except for coronary arterial occlusion. Observations were conducted at 1, 5, 10, and 30 days after coronary artery occlusion with a sample size of six to eight mice at each time point (the sample size was determined by power analysis). The experimental procedures used in this investigation were approved by the Institutional Animal Care and Use Committee of Northwestern University.

Measurement of myocardial infarcts. Myocardial infarcts were measured from the 2,3,5-triphenyltetrazolium chloride (TTC) assay at 1 day (37) and by the AZAN assay (71) at 5, 10, and 30 days. For the TTC assay, the heart was collected from an anesthetized mouse and cut into ~1-mm serial transverse slices. Slices were incubated in 1% TTC-PBS at 37°C for 30 min, fixed in 4% formaldehyde-PBS for 15 min, and photographed for measurements and analyses. The areas of the infarcts (white in color) and intact myocardium (red) were measured from each slice. The volumes of the infarcts and intact myocardium below the coronary artery occlusion were calculated based on the measured areas and specimen thickness. The fraction of myocardial infarcts was calculated in reference to the left ventricular (LV) wall volume below the coronary artery occlusion.

For the AZAN assay, the mouse heart was fixed by a perfusion of 4% formaldehyde-PBS at 120-mmHg pressure and cut into 50-μm serial transverse cryosections. One of every five specimen sections was collected from the LV and stained with AZAN reagents. The areas of fibrotic regions (blue in color) and intact myocardium (red) were measured from each slice. The volumes of the infarcts and intact myocardium below the coronary artery occlusion were calculated based on the measured areas and specimen thickness. The fraction of myocardial infarcts was calculated in reference to the LV wall volume below the coronary artery occlusion.

Cell death in the ischemic myocardium. A TUNEL assay was used to assess the time course of cell death, which was used to determine the time window for administering cardioprotection secretory proteins. At a selected time, the heart of an anesthetized mouse was fixed by a perfusion of 4% formaldehyde-PBS. Specimens were collected from ischemic and sham control myocardium and cut into 10-μm cryosections. The TUNEL assay was carried out as previously described (46). At least six specimen sections, equally spaced through an ischemic region, were collected from each mouse for measurements. TUNEL-positive cell nuclei were measured from six randomly selected regions of each specimen section. The TUNEL index was calculated as the percentage of TUNEL-positive cell nuclei in reference to Hoechst 33258-labeled cell nuclei.

Echocardiography. The contractile activity of the LV was measured by echocardiography. Mice were anesthetized by an intraperitoneal injection of tribromoethanol (250 mg/kg body wt at a concentration of 1.25%). Systolic and diastolic diameters of the LV were measured at the middle point of the LV using a GE LOGIQ-9 Healthcare ultrasound system with a 14-MHz high-resolution M12L matrix probe. The location of the measurement was identified by two-dimensional B-mode echocardiography. Fractional shortening of the LV was calculated as follows: [(diastolic diameter − systolic diameter)/diastolic diameter] × 100.

LV dP/dt and −dP/dt. LV dP/dt and −dP/dt were analyzed based on pressure measurements. Mice were anesthetized by an intraperitoneal injection of tribromoethanol (250 mg/kg body wt at a concentration of 1.25%). A Millar catheter pressure transducer was inserted into the LV via the right carotid artery. LV pressure, dP/dt, and −dP/dt were recorded and measured from sham control and myocardial ischemic mice.

Identification of cardioprotective secretory proteins. To identify the cardioprotective secretory proteins from the myocardial ischemia-induced secretory proteins, including AGP2, BMPER, FGF21, CXCL13, NRG4, PRG4, and TFF3, we administered the recombinant mouse form of each protein to mice immediately after myocardial ischemia (50 ng/g body wt, intravenously, single dose) and tested the fraction of myocardial infarcts at 24 h. The dose of 50 ng/g was used on the basis of dose-response tests for FGF21, which reduced the fraction of myocardial infarcts from the PBS control level from 48% ± 6% (P = 0.05), 39% ± 6% (P = 0.0457), or 32% ± 8% (P < 0.01) at doses of 12.5, 25, or 50 ng/g, respectively, at 24 h after myocardial ischemia. Recombinant mouse AGP2, NRG4, PRG4, and TFF3 were obtained from Abnova, BPER and CXCL13 were from R&D Systems, and FGF21 was from Prospector. PBS was used as a control. The fraction of myocardial infarcts was tested by the TTC assay. A secretory protein was considered cardioprotective if its administration resulted in a significant reduction in the fraction of myocardial infarcts (P < 0.05) compared with PBS administration. A combination of the identified cardioprotective proteins was also administered with the same strategy (50 ng/g each) to test and compare the cardioprotective efficacy of single and multiple proteins.

