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Cardiac Ischemic Preconditioning Promotes MG53 Secretion Through H_2O_2-Activated Protein Kinase C-δ Signaling

Dan Shan, PhD*
Sile Guo, PhD*
Hong-Kun Wu, PhD
Fengxiang Lv, PhD
Li Jin, BS
Mao Zhang, PhD
Peng Xie, BS
Yimei Wang, PhD
Ying Song, BS
Fujian Wu, PhD
Feng Lan, PhD
Xinli Hu, PhD
Chun-Mei Cao, MD, PhD
Yan Zhang, MD, PhD
Rui-Ping Xiao, MD, PhD

BACKGROUND: Ischemic heart disease is the leading cause of morbidity and mortality worldwide. Ischemic preconditioning (IPC) is the most powerful intrinsic protection against cardiac ischemia/reperfusion injury. Previous studies have shown that a multifunctional TRIM family protein, MG53 (mitsugumin 53; also called TRIM72), not only plays an essential role in IPC-mediated cardioprotection against ischemia/reperfusion injury but also ameliorates mechanical damage. In addition to its intracellular actions, as a myokine/cardiokine, MG53 can be secreted from the heart and skeletal muscle in response to metabolic stress. However, it is unknown whether IPC-mediated cardioprotection is causally related to MG53 secretion and, if so, what the underlying mechanism is.

METHODS: Using proteomic analysis in conjunction with genetic and pharmacological approaches, we examined MG53 secretion in response to IPC and explored the underlying mechanism using rodents in in vivo, isolated perfused hearts, and cultured neonatal rat ventricular cardiomyocytes. Moreover, using recombinant MG53 proteins, we investigated the potential biological function of secreted MG53 in the context of IPC and ischemia/reperfusion injury.

RESULTS: We found that IPC triggered robust MG53 secretion in rodents in vivo, perfused hearts, and cultured cardiac myocytes without causing cell membrane leakage. Mechanistically, IPC promoted MG53 secretion through H_2O_2-evoked activation of protein kinase-C-δ. Specifically, IPC-induced myocardial MG53 secretion was mediated by H_2O_2-triggered phosphorylation of protein kinase-C-δ at Y311, which is necessary and sufficient to facilitate MG53 secretion. Functionally, systemic delivery of recombinant MG53 proteins to mimic elevated circulating MG53 not only restored IPC function in MG53-deficient mice but also protected rodent hearts from ischemia/reperfusion injury even in the absence of IPC. Moreover, oxidative stress by H_2O_2 augmented MG53 secretion, and MG53 knockdown exacerbated H_2O_2-induced cell injury in human embryonic stem cell–derived cardiomyocytes, despite relatively low basal expression of MG53 in human heart.

CONCLUSIONS: We conclude that IPC and oxidative stress can trigger MG53 secretion from the heart via an H_2O_2–protein kinase-C-δ–dependent mechanism and that extracellular MG53 can participate in IPC protection against cardiac ischemia/reperfusion injury.

*Drs Shan and Guo contributed equally.

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Clinical Perspective

What Is New?

- We have provided multiple lines of evidence that MG53 (mitsugumin 53) is secreted from the heart in rodents in response to ischemic preconditioning or oxidative stress and that the secreted MG53 protects the heart against ischemia/reperfusion injury.
- Ischemic preconditioning–induced MG53 secretion is dependent on H_2O_2-evoked, Src-mediated phosphorylation of protein kinase-C-δ-Y311.
- In human heart, MG53 is expressed at a level ≈1/10 of its skeletal muscle counterpart. MG53 secretion is triggered by oxidative stress in human embryonic stem cell–derived cardiomyocytes, whereas deficiency of MG53 exacerbates oxidative injury in these cells.

What Are the Clinical Implications?

- Our in vivo and ex vivo results define secreted MG53 as an essential factor conveying ischemic preconditioning–induced cardioprotection.
- Because systemic delivery of MG53 protein restores ischemic preconditioning–mediated cardioprotection in MG53-deficient mice, recombinant human MG53 protein can be developed into a novel treatment for various diseases of human heart in which the endogenous MG53 is low.

Ischemic heart disease has become the most important cause of death around the world.[1] Because adult mammalian cardiomyocytes are terminally differentiated cells with very limited ability for proliferation, cardiomyocyte death induced by ischemic injury and other insults leads to permanent loss of the cardiac functional unit, resulting in depressed cardiac function, arrhythmia, heart failure, and sudden death. The best way to reduce cardiac ischemic injury–induced cell loss is timely restoration of coronary blood flow, that is, reperfusion. However, myocardial reperfusion leads to further damage of the heart, called ischemia/reperfusion (I/R) injury.[2–4] Ischemic preconditioning (IPC), in which nonlethal ischemic stress to the heart prevents the subsequent lethal cardiac I/R injury, is the most powerful intrinsic protection against I/R injury of the heart,[5,6] as well as other organs.[7–9] Although multiple mechanisms,[10] including alterations in metabolism,[11] mitochondrial permeability transition pore,[12,13] osmotic swelling,[14] and free radicals,[15] have been implicated in cardiac IPC, our previous studies place MG53 (mitsugumin 53; also called TRIM72) in the central position of IPC cardioprotection. Intracellular MG53 is essential for the activation of reperfusion injury salvage kinase cardioprotective signaling, thus contributing to IPC and postconditioning protection.[16,17]

The heart has both mechanical and endocrine functions, and its cells—including cardiomyocytes, fibroblasts, vascular smooth muscle cells, and endothelial cells—secrete a variety of regulatory proteins in response to alterations in myocardial oxygen or nutrient supply.[18–20] Proteins secreted from the heart, namely cardiokines, participate in pathological and physiological processes.[21–23] For example, both ANP (atrial natriuretic peptide) and BNP (brain natriuretic peptide) are secreted from the heart in response to myocardial ischemia, which, in turn, exhibit cardiac protective effects.[24–26] Recently, we demonstrated that under metabolic stress MG53 is secreted into the extracellular space and acts as a myokine/cardiokine to induce systemic insulin resistance and metabolic disorders through disrupting insulin receptor signaling.[27] However, it is unclear whether MG53 secretion is sensitive to ischemia and whether secreted MG53 is functionally similar to its intracellular counterpart, conveying cardiac protective effects. Here, we show that IPC markedly induces MG53 secretion through activating H_2O_2–protein kinase-C-δ (PKC-δ dependent signaling and that extracellular MG53 can participate in IPC and protect the heart from I/R injury.

METHODS

The data, analytical methods, and study materials are available to other researchers for purposes of reproducing the results or replicating the procedure. All materials are available in our laboratory at Peking University. Detailed methods are provided in the Data Supplement.

Animal Models

Animals were maintained in the Laboratory Animal Center (an animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care) at Peking University, Beijing, China. The animals were randomly allocated to experimental groups. Male animals were used unless otherwise noted in specific experiments. We did not use noninclusion or exclusion parameters. We did not blind investigators to treatments, but no subjective assessments were made. All procedures involving experimental animals (mice and rats) were performed following protocols approved by the Committee for Animal Research of Peking University and conformed to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication no. 86-23, revised 2011).

The generation of *mg53*$^{-/-}$[16,28] and *mg53 h-TG*[29] mice was described previously. *PKC*-δ$^{-/-}$ mice were purchased from RIKEN BRC (catalog no. 00457). Sprague-Dawley rats (8 weeks old) were purchased from Vital River Laboratories (Beijing, China).

Isolated Rodent Heart Langendorff Perfusion and Perfusate Collection

Langendorff perfusion of the heart was performed as described previously.[16,27] Briefly, adult rats (200–250 g) or mice (20–30 g) were anesthetized by intraperitoneal injection of pentobarbital (70 mg/kg). The heart was excised and

perfused on a Langendorff apparatus at a constant pressure of 55 mm Hg with Krebs-Henseleit solution (in mmol/L: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, and glucose 11.1). The buffer was continuously gassed with 95% O_2/5% CO_2 (pH 7.4) and warmed by a heating bath/circulator. Heart temperature was continuously monitored and maintained at 37±0.5°C.

Global ischemia was induced by cessation of perfusion. IPC was achieved by 4 episodes of 5 minutes of ischemia followed by 5 minutes of reperfusion. Control hearts were continuously perfused (Figure 1A and Figure IA in the Data Supplement).

The outlet perfusate was collected for different periods of time as indicated. For catalase treatment, PEG-catalase (300 U/mL for 10 minutes before IPC) was included in the perfusion solution. The collected perfusate samples were centrifuged at 3000 rpm with Amicon Ultra-15 10K Centrifugal Filter Devices (Millipore, catalog no. UFC801096) 3 times for 15 minutes. The concentrated perfusate samples were used for subsequent analysis. Nonspecific (total) bands were stained with brilliant green dye.

In Vivo Rodent Myocardial I/R and IPC Model

For the setup of experimental models of myocardial ischemia and IPC and the selection of the variables for the assessment of cardiac injury, we were referred to the previous guidelines.[30,31] In vivo rodent I/R injury was performed as previously described.[16] Specifically, Sprague-Dawley rats (200–250 g) and male *mg53*$^{-/-}$ mice (20–30 g) were anesthetized with pentobarbital (40 mg/kg IP) and ventilated through a tracheostomy on a Harvard rodent respirator. A midline sternotomy was performed, and a reversible coronary artery snare occluder was placed around the left anterior descending coronary artery. Myocardial I/R was performed by tightening the snare for 45 minutes (in rats) or 30 minutes (in mice) and then loosening it (reperfusion) for 24 hours (for protein extraction, cardiac function assessment, and infarct size measurement). Blood samples for lactate dehydrogenase (LDH) measurement were collected 10 minutes after reperfusion from rodents subjected to I/R and centrifuged for 10 minutes at 3000 rpm for serum.

For recombinant human MG53 protein (rhMG53) treatment, the rats subjected to in vivo I/R injury were treated with double intravenous injection of rhMG53/BSA (3 mg/kg, 1 injection at 5 minutes before the start of ischemia and the other at 5 minutes before the start of reperfusion); the *mg53*$^{-/-}$ mice were treated with intravenous injection of rhMG53/BSA (6 mg/kg, 40 minutes before IPC procedure).

Determination of myocardial injury by infarct size measurement and LDH release was performed as previously described.[16,32] Cardiac troponin T release was assessed by the ELISA kits from Flarebio Biotech (catalog no. CSB-E16443r for rats; and catalog no. KE1753, for mice).

IPC of the in vivo rodent hearts was induced by 4 episodes of 5 minutes of ischemia followed by 5 minutes of reperfusion. Blood samples for serum MG53 measurement were collected before IPC (0 minutes) and 5/10/30 minutes after the last ischemia from rats subjected to IPC and centrifuged for 10 minutes at 3000 rpm for serum.

Rat serum MG53 was measured with an ELISA kit from Flarebio Biotech (catalog no. CSB-EL024511RA).

Adenoviral Infection of Neonatal Rat Ventricular Myocytes

Neonatal rat ventricular cardiomyocytes (NRVMs) were isolated from 1-day-old Sprague-Dawley rats and implemented by adenovirus-mediated gene transfer by methods described previously.[16] Adenoviral vector expressing the β-gal, PKC-δ, or PKC-δ-Y311F mutation was generated and amplified by Sinogenomax. The collected medium samples were centrifuged at 4000 rpm in Amicon Ultra-4 10K Centrifugal Filter Devices (Millipore) for 20 minutes for further Western blot detection of MG53.

Western Blots

Western blots were performed as previously described.[16,27,28]

Human Donor and Criteria

Normal human ventricular tissue was obtained from the US National Institutes of Health NeuroBioBank at the University of Maryland (Baltimore).

Human Embryonic Stem Cells Induced Cardiomyocytes

Human cardiomyocytes were prepared as previously described.[33] Human embryonic stem (ES) cells H9 were differentiated into cardiomyocytes with a chemical-defined, xeno-free, small molecule–based method as previously reported. H9 cells were maintained on a 6-well plate precoated with Matrigel (BD Biosciences, catalog no. 354277) in E8 medium (basal medium, Life Technologies, catalog no. A1517001). When the cells reached 70% confluence, the medium was replaced with basal medium supplemented with 6 μmol/L CHIR99021 (Selleckchem, catalog no. S1263-25 mg). Forty-eight hours later, the medium was changed to basal medium supplied with 2 μmol/LWnt-C59 (Biorbyt, catalog no. orb181132) for another 48 hours. The cells were then maintained in basal medium, changing to fresh media every 48 hours. Beating cardiomyocytes emerged around day 8. On the 10th day, basal medium was replaced with RPMI 1640 without glucose (Life Technologies, catalog no. 11879020) to purify the cardiomyocytes. Two days later, the cardiomyocytes were associated with TrypLE (Life Technologies) for 10 minutes and passaged to a Matrigel-precoated well plate.

Materials

For Western blotting, the anti–total-PKC-δ antibody (catalog no. 610397; 1:1000) was from BD Biosciences. Anti–phospho-PKC-δ antibody (phosphorylation at Y311; catalog no. 2055; 1:1000), anti–phospho-Src antibody (phosphorylation at Y416; catalog no. 6943; 1:1000), anti-Src antibody (catalog no. 2109; 1:1000), and anti–cleaved caspase-3 antibody (catalog no. 9661; 1:1000) were from Cell Signaling Technology. Flag antibody (catalog no. F1804; 1:5000) was from Sigma-Aldrich. GAPDH antibody (catalog no. BE0023; 1:10000) was from Bioeasy Technology. A high-affinity

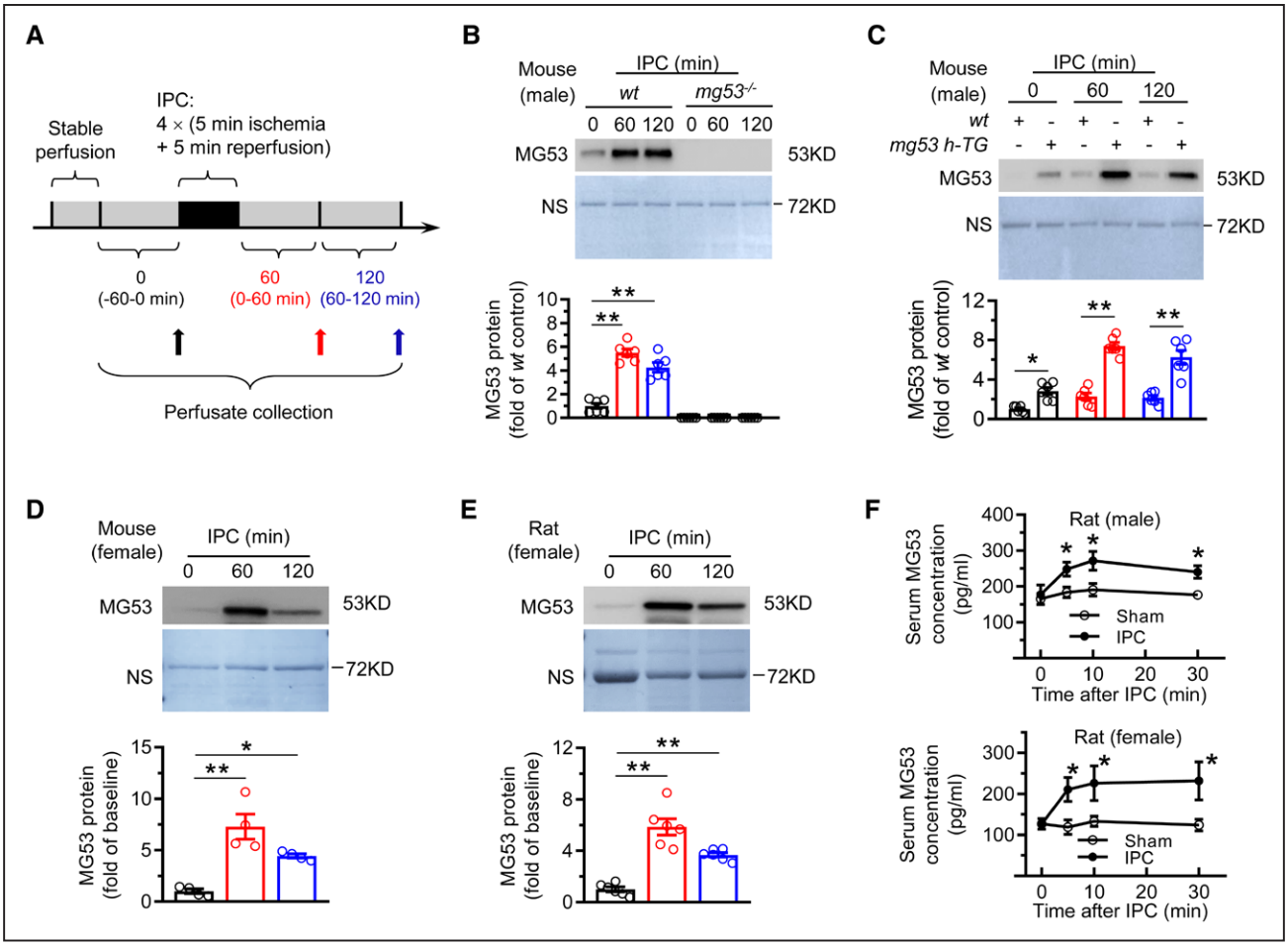

Figure 1. Cardiac ischemic preconditioning induces MG53 (mitsugumin 53) secretion.
A, Schematic diagram showing the protocol for perfusate collection from ischemic preconditioning (IPC) perfused rodent hearts. **B** and **C**, Representative Western blots and averaged data of the MG53 from the perfusate of perfused MG53-deficient (*mg53*$^{-/-}$; **B**) and MG53 heart-specific overexpression transgenic (*mg53 h-TG)* male mouse hearts (**C**), together with their respective male wild-type (*wt*) littermates, during control (–60 to 0 minutes), 0 to 60 minutes, and 60 to 120 minutes after IPC; n=6. *P<0.05, **P<0.01 as indicated. **D**, Representative Western blots and averaged data of MG53 from the perfusate of perfused female mouse hearts during control (–60 to 0 minutes), and 0 to 60 or 60 to 120 minutes after IPC; n=4. *P<0.05, **P<0.01 as indicated. **E**, Representative Western blots and averaged data of MG53 from the perfusate of perfused female rat hearts during control (–60 to 0 minutes), and 0 to 60 or 60 to 120 minutes after IPC; n=6. **P<0.01 as indicated. **F**, Averaged data of the MG53 concentrations in the serum of male and female rats subjected to sham or IPC operation in vivo (male rats: n=8 for sham, 10 for IPC; female rats: n=6). *P<0.05 vs baseline. In all bar graphs, data are normalized to the corresponding nonspecific bands (NS), which were obtained through brilliant green staining, and are presented as mean±SEM.

custom monoclonal antibody (anti-MG53; final concentration, 0.5 μg/mL) was used to assess MG53 in heart perfusate and NRVM culture medium.

H_2O_2 (catalog no. 34,988-7), PEG-catalase (catalog no. C4963), (–)–isoproterenol hydrochloride (catalog no. I6504), H-89 dihydrochloride hydrate (catalog no. B1427), phorbol 12-myristate 13-acetate (catalog no. P8139), and chelerythrine chloride (catalog no. C2932) were from Sigma-Aldrich. Protein kinase A (PKA) inhibitor 14 to 22 amide (catalog no. 476485) was from Calbiochem. Go6983 (catalog no. S2911), PP1 (catalog no. S7060), and Bosutinib (SKI-606; catalog no. S1014) were from Selleck. Brilliant green (catalog no. 5141-20-8) for staining of the nonspecific (total) bands was from Amresco.

Statistical Analysis

Data are expressed as mean±SEM. Statistical analysis was performed with GraphPad Prism version 8.0.1 (GraphPad Software, Inc) and the SPSS 18.0 software package (SPSS Inc). Data groups (2 groups) with normal distributions were compared by use of the 2-sided unpaired Student *t* test. Comparisons between multiple groups were assessed using a 1-way ANOVA with Bonferroni correction. For grouped analyses, either multiple unpaired *t* tests or 2-way ANOVA with the Tukey multiple-comparisons test was performed. Death rates of rats were compared with the χ^2 test. No statistical method was used to predetermine sample size.

RESULTS

Cardiac IPC Induces MG53 Secretion

To identify IPC-sensitive myocardial secreted proteins, we performed IPC (4 episodes of 5-minute ischemia followed by 5-minute reperfusion) in isolated Langendorff-perfused rat hearts. The perfusate of IPC was

collected during 0 to 30 minutes after the last episode of ischemia; the control perfusate was collected from the heart without IPC during the same period (Figure IA in the Data Supplement). After concentration, the perfusate samples were subjected to SDS-PAGE and stained with Coomassie blue. Compared with the control group, the IPC group was associated with an increase in the density of a band of ≈55 kDa (Figure IB in the Data Supplement), which was subjected to mass spectrum analysis. The repeated short-term I/R cycles of IPC did not cause cardiac injury as indexed by unaltered LDH concentration in the perfusate (Figure IIA in the Data Supplement).

In 3 independent experiments, 91 proteins with a high score (>80 defined as positive) were identified by at least 1 experiment, and 22 of them were positive in at least 2 experiments as listed in Table I in the Data Supplement (see also Figure IC in the Data Supplement). In particular, 5 proteins were positive in all of the 3 experiments (Figure IC and ID in the Data Supplement), and among them, MG53, which was previously demonstrated to mediate cardiac IPC protection intracellularly[16] and identified as a myokine/cardiokine,[27] was selected for further analysis.

Consistent with the mass spectrum data from isolated rat hearts, IPC profoundly increased perfusate MG53 levels in Langendorff-perfused male wild-type mouse hearts by 5.50±0.32- and 4.26±0.40-fold from baseline over 0 to 60 and 60 to 120 minutes of reperfusion, respectively (Figure 1A and 1B), without inducing myocardial membrane leakage as indexed by LDH and cardiac troponin T concentration (Figure IIB in the Data Supplement). The identity of the detected band in the perfusate was verified with the mice lacking MG53 (*mg53*$^{-/-}$)[16,28] or mice with cardiac-specific overexpression of MG53 (*mg53 h-TG*).[29] IPC could not induce MG53 release in hearts from *mg53*$^{-/-}$ mice, whereas IPC-induced MG53 release was markedly augmented in the hearts of *mg53 h-TG* mice (Figure 1B and 1C) in the absence of obvious cardiac injury (Figure IIC and IID in the Data Supplement). IPC-induced MG53 secretion was also observed in the isolated hearts of female mice (Figure 1D) and rats (Figure 1E). IPC was able to increase serum MG53 concentration in both male and female rats in vivo compared with the sham group (Figure 1F) without altering serum LDH and cardiac troponin T level (Figure IIE and IIF in the Data Supplement). These data indicate that IPC can induce MG53 release from the heart in both in vivo and ex vivo models without causing cardiac damage.

H_2O_2 Is Required for IPC-Induced MG53 Secretion

Next, we aimed to determine the mechanism underlying the IPC-induced release of MG53. Cardiac reactive oxidative species (ROS) has been implicated in IPC signaling.[34,35] H_2O_2, a key component of ROS, was increased in the IPC group compared with the control group (Figure 2A). Pretreatment of PEG-catalase, an H_2O_2 scavenger, prevented IPC-induced MG53 release in perfused rat hearts (Figure 2B). In cultured cardiomyocytes, we used repetitive short-term hypoxia/reoxygenation cycles, that is, hypoxia preconditioning, to mimic IPC in the absence of cardiomyocyte injury (Figure IIG in the Data Supplement). Similar to IPC, hypoxia preconditioning in NRVMs also augmented MG53 abundance in the culture medium, which was abolished by PEG-catalase pretreatment (Figure 2C). It is notable that short-term pretreatment (10 minutes) of NRVMs with H_2O_2 at concentrations ranging from 100 to 200 μmol/L protected cardiomyocytes against hypoxia/reoxygenation–induced injury, whereas prolonged treatment of H_2O_2 (24 hours) triggered cell death even at a concentration as low as 50 μmol/L (Figure IIH and III in the Data Supplement), indicating that a short exposure of cardiomyocytes to H_2O_2 is protective and sustained H_2O_2 treatment of cells is detrimental. Short-term treatment of H_2O_2 (100 μmol/L) not only protected cultured NRVMs but also induced robust MG53 release in a dose- and time-dependent manner (Figure 2D and 2E), which was blocked by PEG-catalase pretreatment (Figure 2F). These results strongly suggest that H_2O_2 is both necessary and sufficient for IPC-mediated MG53 release in the heart.