Expression of cardioprotective secretory proteins. The relative expression of the cardioprotective secretory proteins in hepatocytes was assessed by immunoprecipitation and immunoblot analysis at 0.5, 1, 3, 5, 10, 20, and 30 days after myocardial ischemia as described in previous reports (38, 39). Hepatocytes were isolated from the liver of sham control and myocardial ischemic mice by collagenase treatment and Percoll-mediated density gradient centrifugation as previously described (51). β-Actin was tested and used as a control. Antibodies for the tests were obtained from Santa Cruz Biotechnology. For each test, an equal amount of total proteins was loaded from each sample for electrophoresis. The relative expression of each protein was assessed in reference to the relative expression of the protein from a healthy mouse. Each test was repeated for three times.

Serum levels of cardioprotective secretory proteins. ELISA was carried out to measure the serum levels of ischemia-induced cardioprotective secretory proteins at 0.5, 1, 3, 5, 10, 20, and 30 days after myocardial ischemia. Serum was produced from blood samples of mice with sham operation or myocardial ischemia. The Invitrogen Amplex ELISA development kit with the Ultrared reagent was used for the test based on the manufacturer’s instruction. ELISA samples were measured using a BioTek Synergy4 plate reader. The relative serum level of each tested protein was calculated in reference to the serum level of the protein from healthy mice.

Testing the liver contribution to the serum elevation of cardioprotective secretory proteins. The liver contribution to the serum elevation of the cardioprotective secretory proteins was tested by partial hepatectomy. To introduce partial hepatectomy, an incision was made in the upper abdominal area immediately after the coronary artery ligation. The median and left lateral lobes of the liver were ligated and removed at the common pedicle, resulting in ~60% removal of the liver mass (43). The abdominal cavity was closed, and the mouse was allowed to recover. Myocardial ischemic mice with identical surgical procedures except for liver ligation/removal were used as controls. In addition, mice with sham heart and liver operation were used as sham controls. The partial hepatectomy model has been shown to induce minimal impairment of liver function, such as metabolism and detoxification (43, 56). The serum level of the cardioprotective secretory proteins was tested by ELISA as described above.
Small interfering RNA-mediated gene silencing. To evaluate the role of endogenous AGP2, BMPER, NRG4, PRG4, or TFF3 in myocardial protection, the expression of each protein was suppressed by small interfering (si)RNA-mediated gene silencing. Previous investigations (1, 8, 19, 70) have demonstrated that the majority of circulating siRNAs accumulates in the liver, inducing gene silencing in hepatocytes. We administered a single dose of AGP2, BMPER, NRG4, PRG4, or TFF3 siRNA to mice intravenously [2.5 μg/g for each siRNA (70) mixed in 100-μl siRNA transfection medium with 3% siRNA transfection reagent]. In addition, siRNAs specific to AGP2, BMPER, NRG4, PRG4, and TFF3 mRNAs were administered in combination with the same strategy as described above (2.5 μg/g for each siRNA). A scrambled siRNA that did not react with any known miRNAs was used as a control at the same dose. All siRNAs and reagents were from Santa Cruz Biotechnology. Myocardial ischemia was induced at 3 days after siRNA administration. At 1 day after myocardial ischemia or 4 days after siRNA administration, serum samples were prepared for ELISA tests of the cardioprotective secretory proteins. Hepatocytes were isolated from the liver by collagenase treatment and Percoll-mediated density gradient centrifugation to test the expression of cardioprotective secretory proteins by immunoprecipitation and immunoblot analysis. The heart was prepared to test the effect of single or multiple siRNA administration on the fraction of myocardial infarcts by the TTC assay.