PKC, But Not PKA, Is Required for IPC-/H_2O_2–Induced MG53 Secretion

Previous studies have demonstrated that a wide range of serine/threonine and tyrosine protein kinases regulate cell secretion, among which PKA and PKC are the most important.[36,37] Therefore, we next determined which protein kinase is responsible for MG53 secretion. Activation of cAMP-dependent PKA signaling by β-adrenergic stimulation with isoproterenol (at 10–100 μmol/L) did not elicit secretion of MG53, whereas H_2O_2 (100 μmol/L) induced profound MG53 secretion in cultured NRVMs (Figure 3A). In addition, inhibition of PKA with either a peptide inhibitor, PKA inhibitor 14 to 22 amide, or small-molecule inhibitor, H-89 dihydrochloride hydrate, did not affect H_2O_2-induced MG53 secretion (Figure 3B and 3C), suggesting that PKA is not involved in the regulation of MG53 secretion. In sharp contrast, PKC activation by phorbol 12-myristate 13-acetate overtly increased H_2O_2-induced MG53 secretion in a dose-dependent manner (Figure 3D), whereas inhibition of PKC with either chelerythrine chloride or Go6983 largely blocked both H_2O_2- and hypoxia preconditioning–induced MG53 secretion (Figure 3E through 3G). Thus, MG53 secretion from cardiomyocytes is dependent on activation of PKC rather than PKA.

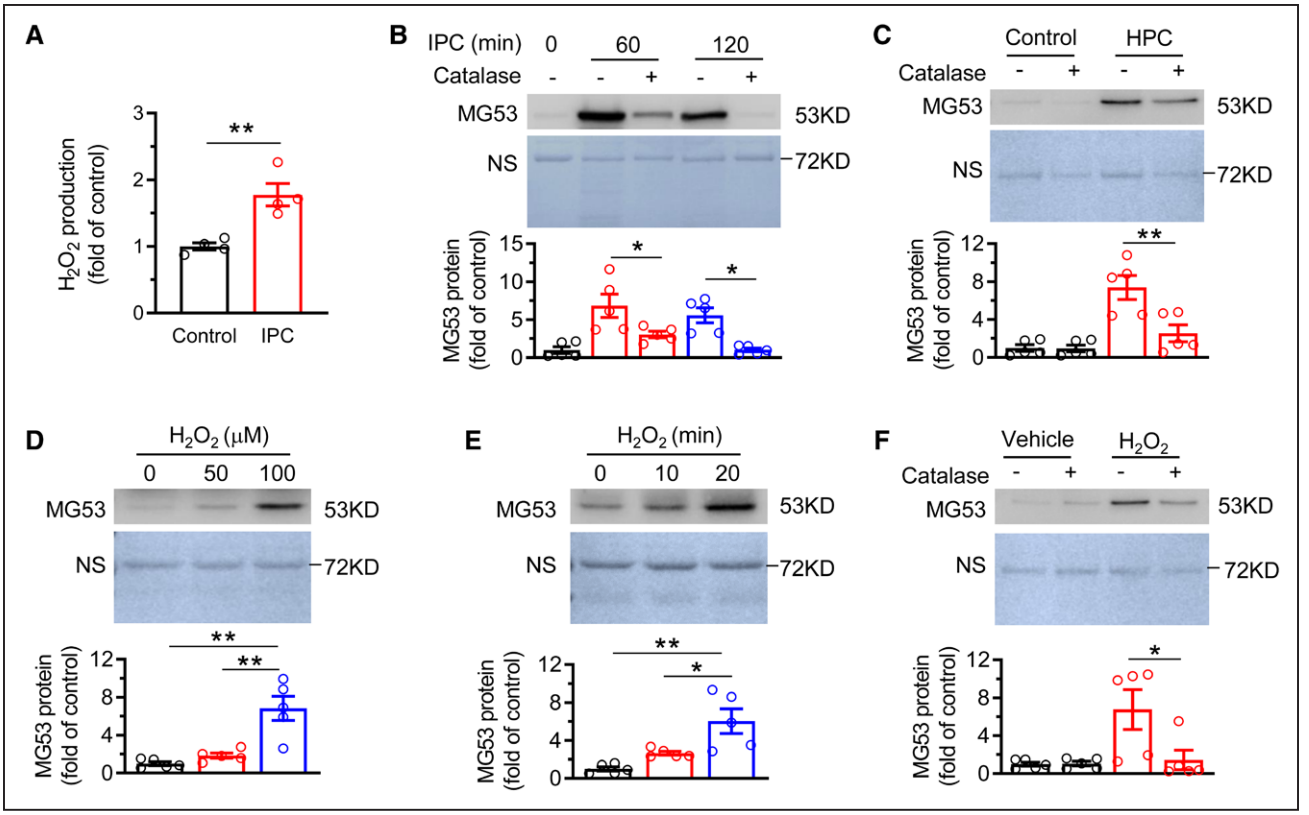

Figure 2. H_2O_2 is required for ischemic preconditioning (IPC)–induced MG53 (mitsugumin 53) secretion.
A, Average data of H_2O_2 production in perfused rat hearts with or without IPC, ex vivo; n=4. ***P*<0.01 as indicated. **B**, Representative Western blots and averaged data of MG53 from the perfusate from perfused rat hearts during control (–60 to 0 minutes), 0 to 60 minutes, and 60 to 120 minutes after IPC with or without PEG-catalase pretreatment (300 U/mL for 10 minutes before IPC); n=5. **P*<0.05 as indicated. **C**, Representative Western blots and averaged data of MG53 from the culture medium of neonatal rat ventricular cardiomyocytes (NRVMs) with hypoxia preconditioning (30 minutes of hypoxia followed by 30 minutes of reoxygenation, 3 cycles) treatment; n=5. ***P*<0.01 as indicated. **D** and **E**, Representative Western blots and averaged data of MG53 from the culture medium of NRVMs with H_2O_2 treatment at different dosages and time points; n=5. **P*<0.05, ***P*<0.01 as indicated. **F**, Representative Western blots and averaged data of MG53 from the culture medium of NRVMs with H_2O_2 (100 μmol/L for 20 minutes) treatment with or without PEG-catalase pretreatment (500 U/mL); n=5. **P*<0.05, as indicated. For **B** through **F**, data are normalized to the corresponding nonspecific bands (NS), which were obtained through brilliant green staining, and are presented as mean±SEM.

IPC Triggers MG53 Secretion Through H_2O_2-Activated PKC-δ Signaling

The PKC family consists of ≈10 members,[38] and among them, PKC-δ has been identified as one of the most important mediators of cardiac IPC protection.[39] In addition, previous studies have reported that PKC-δ can be activated by ROS-related signaling.[40,41] It is notable that we showed that downregulation of PKC-δ with siRNA abolished H_2O_2-induced MG53 secretion in NRVMs (Figure 4A and 4B). Concomitantly, deficiency of PKC-δ markedly attenuated IPC-induced MG53 secretion in perfused mouse hearts lacking PKC-δ (*PKC*-δ$^{-/-}$; Figure 4C and Figure III in the Data Supplement), substantiating the important role of PKC-δ in IPC-induced MG53 secretion. In contrast, adenoviral gene transfer of PKC-δ exaggerated H_2O_2-induced MG53 secretion in NRVMs (Figure 4D and 4E). Furthermore, overexpression of PKC-δ in vivo with coronary artery delivery of adenovirus also significantly enhanced IPC-induced MG53 secretion in perfused rat hearts (Figure 4F and Figure IV in the Data Supplement).

Because phosphorylation of PKC-δ at Y311, a highly conserved residue among species (Figure V in the Data Supplement), is involved in ROS-dependent PKC-δ activation,[42,43] we next examined whether IPC-triggered MG53 secretion is related to ROS-induced phosphorylation of PKC-δ Y311. In cultured NRVMs, phosphorylation of PKC-δ Y311 was elevated in response to H_2O_2 treatment, which was attenuated by pretreatment of cells with PEG-catalase (Figure 4G). More important, IPC was able to increase PKC-δ Y311 phosphorylation in rat hearts, implying a crucial role of PKC-δ Y311 phosphorylation in IPC-induced MG53 secretion (Figure 4H). This possibility was further confirmed by the fact that enforced expression of the PKC-δ-Y311F mutant failed to enhance H_2O_2-induced MG53 secretion (Figure 4I). Furthermore, treatment of cells with either PP1 or bosutinib, Src kinase inhibitors,[42] fully blocks H_2O_2-induced PKC-δ Y311 phosphorylation in NRVMs (Figure VI in the Data Supplement). As for the intracellular distribution of phosphorylated PKC-δ Y311, we found that under basal conditions phosphorylated PKC-δ Y311 is localized mainly in the cytosol but

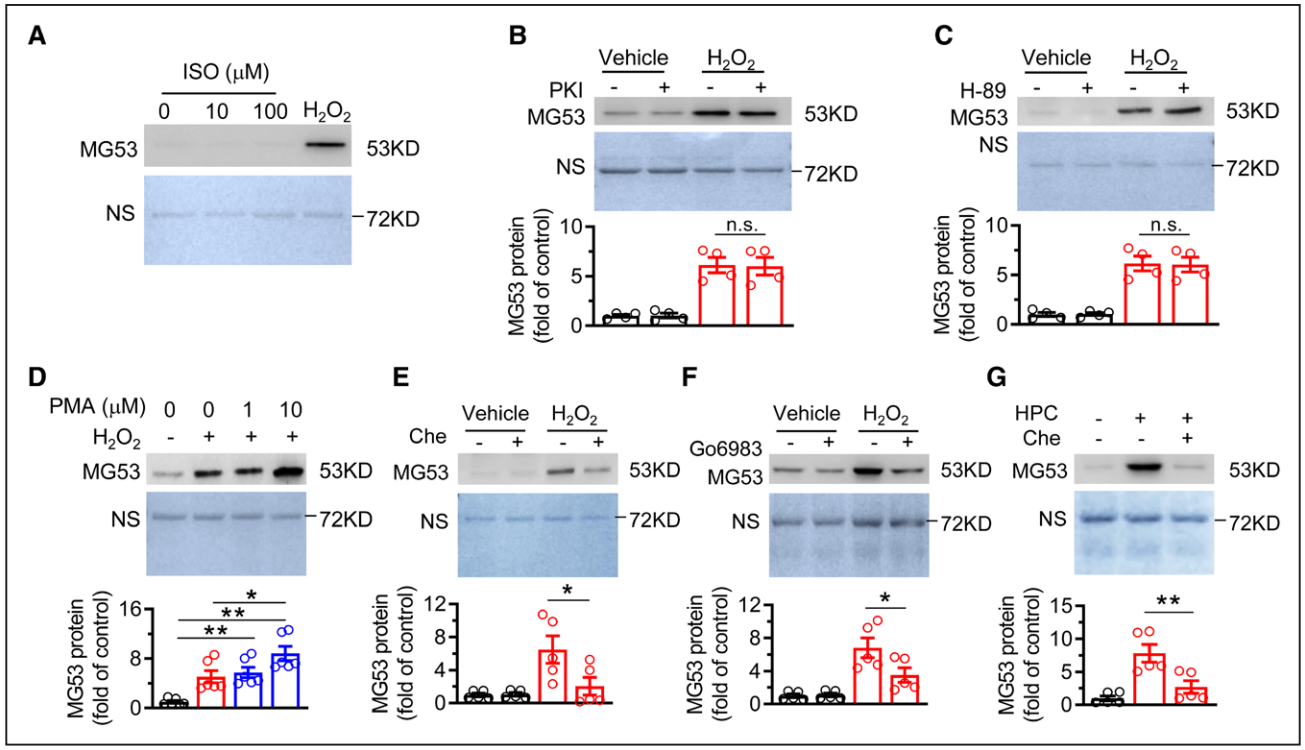

Figure 3. Protein kinase-C (PKC), but not protein kinase-A (PKA), is required for ischemic preconditioning–/H_2O_2-induced MG53 (mitsugumin 53) secretion.

A, Representative Western blots of MG53 from the culture medium of neonatal rat ventricular cardiomyocytes (NRVMs) treated with different dosages of isoproterenol (ISO) for 1 hour. The medium from NRVMs treated with H_2O_2 (100 μmol/L for 20 minutes) was used as positive control. **B** and **C**, Representative Western blots and averaged data of MG53 from the culture medium of NRVMs with H_2O_2 (100 μmol/L for 20 minutes) with or without PKA inhibitor 14 to 22 amide (PKI; 5 μmol/L for 6 hours, n=4; **B**) or H-89 dihydrochloride hydrate (H-89; 1 μmol/L for 6 hours, n=4; **C**) pretreatment. **D**, Representative Western blots and averaged data of MG53 from the culture medium of NRVMs with H_2O_2 (100 μmol/L for 20 minutes) with or without phorbol 12-myristate 13-acetate (PMA; 1 hour) pretreatment; n=6. *P<0.05, **P<0.01 as indicated. **E** and **F**, Representative Western blots and averaged data of MG53 from the culture medium of NRVMs with H_2O_2 (100 μmol/L for 20 minutes) with or without chelerythrine chloride (Che; 10 μmol/L for 1 hour, n=5; **E**) or Go6983 (100 nmol/L for 1 hour, n=5; **F**) pretreatment. *P<0.05 as indicated. **G**, Representative Western blots and averaged data of MG53 from the culture medium of NRVMs with hypoxia preconditioning (HPC; 30 minutes of hypoxia followed by 30 minutes of reoxygenation, 3 cycles) treatment with or without Che pretreatment (10 μmol/L for 1 hour before HPC); n=5. **P<0.01 as indicated. For **B** through **G**, data are normalized to the corresponding nonspecific bands (NS), which were obtained through brilliant green staining, and are presented as mean±SEM. n.s. Indicates not significant.

translocated to the plasma membrane and mitochondria compartment in response to H_2O_2 treatment in NRVMs (Figure VII in the Data Supplement), consistent with the previous notion.[44] Taken together, our in vitro and in vivo data indicate that IPC triggers MG53 secretion by H_2O_2-evoked, Src-mediated PKC-δ phosphorylation at Y311.

Cardiac I/R Injury Is Alleviated by Systemic Administration of Recombinant MG53 Protein in Rats

Next, we aimed to investigate the role of the secreted MG53 from cardiomyocytes. Because it is well established that IPC is the most powerful intrinsic protective mechanism against cardiac I/R injury[6] and that intracellular MG53 protects the heart through both membrane repair[45] and activation of prosurvival pathways,[16,17] we hypothesized that secreted MG53 may exert a cardioprotective effect and act as an effector of IPC protection. To test our hypothesis, we evaluated the potential cardiac protective effect of systemic delivery of rhMG53 (Figure VIII in the Data Supplement) in male rats subjected to I/R injury (Figure 5A). Treatment of rhMG53 overtly decreased the infarct size (infarct size/area at risk) induced by I/R compared with the BSA-treated control group (11.9±1.8% versus 27.3±2.0%; P<0.01) without altering the area at risk/left ventricle ratio (Figure 5B and 5C). The in vivo administration of rhMG53 reduced the mortality from 44.7% (17 of 38 animals) to 5.3% (1 of 19 animals) in rats, suggesting that elevation of blood MG53 concentration can markedly ameliorate I/R-induced cardiac injury and animal death (Figure 5D). Cardioprotection by rhMG53 was also evidenced by decreased blood LDH concentration, reduced numbers of terminal deoxynucleotidyl transferase dUTP nick-end labeling–positive cardiac cells, and restored cardiac function in the rhMG53 group relative to the control (Figure 5E through 5G). Similarly, rhMG53 treatment effectively protected female rats from cardiac I/R injury (Figure IX in the Data Supplement).

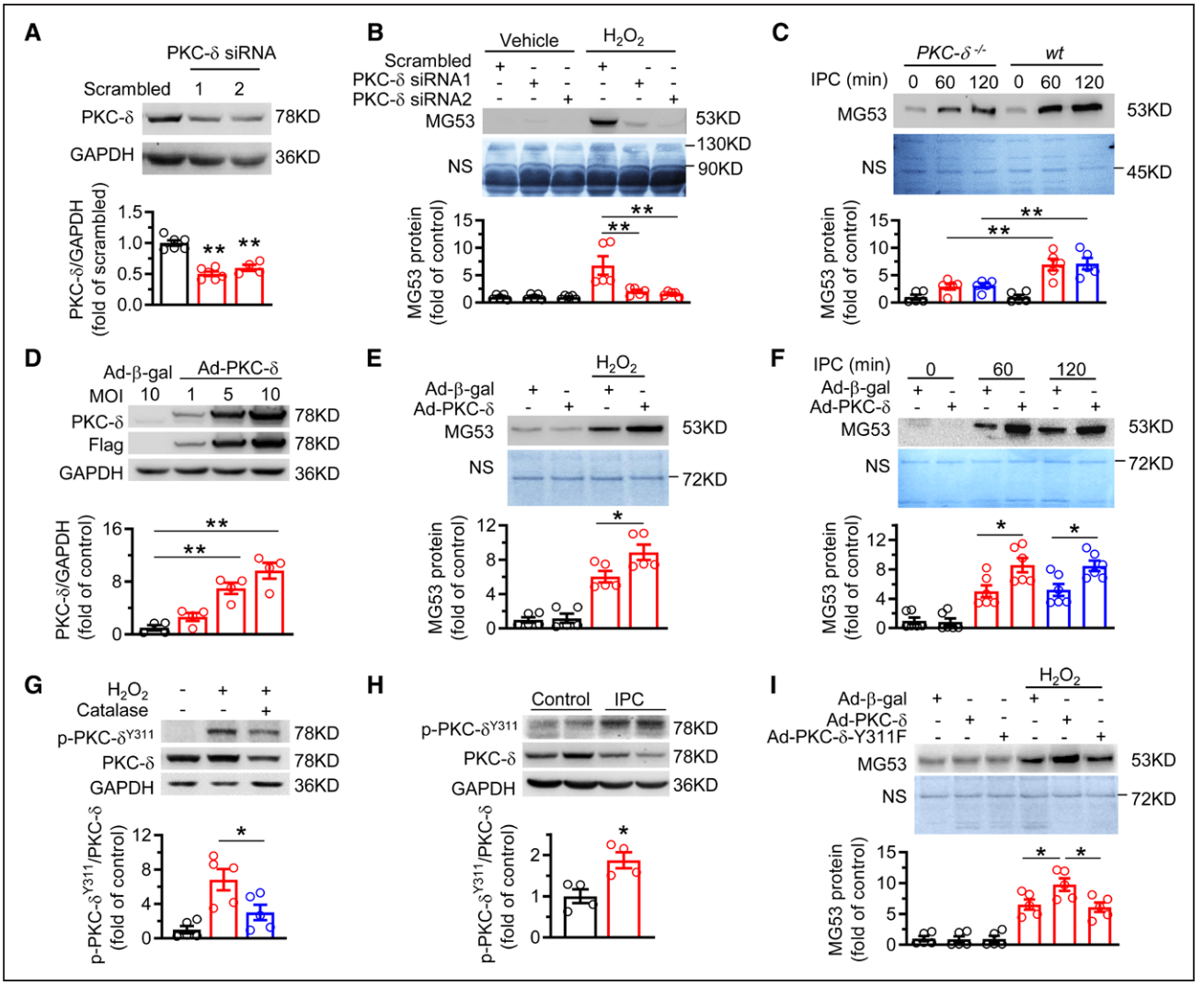

Figure 4. Ischemic preconditioning (IPC) triggers MG53 (mitsugumin 53) secretion through H_2O_2-activated protein kinase-C-δ (PKC-δ) signaling. **A**, Representative Western blots and averaged data of PKC-δ from cultured neonatal rat ventricular cardiomyocytes (NRVMs) transfected with scrambled or 2 sets of PKC-δ siRNAs; n=4 for siRNA2, 6 for Scrambled and siRNA1. ***P*<0.01 vs scrambled group. **B**, Representative Western blots and averaged data of MG53 from the culture medium of NRVMs with H_2O_2 treatment (100 μmol/L for 20 minutes) transfected with scrambled or two sets of PKC-δ siRNAs; n=5. ***P*<0.01 as indicated. **C**, Representative Western blots and averaged data of MG53 from the perfusate from perfused hearts of wild-type (*wt*) and *PKC-δ*$^{-/-}$ mice during control (–60 to 0 minutes), 0 to 60 minutes, and 60 to 120 minutes after IPC; n=5. ***P*<0.01 as indicated. **D**, Representative Western blots and averaged data of adenovirus (Ad)-PKC-δ overexpression in NRVMs; n=4. ***P*<0.01 as indicated. **E**, Representative Western blots and averaged data of MG53 from the culture medium of NRVMs with or without H_2O_2 treatment (100 μmol/L for 20 minutes) in the presence or absence of Ad-PKC-δ infection; n=5. **P*<0.05 as indicated. **F**, Representative Western blots and averaged data of MG53 from the perfusate from perfused rat hearts during control (–60 to 0), 0 to 60 minutes, and 60 to 120 minutes after IPC with or without PKC-δ overexpression (coronary delivery of Ad-PKC-δ); n=6. **P*<0.05 as indicated. **G**, Representative Western blots and averaged data of phosphorylated Y311 site of PKC-δ and total PKC-δ from cultured NRVMs with or without H_2O_2 treatment (100 μmol/L for 20 minutes) in the presence or absence of PEG-catalase pretreatment; n=5. **P*<0.05 as indicated. **H**, Representative Western blots and averaged data of p-PKC-δ^{Y311} and total PKC-δ from perfused rat hearts with or without IPC (10 minutes after the last reperfusion); n=4. **P*<0.05 vs control group. **I**, Representative Western blots and averaged data of MG53 from the culture medium of NRVMs infected with Ad-β-gal, Ad-PKC-δ or Ad-PKC-δ-Y311F mutant with or without H_2O_2 treatment (100 μmol/L for 20 minutes); n=5. **P*<0.05 as indicated. For **A** through **I**, data are presented as mean±SEM. For **B**, **C**, **E**, **F**, and **I**, data are normalized to the corresponding nonspecific bands (NS), which were obtained through brilliant green staining.