Administration of recombinant proteins. To test the long-term effect of the cardioprotective secretory proteins (AGP2, BMPER, NRG4, PRG4, and TFF3) at 5, 10, and 30 days, the recombinant mouse form of these proteins in combination was administered intravenously to mice 2 times/day for 3 days with the first dose given immediately after myocardial ischemia. The 3-day dosing strategy was based on the observation that cardiac cell death occurred primarily during the early 3 days after myocardial ischemia. At a scheduled time, the mouse was used to test the contractile function of the LV, and LV specimens were prepared to test the fraction of myocardial infarcts.

Statistics. Means ± SD were calculated for each measured parameter. The two-sided Student’s t-test was used for analyzing differences between two groups, and ANOVA was used for comparisons between more than two groups. Sample size in each group was estimated by power analysis at α = 0.05 and 1 – β = 0.8. Differences were considered statistically significant at P < 0.05.

RESULTS

Identification of cardioprotective secretory proteins. Myocardial ischemia induced the upregulation of genes encoding secretory proteins, including AGP2, BMPER, CXCL13, FGF21, NRG4, PRG4, and TFF3, in hepatocyte (37). To identify cardio-
protective proteins from this population, the recombinant form of each protein was administered to mice immediately after myocardial ischemia, and the fraction of acute myocardial infarcts was measured and analyzed in reference to that with PBS administration. As shown in Fig. 1, administration of the recombinant form of individual secretory proteins resulted in various levels of cardioprotection. Five of the proteins, including AGP2, BMPER, FGF21, NRG4, and TFF3, when administered individually, significantly reduced the fraction of acute myocardial infarction. These proteins were identified as cardioprotective proteins, further tested for protein expression in hepatocytes and serum, and evaluated for long-term cardioprotective efficacy as described below.

Upregulation of cardioprotective secretory proteins. A previous investigation (37) demonstrated the upregulation of genes encoding secretory proteins in the hepatocyte in response to myocardial ischemia. Here, we tested the relative expression of cardioprotective secretory proteins, in hepatocytes and serum, and evaluated for long-term cardioprotective efficacy as described below.

A

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<th>Protein</th>
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Fig. 2. Relative levels of cardioprotective secretory proteins AGP2, BMPER, FGF21, NRG4, and TFF3 in hepatocytes and serum. A: immunoblot analyses of AGP2, BMPER, FGF21, NRG4, and TFF3 expression in hepatocytes from mice with sham operation and MI. Specimens at time 0 were prepared from healthy mice. β-Actin was used as a control. B: serum levels of AGP2, BMPER, FGF21, NRG4, and TFF3 in mice with sham operation (open bars) or MI (shaded bars) by ELISA. Data at time 0 were from healthy mice. The relative level was calculated in reference to the value at time 0. Values are means ± SD; n = 6 for each time. P values by ANOVA for comparisons between sham controls and MI were as follows: AGP2, P < 0.001 (for the first 10 days); BMPER, P < 0.001 (for the first 10 days); FGF21, P < 0.001 (for the first 5 days); NRG4, P < 0.001 (for the first 10 days); and TFF3, P < 0.01 (for the entire observation period of 30 days).
Serum elevation of cardioprotective secretory proteins. In myocardial ischemia, the upregulated secretory proteins might be released into the circulatory system to reach the ischemic myocardium for cardioprotection. Thus, we tested the serum level of these proteins by ELISA. As shown in Fig. 2B, serum levels of AGP2, BMPER, FGF21, NRG4, and TFF3 increased significantly at selected times after myocardial ischemia. The time course of change in the serum levels of these proteins was consistent with the time course of protein expression in hepatocytes, suggesting a contribution of the liver to the serum elevation of these proteins. Note that sham operation also induced an elevation in the serum levels of the secretory proteins, but the level of change was significantly lower than that in myocardial ischemia (Fig. 2B).

To demonstrate the liver contribution to the serum elevation of AGP2, BMPER, FGF21, NRG4, and TFF3, we introduced partial hepatectomy (~60% of the liver removed) to mice with myocardial ischemia, a model inducing a reduction in the expression of secretory proteins in the liver. As shown in Fig. 3, while myocardial ischemia with sham liver operation induced a significant increase in the serum levels of AGP2, BMPER, FGF21, NRG4, and TFF3, partial hepatectomy resulted in a significant drop of these proteins. These observations confirmed the contribution of the liver to the serum elevation of AGP2, BMPER, FGF21, NRG4, and TFF3.

Influence of siRNA treatment on myocardial infarction. To evaluate the role of naturally expressed cardioprotective secretory proteins in myocardial protection, we tested the fraction of myocardial infarcts when the serum levels of AGP2, BMPER, FGF21, NRG4, and/or TFF3 were suppressed by siRNA-mediated gene silencing. Administration of AGP2, BMPER, FGF21, NRG4, or TFF3 siRNA reduced protein levels by 88.4%, 78.9%, 81.4%, 77.5%, or 62.7%, respectively, in hepatocytes (Fig. 4A) at 4 days after siRNA administration or 1 day after myocardial ischemia compared with control siRNA administration. These changes were associated with a significant reduction in the serum levels of each protein, as shown by ELISA (Fig. 4B). Suppression of each cardioprotective secretory protein by siRNA treatment resulted in an increase in the fraction of acute myocardial infarcts (Fig. 5), although AGP2 or NRG4 siRNA did not cause a statistically significant change. Administration of AGP2, BMPER, FGF21, NRG4, and TFF3 siRNAs in combination further intensified myocardial infarction compared with the administration of a single siRNA (Fig. 5). These observations supported the notion that naturally expressed secretory proteins contribute to myocardial protection.

Time course of cardiac cell death. Myocardial cell death is a time-dependent process after myocardial ischemia. To protect the myocardium from death, a protective agent must be administered before cell death occurs. To establish a strategy for the administration of cardioprotective secretory proteins, we tested the time course of cardiac cell death using the TUNEL assay. As shown in Fig. 6, A and B, the rate of cardiac cell death increased rapidly after myocardial ischemia, reached a peak at ~1 day, and returned toward the control level after 3
days. These observations demonstrate that cardiac cell death occurred primarily during the early 3 days and that a cardioprotective agent should be administered to cover at least the first 3 days after myocardial ischemia.

Mitigation of myocardial infarction in response to the administration of cardioprotective secretory proteins. The aforementioned investigations provided two lines of evidence suggesting that multiple secretory proteins might be required for effective myocardial protection: 1) multiple proteins, including AGP2, BMPER, FGF21, NRG4, and TFF3, were upregulated in response to myocardial ischemia and 2) suppression of these proteins by siRNA treatment intensified myocardial infarction more than the suppression of a single protein. Thus, we tested whether the administration of AGP2, BMPER, FGF21, NRG4, and TFF3 in combination was a more effective approach to alleviate acute myocardial infarction than the administration of a single protein. As shown in Fig. 1, whereas the administration of a single protein (AGP2, BMPER, FGF21, NRG4, or TFF3) mitigated acute myocardial infarction, administration of these proteins in combination further reduced acute myocardial infarction. These observations support the necessity of administering multiple secretory proteins for effective myocardial protection.