Extracellular MG53 Restores IPC Cardioprotection in *mg53*$^{-/-}$ Mice

Previous studies have shown that IPC cardioprotection is totally abolished in *mg53*$^{-/-}$ mouse hearts.[16] Therefore, we sought to determine whether delivery of rhMG53 can restore IPC protection in the MG53 knockout mice. MG53 was detected in the hearts of *mg53*$^{-/-}$ mice 1 hour after intravenous injection of rhMG53 (Figure 6A and 6B), suggesting that extracellular delivery of MG53 protein can be deposited to the hearts. IPC did not exert any cardioprotective effects in *mg53*$^{-/-}$ mice treated with BSA, consistent with the previous reports.[16] Although pretreatment with rhMG53 alleviated cardiac I/R injury in *mg53*$^{-/-}$ mice subjected to I/R, IPC combined with rhMG53 pretreatment further reduced I/R-induced myocardial infarction, cardiomyocyte death, and cardiac dysfunction (Figure 6C through 6E), indicating that

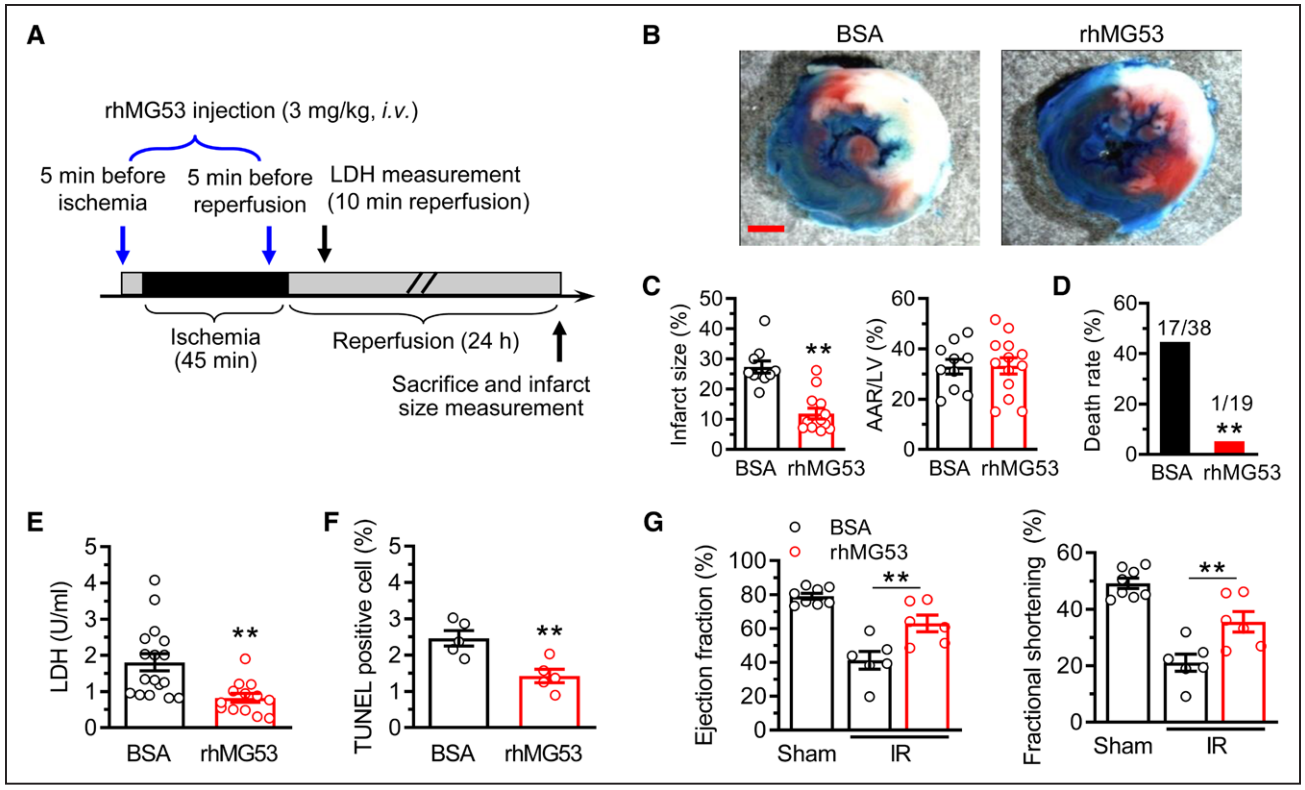

Figure 5. Cardiac ischemia/reperfusion (I/R) injury is alleviated by systemic administration of recombinant MG53 (mitsugumin 53) protein in male rats.

A, Schematic diagram showing the protocol for recombinant human MG53 protein (rhMG53) protection against cardiac I/R injury in vivo. **B** and **C**, Representative photos (**B**) and averaged data (**C**) of the infarct size and area at risk (AAR) of the rats subjected to cardiac I/R injury with BSA or rhMG53 protein treatment in vivo; n=10 for BSA, 13 for rhMG53. ***P*<0.01 as indicated. Scale bar is 2 mm. **D** through **F**, Death rates (**D**), serum lactate dehydrogenase (LDH) concentration (n=17 for BSA, 13 for rhMG53; **E**), and cardiomyocyte apoptosis detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining (n=5; **F**) of the rats subjected to cardiac I/R injury with BSA or rhMG53 protein treatment in vivo. **G**, Cardiac function accessed by echocardiography in male rats subjected to cardiac sham or I/R injury with BSA or recombinant MG53 protein treatment; n=8 for sham, 6 for BSA and rhMG53. ***P*<0.01 as indicated. For **C** through **G**, data are presented as mean±SEM. In **D**, the numbers above the bar indicate the number of the animals that died during ischemia and reperfusion/the number of the animals subjected to surgery. LV indicates left ventricle.

the presence of extracellular MG53 is able to restore the cardioprotection of IPC in *mg53*$^{-/-}$ mice and that extracellular MG53, similar to its intracellular species, participates in IPC-mediated myocardial protection.

MG53 Is Present in Human Heart and Enables the Heart to Resist Oxidative Injury

Although previous studies have reported that there is little or no MG53 present in human heart,[46] using a highly specific MG53 monoclonal antibody, we detected low expression levels of MG53 in human myocardial tissue (Figure 7A and Figure X in the Data Supplement). The identity of the band recognized by the MG53 antibody was verified by mass spectrum analysis, in which multiple peptides of MG53 were detected (Figure 7B). Regardless of its low expression levels in human heart, knockdown of MG53 with siRNA exaggerated H_2O_2-induced death of cardiomyocytes differentiated from human ES cells (Figure 7C and 7D), suggesting that MG53 may be required for the human heart to resist oxidative stress. Similar to NRVMs, the secretion of MG53 was induced in human ES cell–derived cardiomyocytes in response to short-term, low-dose H_2O_2 stimulation in the absence of a change in LDH concentration in the culture medium (Figure 7E and Figure XI in the Data Supplement). Last, rhMG53 treatment ameliorated H_2O_2-induced damage in human ES cell–derived cardiomyocytes (Figure 7F), further underscoring the potentially important biological function of MG53 in human heart.

DISCUSSION

Although numerous cardiokines have been identified and characterized over the past 3 decades, here we have provided multiple lines of evidence to demonstrate that the most powerful intrinsic means of cardioprotection, IPC, elicits MG53 secretion via H_2O_2-evoked PKC-δ signaling, which, in turn, participates in IPC-mediated cardioprotection. First, we show that IPC-induced MG53 secretion is accompanied by increased H_2O_2 production and PKC-δ activation in rodents in

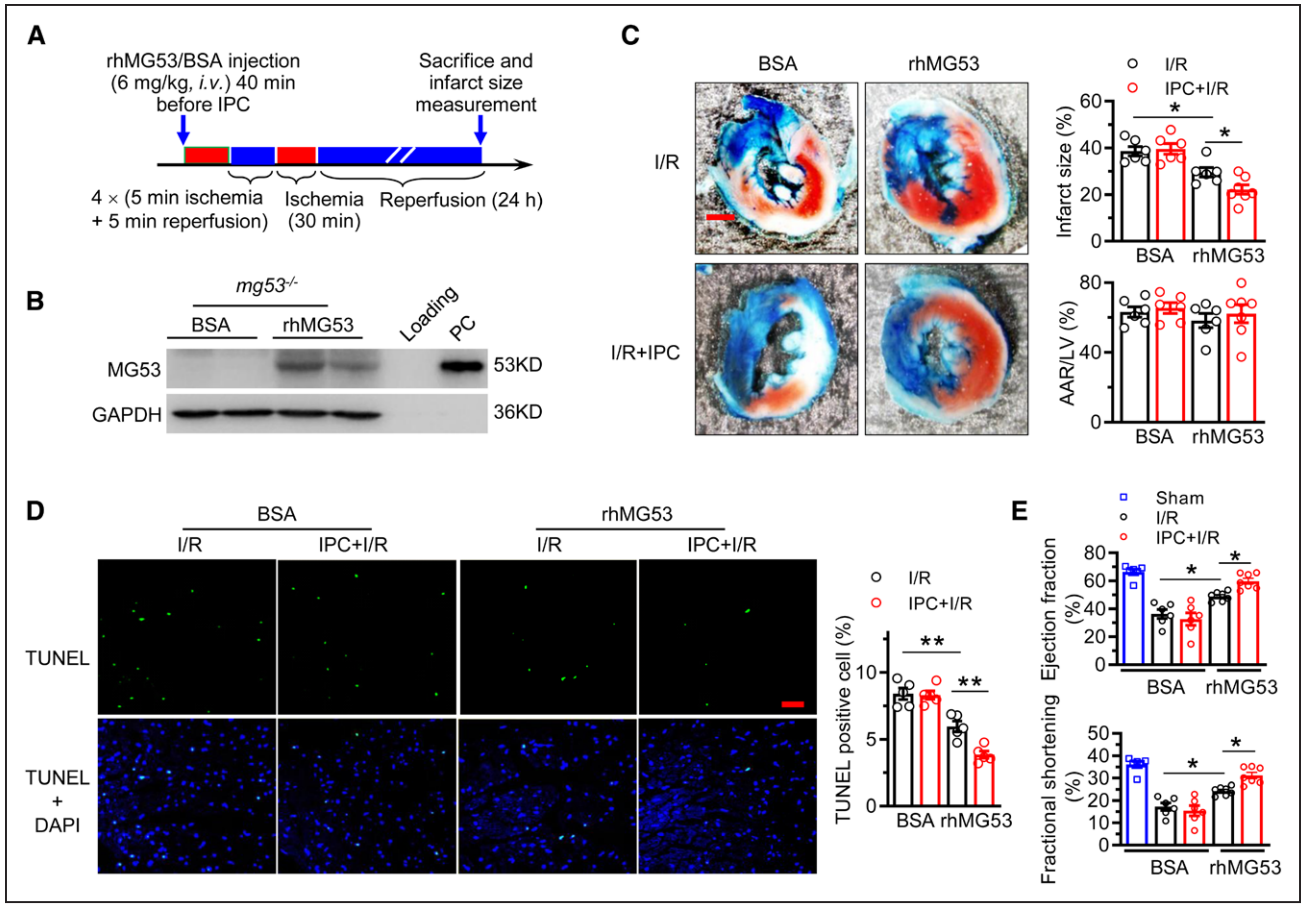

Figure 6. Extracellular MG53 (mitsugumin 53) restores ischemic preconditioning (IPC) in *mg53*$^{-/-}$ mice.
A, Schematic diagram showing the protocol for recombinant human MG53 protein (rhMG53) restoration of IPC protection against cardiac ischemia/reperfusion (I/R) injury in vivo. **B**, Representative Western blot of MG53 from the hearts of *mg53*$^{-/-}$ mice with BSA or rhMG53 protein treatment. Purified rhMG53 was used as a positive control (PC). **C**, Representative photos and averaged data of infarct size and area at risk (AAR) of the *mg53*$^{-/-}$ mice subjected to cardiac I/R injury with or without IPC and with BSA or rhMG53 protein treatment in vivo; n=6 for BSA+I/R, rhMG53+I/R and BSA+IPC+I/R, n=7 for rhMG53+IPC+I/R. *P<0.05 as indicated. Scale bar is 1 mm. **D**, Representative photos and averaged data of cardiomyocyte apoptosis detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining of the *mg53*$^{-/-}$ mice subjected to cardiac I/R injury with or without IPC and with BSA or rhMG53 protein treatment in vivo; n=5. **P<0.01 as indicated. Scale bar is 20 μm. **E**, Cardiac function accessed by echocardiography in *mg53*$^{-/-}$ mice subjected to cardiac sham or I/R injury with or without IPC and with BSA or rhMG53 protein treatment in vivo; n=6 for sham, BSA+I/R, and BSA+IPC+I/R; n=7 for rhMG53+I/R and rhMG53+IPC+I/R. *P<0.05 as indicated. For **C** through **E**, data are presented as mean±SEM. LV indicates left ventricle.

vivo and in perfused isolated hearts. Second, H_2O_2 increases and its specific scavenger catalase blocks MG53 secretion and phosphorylation of PKC-δ at the Y311 site. Last, upregulation of PKC-δ enhances whereas its deficiency or the disruption of its Y311 phosphorylation prevents IPC- and H_2O_2-induced MG53 secretion. These results indicate that IPC-triggered MG53 secretion is attributable mainly to H_2O_2-activated PKC-δ signaling (Figure 8).