The multiprotein approach was further used for long-term cardioprotective tests at 5, 10, and 30 days after myocardial ischemia. A dose-response test was conducted to establish a dose level for the administration of AGP2, BMPER, FGF21, NRG4, and TFF3 in combination. Based on the ratio of the ELISA-derived maximal serum protein levels, i.e., AGP2:BMPER:FGF21:NRG4:TFF3 = 1:1:1.38:1.23:1.55, three complex dose levels were established and tested for these proteins: 1) 12.5, 12.5, 17.25, 15.4, and 19.4 ng/g body wt; 2) 25, 25, 34.5, 30.8, and 38.8 ng/g; and 3) 50, 50, 69, 61.6, and 77.6 ng/g for AGP2, BMPER, FGF21, NRG4, and TFF3, respectively. For each complex dose level, the five cardioprotective secretory proteins were administered in combination immediately after myocardial ischemia (single intravenous injection). Administration of complex dose levels 1, 2, or 3 reduced the fraction of acute myocardial infarcts by 23.4% (p < 0.05), 34.1% (p < 0.01), or 41.5% (p < 0.001) of the PBS control value, respectively. Dose level 3 did not induce a further significant reduction in myocardial infarction compared with dose level 2. Thus, dose level 2 was used for long-term cardioprotective tests. Note that the cardioprotective effect of dose level 1 fluctuated more than that of the higher doses among different animals, as indicated by the larger range of SDs.

To test the long-term effects of the five cardioprotective secretory proteins, including AGP2, BMPER, FGF21, NRG4, and TFF3, a mixture of these proteins (complex dose level 2) was intravenously administered immediately after myocardial ischemia followed by an injection every 12 h for a total period of 3 days, while PBS was used as a control. The 3-day administration strategy was based on the observation that
ischemic myocardial death occurred primarily during the early 3 days (Fig. 6, A and B) and the protective proteins were no longer effective after 3 days after myocardial ischemia. As shown in Fig. 6, C and D, administration of AGP2, BMPER, FGF21, NRG4, and TFF3 in combination significantly reduced the fraction of myocardial infarcts at 5, 10, and 30 days after myocardial ischemia compared with PBS administration. These observations suggest that the administration of the ischemia-induced secretory proteins protected the ischemic myocardium from injury.

Improvement of myocardial contractile function in response to the administration of cardioprotective secretory proteins. To demonstrate the influence of cardioprotective secretory proteins on the contractile activity of the ischemic myocardium, we measured and analyzed the hemodynamic and dimensional changes of the LV. As shown in Fig. 7A, the heart beating rate of mice with myocardial ischemia was slightly increased compared with that of the sham controls, whereas administration of the five cardioprotective secretory proteins resulted in a mild decrease in the heart beating rate, but the changes were not statistically significant. The LV systolic blood pressure was slightly decreased in myocardial ischemia compared with that in sham controls, and administration of the five cardioprotective secretory proteins partially prevented this change (Fig. 7A). A significant increase was found in the end-diastolic blood pressure of the LV in myocardial ischemia. Administration of the five cardioprotective secretory proteins significantly lowered the end-diastolic blood pressure at 5, 10, and 30 days after myocardial ischemia (Fig. 7A). LV dP/dt was significantly reduced in myocardial ischemia compared with that in sham controls. Administration of the five cardioprotective secretory proteins significantly improved LV dP/dt at 5, 10, and 30 days.
after myocardial ischemia (Fig. 7, B and C). Furthermore, myocardial ischemia induced a significant reduction in the fractional shortening of the LV, as measured by echocardiography (Fig. 8). Administration of the five cardioprotective secretory proteins significantly improved the fractional shortening of the ischemic LV (Fig. 8). Taken together, these tests suggest that the administration of the ischemia-induced cardioprotective secretory proteins improved the contractile function of the ischemic myocardium.

**DISCUSSION**

**Potential impacts.** The significance of this investigation is that ischemia-induced secretory proteins, including AGP2, BMPER, FGF21, NRG4, and TFF3, contribute to cardioprotection in myocardial ischemia and that prompt administration of these proteins mitigates myocardial infarction. Several lines of evidence support the cardioprotective role of these secretory proteins. First, the secretory proteins were upregulated in response to myocardial ischemia. The mRNA levels of AGP2, BMPER, FGF21, NRG4, and TFF3 were increased by 18, 6.3, 10.9, 6.1, and 19 times, respectively, in myocardial ischemia compared with that in sham controls (37). These gene-level changes were associated with protein-level upregulation of the proteins in hepatocytes and serum, suggesting that the proteins were released into the circulatory system, an effective means for remote myocardial protection. Second, suppression of the ischemia-induced secretory proteins by siRNA-mediated gene silencing intensified myocardial infarction. The third line of evidence was that administration of ischemia-induced secretory proteins mitigated myocardial infarction and improved LV contractile function. These observations strongly suggest a cardioprotective role for ischemia-induced secretory proteins. A potential clinical impact is that these proteins may be used as therapeutic agents for the mitigation of myocardial infarction.