Extracellular MG53 has previously been explained by passive leakage caused by cardiac damage.[46,47] However, this work and recent studies have revealed that MG53 can be secreted in response to physiological or pathological stress signals. For example, under metabolic stress conditions such as high glucose or high insulin stimulation, MG53 is secreted from the heart and skeletal muscle as a cardiokine/myokine, contributing to the pathogenesis of systemic insulin resistance.[27] Here, we have illustrated a robust response of MG53 secretion to IPC or oxidative stress. Furthermore, we have elucidated that MG53 secretion is mediated by a canonical secretory pathway[27] that requires the activation of H_2O_2–PKC-δ signaling in the absence of cardiac injury or cell membrane breakage. Functionally, the secreted MG53 participates in IPC-induced cardioprotective signaling and markedly ameliorates I/R injury. Thus, extracellular MG53, as a cardiokine/myokine rather than a passively leaked protein, regulates both cardiac function and myocyte viability in addition to its role in modulating insulin signaling and energy metabolism.

Several studies have reported that recombinant MG53 protein ameliorates various harmful stimulus-caused injuries to the heart, skeletal muscle, and some nonmuscle organs.[47–54] Although intracellular MG53 protects the heart through the activation of reperfusion injury salvage kinase signaling,[16] the mechanism underlying the protective effect of recombinant MG53 protein remained elusive until the present study. Here,

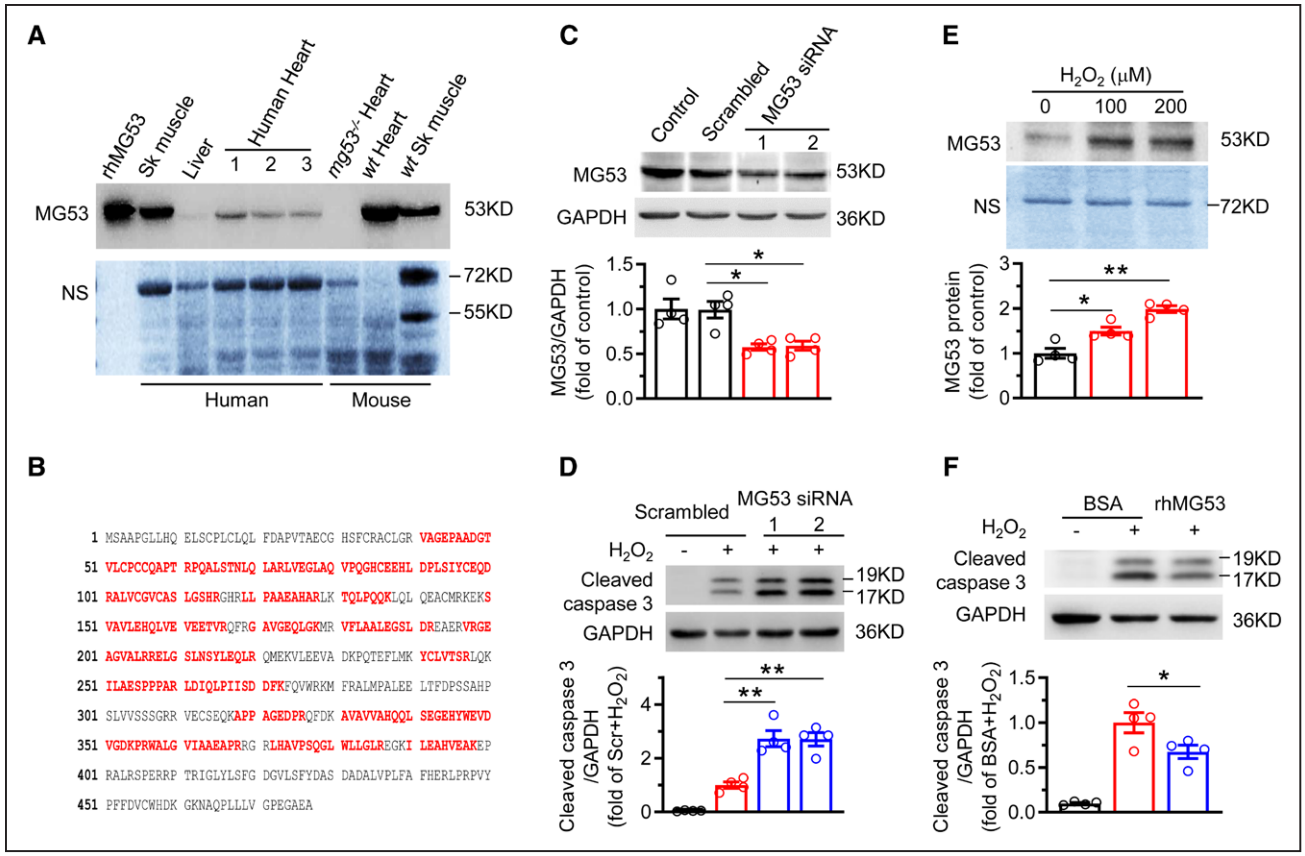

Figure 7. MG53 (mitsugumin 53) is present in human heart and enables the heart to resist oxidative injury.
A, Representative Western blots of lysates (50 μg) from the heart, liver, and skeletal muscle from normal human heart and the heart and skeletal muscle of wild-type *(wt)* or *mg53*$^{-/-}$ mice. Purified recombinant human MG53 protein (rhMG53; 20 ng) was used as a positive control. Nonspecific bands (NS), which were obtained through brilliant green staining, were used as loading controls. **B**, Human heart whole lysates were immunoprecipitated with the MG53 antibody and subjected to mass spectrum (MS) analysis. The peptides detected by MS experiments matched with MG53 sequence are shown in bold red. **C**, Representative Western blots and averaged data of MG53 from cultured human cardiomyocytes derived from human embryonic stem cells transfected with scrambled or 2 sets of MG53 siRNAs; n=4. *P<0.05 as indicated. **D**, Representative Western blots and averaged data of cleaved caspase-3 from the lysates of cultured human cardiomyocytes with H_2O_2 treatment transfected with scrambled or MG53 siRNAs; n=4. **P<0.01 as indicated. **E**, Representative Western blots and averaged data of MG53 from the culture medium of human cardiomyocytes with H_2O_2 treatment (at indicated dosages for 2 hours); n=4. *P<0.05, **P<0.01 as indicated. **F**, Representative Western blots and averaged data of Cleaved caspase-3 from the lysates of cultured human cardiomyocytes with H_2O_2 treatment (200 μmol/L for 20 hours) preincubation of BSA or rhMG53 protein (0.1 μg/mL for 3 hours); n=4. *P<0.05. Data are presented as mean±SEM. For **E**, data are normalized to the corresponding nonspecific bands, which were obtained through brilliant green staining.

we have found, for the first time, that, as an endocrine/paracrine organ, the heart secretes MG53 in response to IPC, which in turn participates in IPC-induced cardioprotection, as evidenced by the restoration of IPC protection through systemic delivery of rhMG53 in MG53-deficient mice (Figure 6). The ability of recombinant MG53 protein to rescue IPC cardioprotection in *mg53*$^{-/-}$ mice implies that exogenous MG53 may enhance or mimic IPC-mediated cardioprotection in human heart in which endogenous MG53 expression is limited. Taken together, these in vitro and in vivo studies not only shed new light on our fundamental understanding of the mechanism responsible for IPC cardioprotection but also underscore the high potential of using circulating MG53 as a novel therapeutic target to treat various human heart diseases.

It is noteworthy that either ROS or PKC-δ acts as a double-edged sword for the heart, although both are crucial for IPC-induced MG53 secretion and the resultant cardioprotection. Specifically, massive ROS production leads to cell injury and death, but a moderate short-term increase of intracellular ROS is an important mediator of cardiac preconditioning and indispensable for IPC-mediated cardioprotection.[35,55,56] Mitochondria are the major source of H_2O_2 involved in preconditioning-triggered cardiac protection.[15,57] Specifically mitochondrial respiration produces superoxide, a major component of ROS, which undergoes dismutation to generate H_2O_2. In line with the previous notion, we have shown here that IPC elevates H_2O_2 production, resulting in H_2O_2-dependent MG53 secretion and its participation in IPC protection. We have found that short exposure of cardiomyocytes to H_2O_2 is protective, whereas prolonged H_2O_2 treatment of cells is detrimental regardless of its concentration (50–200 μmol/L).

Similar to ROS, PKC-δ is essentially involved in cardiac IPC protection,[39,58] but it also induces cardiomyocyte apoptosis under some circumstances.[59,60] The different

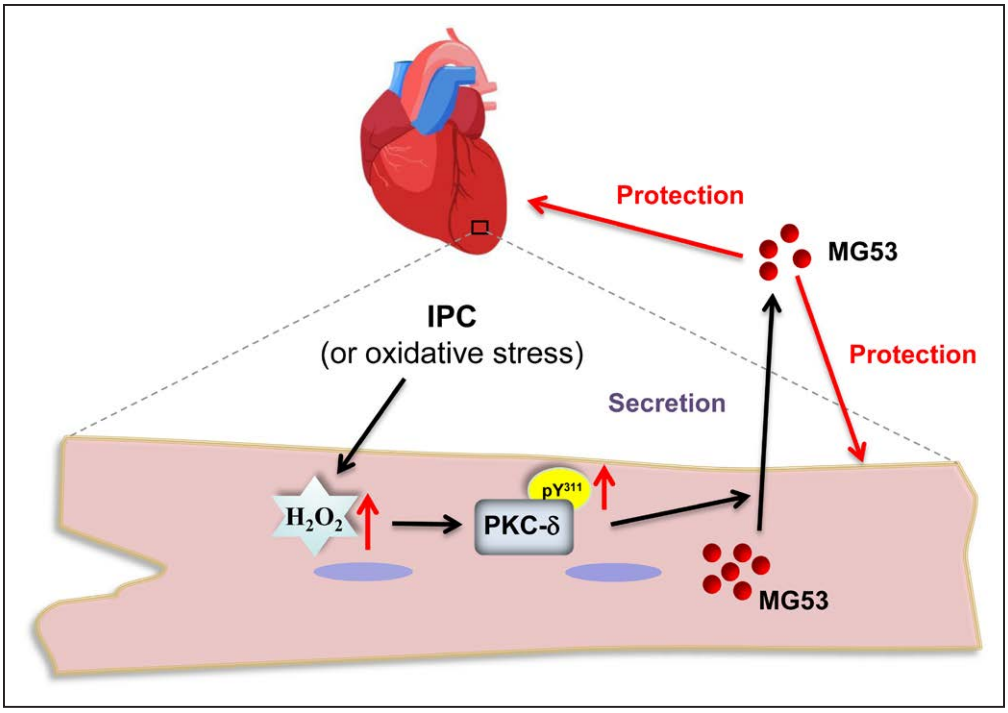

Figure 8. Schematic presentation showing that ischemic preconditioning (IPC) induces MG53 (mitsugumin 53) secretion through H_2O_2-protein kinase-C-δ (PKC-δ)–dependent pathway to mediate IPC cardioprotection.
In response to repetitive short-term IPC, cardiac reactive oxidative species (H_2O_2) was increased, which induced the phosphorylation of Y311 site of PKC-δ and subsequently led to the secretion of intracellular MG53. The extracellular MG53, similar to its intracellular species, participates in cardiac IPC protection.

outcomes of PKC-δ activation may be related to the form and extent of its activation. For example, caspase cleavage of PKC-δ is an important mechanism for amplification of the apoptotic pathway[61]; however, phosphorylation of PKC-δ at Tyr155 is antiapoptotic in glioma cells.[62] Here, our data show that IPC-induced, Src-mediated phosphorylation of PKC-δ at Y311 protects the heart against I/R injury through promoting MG53 secretion. Because PKC-δ Y311 is a target of Src[42] and because both PKC-δ and Src are translocated to mitochondria in response to IPC or H_2O_2 treatment,[44,63,64] we presume that PKC-δ is phosphorylated by Src on cardiac mitochondria and subsequently evokes MG53 secretion. Nevertheless, this possibility merits future investigation.

In addition to its extracellular versus intracellular distribution, posttranslational modification of MG53 plays an important role in its stability and biological function. Myocardial ischemia triggers MG53 S-nitrosylation at cysteine 144, which prevents oxidation-induced MG53 degradation, leading to cardioprotection.[65] S-nitrosylation of MG53 and other proteins has previously been implicated in both cardiac IPC and postconditioning protection.[66–68] In particular, MG53 cysteine 144 S-nitrosylation has been identified in postconditioning heart.[67] Because the mutation of MG53 cysteine 144 prevents I/R-induced MG53 release from the heart,[69] future investigation is required to determine whether S-nitrosylation of this site is obligated to IPC-mediated MG53 secretion.

Although it has previously been claimed that little or no MG53 is expressed in human heart,[46] in the present study, Western blot, in conjunction with mass spectrum analysis, has revealed that MG53 is present in human left ventricle and its abundance is ≈1/10 of that of human skeletal muscle. As is the case in rodent cardiomyocytes, MG53 is secreted from human ES cell–derived cardiomyocytes in response to oxidative stress, and deficiency of MG53 sensitizes the cells to oxidative stress. Likewise, despite the fact that the amount of MG53 in murine kidney and lung is only 2.5% to 5% of that in skeletal muscle, its deficiency exaggerates tissue injury in mice.[51,52] Thus, MG53 has biological functions in tissues with relatively low expression levels.

CONCLUSIONS

We have provided multiple lines of evidence to define MG53 as an IPC-sensitive cardioprotective cardiokine, marking circulating MG53 as a promising target for the treatment of cardiac I/R injury and other kinds of myocardial damage. Mechanistically, we have shown that MG53 secretion is triggered by IPC-induced increases in ROS, in particular H_2O_2, which subsequently activates PKC-δ via elevating its phosphorylation at Y311. Our present and previous findings indicate that circulating MG53, as a cardiokine, acts as a double-edged sword, protecting multiple organs from acute I/R injury and oxidative stress but, when chronically elevated, inducing

insulin resistance and metabolic disorders. The present and previous studies pave the way for MG53 signaling pathway(s)-based therapies to treat ischemic heart diseases and cardiometabolic diseases.

ARTICLE INFORMATION

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Correspondence

Rui-Ping Xiao, MD, PhD, Yan Zhang, MD, PhD, or Chun-Mei Cao, MD, PhD, Institute of Molecular Medicine, Room 236, New Life Science Bldg, Peking University, 5th Yiheyuan Road, Beijing, China 100871. Email xiaor@pku.edu.cn, zhangyan9876@pku.edu.cn, or caochunmei@pku.edu.cn

Affiliations

State Key Laboratory of Membrane Biology, Institute of Molecular Medicine (D.S., S.G., H.-K.W., F. Lv, L.J., M.Z., P.X., Y.W., Y.S., X.H., C.-M.C., Y.Z., R.-P.X.), and Beijing City Key Laboratory of Cardiometabolic Molecular Medicine (R.-P.X.), Peking University, China. Peking-Tsinghua Center for Life Sciences, Beijing, China (R.-P.X.). Beijing Laboratory for Cardiovascular Precision Medicine, The Key Laboratory of Remodeling-Related Cardiovascular Disease, Ministry of Education, Beijing Collaborative Innovation Center for Cardiovascular Disorders, Anzhen Hospital, Capital Medical University, China (F.W., F. Lan). Beijing Institute of Heart, Lung and Blood Vessel Diseases, China (F.W., F. Lan).

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Disclosures

None.

Supplemental Materials

Expanded Methods
Data Supplement Figures I–XI
Data Supplement Table I

REFERENCES

1. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet.* 2018;392:1736–1788. doi: 10.1016/S0140-6736(18)32203-7
2. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *N Engl J Med.* 2007;357:1121–1135. doi: 10.1056/NEJMra071667
3. Heusch G, Gersh BJ. The pathophysiology of acute myocardial infarction and strategies of protection beyond reperfusion: a continual challenge. *Eur Heart J.* 2017;38:774–784. doi: 10.1093/eurheartj/ehw224
4. Hausenloy DJ, Botker HE, Engstrom T, Erlinge D, Heusch G, Ibanez B, Kloner RA, Ovize M, Yellon DM, Garcia-Dorado D. Targeting reperfusion injury in patients with ST-segment elevation myocardial infarction: trials and tribulations. *Eur Heart J.* 2017;38:935–941. doi: 10.1093/eurheartj/ehw145
5. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation.* 1986;74:1124–1136. doi: 10.1161/01.cir.74.5.1124
6. Yellon DM, Downey JM. Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol Rev.* 2003;83:1113–1151. doi: 10.1152/physrev.00009.2003
7. Chen J, Simon R. Ischemic tolerance in the brain. *Neurology.* 1997;48:306–311. doi: 10.1212/wnl.48.2.306
8. Nilsson B, Friman S, Gustafsson BI, Delbro DS. Preconditioning protects against ischemia/reperfusion injury of the liver. *J Gastrointest Surg.* 2000;4:44–49. doi: 10.1016/s1091-255x(00)80031-1
9. Taha MO, Miranda-Ferreira R, Chang AC, Rodrigues AM, Fonseca IS, Toral LB, Cardoso MR, Simões MJ, Oliveira-Junior IS, Monteiro HP, et al. Effect of ischemic preconditioning on injuries caused by ischemia and reperfusion in rat intestine. *Transplant Proc.* 2012;44:2304–2308. doi: 10.1016/j.transproceed.2012.07.056
10. Heusch G. Molecular basis of cardioprotection: signal transduction in ischemic pre-, post-, and remote conditioning. *Circ Res.* 2015;116:674–699. doi: 10.1161/CIRCRESAHA.116.305348
11. Vogt AM, Poolman M, Ackermann C, Yildiz M, Schoels W, Fell DA, Kubler W. Regulation of glycolytic flux in ischemic preconditioning: a study employing metabolic control analysis. *J Biol Chem.* 2002;277:24411–24419. doi: 10.1074/jbc.M201138200
12. Hausenloy DJ, Maddock HL, Baxter GF, Yellon DM. Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? *Cardiovasc Res.* 2002;55:534–543. doi: 10.1016/s0008-6363(02)00455-8
13. Hausenloy D, Wynne A, Duchen M, Yellon D. Transient mitochondrial permeability transition pore opening mediates preconditioning-induced protection. *Circulation.* 2004;109:1714–1717. doi: 10.1161/01.CIR.0000126294.81407.7D
14. Diaz RJ, Losito VA, Mao GD, Ford MK, Backx PH, Wilson GJ. Chloride channel inhibition blocks the protection of ischemic preconditioning and hypo-osmotic stress in rabbit ventricular myocardium. *Circ Res.* 1999;84:763–775. doi: 10.1161/01.res.84.7.763
15. Kalogeris T, Bao Y, Korthuis RJ. Mitochondrial reactive oxygen species: a double edged sword in ischemia/reperfusion vs preconditioning. *Redox Biol.* 2014;2:702–714. doi: 10.1016/j.redox.2014.05.006
16. Cao CM, Zhang Y, Weisleder N, Ferrante C, Wang X, Lv F, Zhang Y, Song R, Hwang M, Jin L, et al. MG53 constitutes a primary determinant of cardiac ischemic preconditioning. *Circulation.* 2010;121:2565–2574. doi: 10.1161/CIRCULATIONAHA.110.954628
17. Zhang Y, Lv F, Jin L, Peng W, Song R, Ma J, Cao CM, Xiao RP. MG53 participates in ischaemic postconditioning through the RISK signalling pathway. *Cardiovasc Res.* 2011;91:108–115. doi: 10.1093/cvr/cvr029
18. Kakkar R, Lee RT. Intramyocardial fibroblast myocyte communication. *Circ Res.* 2010;106:47–57. doi: 10.1161/CIRCRESAHA.109.207456
19. Tian Y, Morrisey EE. Importance of myocyte-nonmyocyte interactions in cardiac development and disease. *Circ Res.* 2012;110:1023–1034. doi: 10.1161/CIRCRESAHA.111.243899
20. Tirziu D, Giordano FJ, Simons M. Cell communications in the heart. *Circulation.* 2010;122:928–937. doi: 10.1161/CIRCULATIONAHA.108.847731
21. Bordicchia M, Liu D, Amri EZ, Ailhaud G, Dessì-Fulgheri P, Zhang C, Takahashi N, Sarzani R, Collins S. Cardiac natriuretic peptides act via p38 MAPK to induce the brown fat thermogenic program in mouse and human adipocytes. *J Clin Invest.* 2012;122:1022–1036. doi: 10.1172/JCI59701
22. Seki M, Powers JC, Maruyama S, Zuriaga MA, Wu CL, Kurishima C, Kim L, Johnson J, Poidomani A, Wang T, et al. Acute and chronic increases of circulating FSTL1 normalize energy substrate metabolism in pacing-induced heart failure. *Circ Heart Fail.* 2018;11:e004486. doi: 10.1161/CIRCHEARTFAILURE.117.004486
23. Hayakawa S, Ohashi K, Shibata R, Kataoka Y, Miyabe M, Enomoto T, Joki Y, Shimizu Y, Kambara T, Uemura Y, et al. Cardiac myocyte-derived follistatin-like 1 prevents renal injury in a subtotal nephrectomy model. *J Am Soc Nephrol.* 2015;26:636–646. doi: 10.1681/ASN.2014020210
24. Okawa H, Horimoto H, Mieno S, Nomura Y, Yoshida M, Shinjiro S. Preischemic infusion of alpha-human atrial natriuretic peptide elicits myoprotective effects against ischemia reperfusion in isolated rat hearts. *Mol Cell Biochem.* 2003;248:171–177. doi: 10.1023/a:1024148621505
25. Sabatine MS, Morrow DA, de Lemos JA, Omland T, Desai MY, Tanasijevic M, Hall C, McCabe CH, Braunwald E. Acute changes in circulating