A fundamental question is whether it is necessary to administer the secretory proteins for myocardial protection while innate secretory proteins are upregulated in myocardial ischemia. This question can be addressed based on the expression time course of the secretory proteins in relevance to the time course of cell death after myocardial ischemia. While myocardial death occurs immediately after myocardial ischemia, the secretory proteins are not fully expressed during the early period because of the de novo process of gene expression and protein synthesis. Thus, it is necessary to administer the secretory proteins before the occurrence of myocardial death. Furthermore, as cell death lasts for ~3 days after myocardial ischemia, it is necessary to administer the secretory proteins to cover this period.

The liver is an organ known to be responsible for metabolism, detoxification, and production of proteins, cholesterol, and bile. This organ possesses a high capacity of protection and regeneration in response to mechanical and chemical injury.
Liver cells, including hepatocytes, biliary epithelial cells, endothelial cells, Kupffer cells, and Ito cells, are capable of rapid proliferation during liver regeneration (2, 14, 16, 44, 59). The results of the present investigation suggest that the liver has also evolved as an organ contributing to cardioprotection in myocardial ischemia. This discovery establishes a basis for further investigations on cardiohepatic interactions and the mechanisms of liver-mediated myocardial protection.

It should be addressed that, in response to myocardial ischemia, other organs may also express and release cardio-protective secretory proteins, which may or may not be the same as those from the liver. While it is difficult to understand why a remote organ is involved in myocardial protection, the lack of effective protection in the cardiac system may drive the development of systemic protective mechanisms. An investigation on the response of systemic organs to myocardial ischemia will provide a complete database of cardioprotective proteins. Such a database will help develop strategies for myocardial protection.

**Mechanisms of action.** The present observations raise a fundamental question: how the ischemia-induced secretory proteins protect the myocardium from ischemic injury. We hypothesize that these proteins are released into the circulatory system, act on ischemic cardiomyocytes, and activate cell survival signaling pathways, reducing the rate of cardiomyocyte injury. This question was partially addressed by using FGF21 and TFF3 as examples (40, 41). FGF21 is 1 of the 22 FGF family members (17, 49) and is primarily expressed in the liver and, to a lesser degree, in the thymus, adipose tissue (5, 49, 54), and pancreatic β-cells (66). FGF21 has been known to stimulate insulin-independent glucose uptake in adipocytes (13, 30), reduce the level of fasting blood glucose (13, 30), and regulate lipolysis in adipocytes (3, 5, 24, 25). We (40) found that transgenic mice with FGF21 overexpression exhibited...
reduced myocardial infarction compared with wild-type mice under the same ischemic conditions, supporting the cardioprotective role of FGF21. Further tests demonstrated that the administration of FGF21 to healthy wild-type mice induced the phosphorylation of FGF receptor (FGFR1), phosphoinositide 3-kinase (PI3K), Akt1, and Bcl-xL/Bcl-2-associated death promoter (BAD) in cardiomyocytes within 30 min, a signaling process known to support cell survival (11, 12). These signaling molecules were also phosphorylated in cardiomyocytes during the early period of myocardial ischemia. When FGFR1 expression was suppressed by siRNA-mediated gene silencing (direct siRNA delivery into the LV myocardium at multiple locations 3 days before myocardial ischemia), the relative phosphorylation of PI3K, Akt1, and BAD was reduced accordingly, resulting in the intensification of myocardial infarction. These observations suggest that the FGFR1-PI3K-Akt1-BAD signaling pathway potentially mediates the cardioprotective effect of FGF21.

TFF3 was first discovered in the mucus-secreting goblet cells of the gastrointestinal system (52, 55, 60, 67) and has been shown to support mucosal integrity under physiological conditions and facilitate mucosal healing after mechanical and chemical injury (55, 67). We have demonstrated that TFF3/H11002 mice exhibit a significant increase in acute myocardial infarction compared with wild-type mice. The PI3K-Akt1-BAD signaling pathway was also found to mediate the cardioprotective role of TFF3 (41), although the TFF3 receptor has not been identified in cardiomyocytes. An interesting phenomenon for myocardial ischemia-induced TFF3 expression was the presence of two peaks at 3 and 10 days after myocardial ischemia. It is conceivable that the two peaks may be related to two distinct processes. Based on the time course of myocardial injury and liver responses, the first TFF3 expression peak contributes to early myocardial protection, as demonstrated in this investigation, whereas the second peak may contribute to a later process of myocardial repair. However, the mechanisms and significance of the second peak of TFF3 expression remain to be investigated.

These FGF21 and TFF3 investigations have revealed partial mechanisms for the cardioprotective effect of secretory proteins in myocardial ischemia. Further investigations are needed to completely understand the mechanisms of FGF21- or TFF3-mediated myocardial protection and elucidate the mechanisms of action for other secretory proteins, including AGP2, BMPER, and NRG4.

Technical considerations for measuring myocardial infarcts. In this investigation, we used the coronary artery ligation model to test the cardioprotective effects of ischemia-induced secretory proteins. A critical issue is how to establish a method for reproducible measurements of myocardial infarcts. As the area of ischemia or infarcts is dependent on the location of coronary artery ligation, a consistent coronary artery ligation...
location will result in a reproducible area of ischemia or infarcts. However, it is often difficult to ligate the coronary artery at exactly the same anatomic location across different animals. Other factors, such as variations in the heart size and coronary artery anatomy, may also influence the size of myocardial infarcts. Thus, it is necessary to normalize the measured area of myocardial infarcts.

One of the normalization methods is to calculate the ratio of the measured myocardial infarct area to the ischemic area at risk, as estimated using a blood flow tracing method (6, 9, 18, 48, 69). While this method is commonly used, it is only suitable for the assessment of acute myocardial infarcts before substantial angiogenesis occurs. In this investigation, we measured and compared the degree of myocardial infarcts between the acute phase (1 day) and chronic phase of myocardial ischemia (5, 10, and 30 days). The ischemic area at risk by the blood flow tracing method was not used to normalize the area of myocardial infarcts because of new vascular formation in the ischemic area during the chronic phase. Instead, we used the volume of the LV wall below the coronary artery ligation to normalize the volume of myocardial infarcts, a method suitable for analyses at acute as well as chronic times. Although this approach takes into account the location of coronary artery ligation, heart size, and anatomy of coronary artery, it still needs improvement, as the statistical SDs of myocardial infaracts are relatively large (Figs. 1, 5, and 6).

Concluding remarks. The present investigation demonstrated an interorgan protective mechanism: liver-mediated protection of the ischemic myocardium by upregulation of secretory proteins, including AGP2, BMPER, FGF21, NRG4, and TFF3. These molecules can be released into the circulatory system to enhance myocardial resistance to ischemia. These findings may lead to the development of novel cardioprotective strategies as well as research topics on the mechanisms of myocardial protection. While this report brings forward the concept that the liver contributes to myocardial protection, there are various aspects that remain to be addressed, including the mechanisms of secretory protein upregulation and action. Inflammatory mediating factors may be involved in controlling the expression of the secretory proteins in response to myocardial ischemia, and various cell survival signaling pathways may participate in the regulation of the cardioprotective action of the secretory proteins. Genetic approaches, such as transgenic modeling and siRNA-mediated modulation, may be used to understand the role of the inflammatory and signaling molecules in the regulation of liver responses and myocardial protection. Furthermore, the liver response may be a part of a systemic process activated by myocardial ischemia. Investigations on the response of other organs to myocardial ischemia may provide additional insights into the mechanisms of myocardial protection and development of therapeutic strategies.

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DISCLOSURES

A patent application based on this work has been filed through Northwestern University.

AUTHOR CONTRIBUTIONS


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