natriuretic peptide levels in relation to myocardial ischemia. *J Am Coll Cardiol.* 2004;44:1988–1995. doi: 10.1016/j.jacc.2004.07.057
26. D'Souza SP, Yellon DM, Martin C, Schulz R, Heusch G, Onody A, Ferdinandy P, Baxter GF. B-type natriuretic peptide limits infarct size in rat isolated hearts via KATP channel opening. *Am J Physiol Heart Circ Physiol.* 2003;284:H1592–H1600. doi: 10.1152/ajpheart.00902.2002
27. Wu HK, Zhang Y, Cao CM, Hu X, Fang M, Yao Y, Jin L, Chen G, Jiang P, Zhang S, et al. Glucose-sensitive myokine/cardiokine MG53 regulates systemic insulin response and metabolic homeostasis. *Circulation.* 2019;139:901–914. doi: 10.1161/CIRCULATIONAHA.118.037216
28. Song R, Peng W, Zhang Y, Lv F, Wu HK, Guo J, Cao Y, Pi Y, Zhang X, Jin L, et al. Central role of E3 ubiquitin ligase MG53 in insulin resistance and metabolic disorders. *Nature.* 2013;494:375–379. doi: 10.1038/nature11834
29. Liu F, Song R, Feng Y, Guo J, Chen Y, Zhang Y, Chen T, Wang Y, Huang Y, Li CY, et al. Upregulation of MG53 induces diabetic cardiomyopathy through transcriptional activation of peroxisome proliferation-activated receptor α. *Circulation.* 2015;131:795–804. doi: 10.1161/CIRCULATIONAHA.114.012285
30. Lindsey ML, Bolli R, Canty JM Jr, Du XJ, Frangogiannis NG, Frantz S, Gourdie RG, Holmes JW, Jones SP, Kloner RA, et al. Guidelines for experimental models of myocardial ischemia and infarction. *Am J Physiol Heart Circ Physiol.* 2018;314:H812–H838. doi: 10.1152/ajpheart.00335.2017
31. Bøtker HE, Hausenloy D, Andreadou I, Antonucci S, Boengler K, Davidson SM, Deshwal S, Devaux Y, Di Lisa F, Di Sante M, et al. Practical guidelines for rigor and reproducibility in preclinical and clinical studies on cardioprotection. *Basic Res Cardiol.* 2018;113:39. doi: 10.1007/s00395-018-0696-8
32. Zhang T, Zhang Y, Cui M, Jin L, Wang Y, Lv F, Liu Y, Zheng W, Shang H, Zhang J, et al. CaMKII is a RIP3 substrate mediating ischemia- and oxidative stress-induced myocardial necroptosis. *Nat Med.* 2016;22:175–182. doi: 10.1038/nm.4017
33. Burridge PW, Matsa E, Shukla P, Lin ZC, Churko JM, Ebert AD, Lan F, Diecke S, Huber B, Mordwinkin NM, et al. Chemically defined generation of human cardiomyocytes. *Nat Methods.* 2014;11:855–860. doi: 10.1038/nmeth.2999
34. Baines CP, Goto M, Downey JM. Oxygen radicals released during ischemic preconditioning contribute to cardioprotection in the rabbit myocardium. *J Mol Cell Cardiol.* 1997;29:207–216. doi: 10.1006/jmcc.1996.0265
35. Tritto I, D'Andrea D, Eramo N, Scognamiglio A, De Simone C, Violante A, Esposito A, Chiariello M, Ambrosio G. Oxygen radicals can induce preconditioning in rabbit hearts. *Circ Res.* 1997;80:743–748. doi: 10.1161/01.res.80.5.743
36. Turner KM, Burgoyne RD, Morgan A. Protein phosphorylation and the regulation of synaptic membrane traffic. *Trends Neurosci.* 1999;22:459–464. doi: 10.1016/s0166-2236(99)01436-8
37. Leenders AG, Sheng ZH. Modulation of neurotransmitter release by the second messenger-activated protein kinases: implications for presynaptic plasticity. *Pharmacol Ther.* 2005;105:69–84. doi: 10.1016/j.pharmthera.2004.10.012
38. Steinberg SF. Structural basis of protein kinase C isoform function. *Physiol Rev.* 2008;88:1341–1378. doi: 10.1152/physrev.00034.2007
39. Mayr M, Metzler B, Chung YL, McGregor E, Mayr U, Troy H, Hu Y, Leitges M, Pachinger O, Griffiths JR, et al. Ischemic preconditioning exaggerates cardiac damage in PKC-delta null mice. *Am J Physiol Heart Circ Physiol.* 2004;287:H946–H956. doi: 10.1152/ajpheart.00878.2003
40. Konishi H, Tanaka M, Takemura Y, Matsuzaki H, Ono Y, Kikkawa U, Nishizuka Y. Activation of protein kinase C by tyrosine phosphorylation in response to H2O2. *Proc Natl Acad Sci U S A.* 1997;94:11233–11237. doi: 10.1073/pnas.94.21.11233
41. Steinberg SF. Distinctive activation mechanisms and functions for protein kinase Cdelta. *Biochem J.* 2004;384(pt 3):449–459. doi: 10.1042/BJ20040704
42. Rybin VO, Guo J, Sabri A, Elouardighi H, Schaefer E, Steinberg SF. Stimulus-specific differences in protein kinase C delta localization and activation mechanisms in cardiomyocytes. *J Biol Chem.* 2004;279:19350–19361. doi: 10.1074/jbc.M311096200
43. Konishi H, Yamauchi E, Taniguchi H, Yamamoto T, Matsuzaki H, Takemura Y, Ohmae K, Kikkawa U, Nishizuka Y. Phosphorylation sites of protein kinase C delta in H2O2-treated cells and its activation by tyrosine kinase *in vitro*. *Proc Natl Acad Sci U S A.* 2001;98:6587–6592. doi: 10.1073/pnas.111158798
44. Guo J, Cong L, Rybin VO, Gertsberg Z, Steinberg SF. Protein kinase C-{delta} regulates the subcellular localization of Shc in H2O2-treated cardiomyocytes. *Am J Physiol Cell Physiol.* 2010;299:C770–C778. doi: 10.1152/ajpcell.00170.2010
45. Wang X, Xie W, Zhang Y, Lin P, Han L, Han P, Wang Y, Chen Z, Ji G, Zheng M, et al. Cardioprotection of ischemia/reperfusion injury by cholesterol-dependent MG53-mediated membrane repair. *Circ Res.* 2010;107:76–83. doi: 10.1161/CIRCRESAHA.109.215822
46. Lemckert FA, Bournazos A, Eckert DM, Kenzler M, Hawkes JM, Butler TL, Ceely B, North KN, Winlaw DS, Egan JR, et al. Lack of MG53 in human heart precludes utility as a biomarker of myocardial injury or endogenous cardioprotective factor. *Cardiovasc Res.* 2016;110:178–187. doi: 10.1093/cvr/cvw017
47. Weisleder N, Takizawa N, Lin P, Wang X, Cao C, Zhang Y, Tan T, Ferrante C, Zhu H, Chen PJ, et al. Recombinant MG53 protein modulates therapeutic cell membrane repair in treatment of muscular dystrophy. *Sci Transl Med.* 2012;4:139ra85. doi: 10.1126/scitranslmed.3003921
48. Yao Y, Zhang B, Zhu H, Li H, Han Y, Chen K, Wang Z, Zeng J, Liu Y, Wang X, et al. MG53 permeates through blood-brain barrier to protect ischemic brain injury. *Oncotarget.* 2016;7:22474–22485. doi: 10.18632/oncotarget.7965
49. Li H, Duann P, Lin PH, Zhao L, Fan Z, Tan T, Zhou X, Sun M, Fu M, Orange M, et al. Modulation of wound healing and scar formation by MG53 protein-mediated cell membrane repair. *J Biol Chem.* 2015;290:24592–24603. doi: 10.1074/jbc.M115.680074
50. Yao W, Li H, Han X, Chen C, Zhang Y, Tai WL, Xia Z, Hei Z. MG53 anchored by dysferlin to cell membrane reduces hepatocyte apoptosis which induced by ischaemia/reperfusion injury *in vivo* and *in vitro*. *J Cell Mol Med.* 2017;21:2503–2513. doi: 10.1111/jcmm.13171
51. Jia Y, Chen K, Lin P, Lieber G, Nishi M, Yan R, Wang Z, Yao Y, Li Y, Whitson BA, et al. Treatment of acute lung injury by targeting MG53-mediated cell membrane repair. *Nat Commun.* 2014;5:4387. doi: 10.1038/ncomms5387
52. Duann P, Li H, Lin P, Tan T, Wang Z, Chen K, Zhou X, Gumpper K, Zhu H, Ludwig T, et al. MG53-mediated cell membrane repair protects against acute kidney injury. *Sci Transl Med.* 2015;7:279ra36. doi: 10.1126/scitranslmed.3010755
53. Liu J, Zhu H, Zheng Y, Xu Z, Li L, Tan T, Park KH, Hou J, Zhang C, Li D, et al. Cardioprotection of recombinant human MG53 protein in a porcine model of ischemia and reperfusion injury. *J Mol Cell Cardiol.* 2015;80:10–19. doi: 10.1016/j.yjmcc.2014.12.010
54. Chandler HL, Tan T, Yang C, Gemensky-Metzler AJ, Wehrman RF, Jiang Q, Peterson CMW, Geng B, Zhou X, Wang Q, et al. MG53 promotes corneal wound healing and mitigates fibrotic remodeling in rodents. *Commun Biol.* 2019;2:71. doi: 10.1038/s42003-019-0316-7
55. Vanden Hoek TL, Becker LB, Shao Z, Li C, Schumacker PT. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J Biol Chem.* 1998;273:18092–18098. doi: 10.1074/jbc.273.29.18092
56. Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, Heusch G, Cohen MV, Downey JM. Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. *Circ Res.* 2000;87:460–466. doi: 10.1161/01.res.87.6.460
57. Muntean DM, Sturza A, Dănilă MD, Borza C, Duicu OM, Mornoș C. The role of mitochondrial reactive oxygen species in cardiovascular injury and protective strategies. *Oxid Med Cell Longev.* 2016;2016:8254942. doi: 10.1155/2016/8254942
58. Kolár F, Jezková J, Balková P, Breh J, Neckár J, Novák F, Nováková O, Tomásová H, Srbová M, Ost'ádal B, et al. Role of oxidative stress in PKC-delta upregulation and cardioprotection induced by chronic intermittent hypoxia. *Am J Physiol Heart Circ Physiol.* 2007;292:H224–H230. doi: 10.1152/ajpheart.00689.2006
59. Heidkamp MC, Bayer AL, Martin JL, Samarel AM. Differential activation of mitogen-activated protein kinase cascades and apoptosis by protein kinase C epsilon and delta in neonatal rat ventricular myocytes. *Circ Res.* 2001;89:882–890. doi: 10.1161/hh2201.099434
60. Murriel CL, Churchill E, Inagaki K, Szweda LI, Mochly-Rosen D. Protein kinase Cdelta activation induces apoptosis in response to cardiac ischemia and reperfusion damage: a mechanism involving BAD and the mitochondria. *J Biol Chem.* 2004;279:47985–47991. doi: 10.1074/jbc.M405071200
61. Khwaja A, Tatton L. Caspase-mediated proteolysis and activation of protein kinase Cdelta plays a central role in neutrophil apoptosis. *Blood.* 1999;94:291–301.
62. Okhrimenko H, Lu W, Xiang C, Ju D, Blumberg PM, Gomel R, Kazimirsky G, Brodie C. Roles of tyrosine phosphorylation and cleavage of protein kinase Cdelta in its protective effect against tumor necrosis factor-related apoptosis inducing ligand-induced apoptosis. *J Biol Chem.* 2005;280:23643–23652. doi: 10.1074/jbc.M501374200

63. Ping P, Zhang J, Zheng YT, Li RC, Dawn B, Tang XL, Takano H, Balafanova Z, Bolli R. Demonstration of selective protein kinase C-dependent activation of Src and Lck tyrosine kinases during ischemic preconditioning in conscious rabbits. *Circ Res.* 1999;85:542–550. doi: 10.1161/01.res.85.6.542
64. Ge H, Zhao M, Lee S, Xu Z. Mitochondrial Src tyrosine kinase plays a role in the cardioprotective effect of ischemic preconditioning by modulating complex I activity and mitochondrial ROS generation. *Free Radic Res.* 2015;49:1210–1217. doi: 10.3109/10715762.2015.1050013
65. Kohr MJ, Evangelista AM, Ferlito M, Steenbergen C, Murphy E. S-nitrosylation of TRIM72 at cysteine 144 is critical for protection against oxidation-induced protein degradation and cell death. *J Mol Cell Cardiol.* 2014;69:67–74. doi: 10.1016/j.yjmcc.2014.01.010
66. Sun J, Morgan M, Shen RF, Steenbergen C, Murphy E. Preconditioning results in S-nitrosylation of proteins involved in regulation of mitochondrial energetics and calcium transport. *Circ Res.* 2007;101:1155–1163. doi: 10.1161/CIRCRESAHA.107.155879
67. Tong G, Aponte AM, Kohr MJ, Steenbergen C, Murphy E, Sun J. Postconditioning leads to an increase in protein S-nitrosylation. *Am J Physiol Heart Circ Physiol.* 2014;306:H825–H832. doi: 10.1152/ajpheart.00660.2013
68. Sun J, Murphy E. Protein S-nitrosylation and cardioprotection. *Circ Res.* 2010;106:285–296. doi: 10.1161/CIRCRESAHA.109.209452
69. Fillmore N, Casin KM, Sinha P, Sun J, Ma H, Boylston J, Noguchi A, Liu C, Wang N, Zhou G, et al. A knock-in mutation at cysteine 144 of TRIM72 is cardioprotective and reduces myocardial TRIM72 release. *J Mol Cell Cardiol.* 2019;136:95–101. doi: 10.1016/j.yjmcc.2019.09.008