MG53 Constitutes a Primary Determinant of Cardiac Ischemic Preconditioning

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Background—Ischemic heart disease is the greatest cause of death in Western countries. The deleterious effects of cardiac ischemia are ameliorated by ischemic preconditioning (IPC), in which transient ischemia protects against subsequent severe ischemia/reperfusion injury. IPC activates multiple signaling pathways, including the reperfusion injury salvage kinase pathway (mainly PI3K-Akt-glycogen synthase kinase-3β [GSK3β] and ERK1/2) and the survivor activating factor enhancement pathway involving activation of the JAK-STAT3 axis. Nevertheless, the fundamental mechanism underlying IPC is poorly understood.

Methods and Results—In the present study, we define MG53, a muscle-specific TRIM-family protein, as a crucial component of cardiac IPC machinery. Ischemia/reperfusion or hypoxia/oxidative stress applied to perfused mouse hearts or neonatal rat cardiomyocytes, respectively, causes downregulation of MG53, and IPC can prevent ischemia/reperfusion-induced decrease in MG53 expression. MG53 deficiency increases myocardial vulnerability to ischemia/reperfusion injury and abolishes IPC protection. Overexpression of MG53 attenuates whereas knockdown of MG53 enhances hypoxia- and H_2O_2-induced cardiomyocyte death. The cardiac protective effects of MG53 are attributable to MG53-dependent interaction of caveolin-3 with phosphatidylinositol 3 kinase and subsequent activation of the reperfusion injury salvage kinase pathway without altering the survivor activating factor enhancement pathway.

Conclusions—These results establish MG53 as a primary component of the cardiac IPC response, thus identifying a potentially important novel therapeutic target for the treatment of ischemic heart disease. **(*Circulation*. 2010;121:2565-2574.)**

Key Words: hypoxia ■ ischemia ■ myocardial infarction ■ myocytes ■ stress

Ischemic heart disease remains the greatest cause of mortality in Western countries and the predicted leading source of mortality worldwide by 2020.[1] Blockage of heart blood flow leads to myocardial ischemia. Persistent ischemia causes myocardial infarctions, resulting in profound myocyte death, irreversible myocardial damage, and a permanent loss of contractile mass. Timely reperfusion of ischemic heart is the only way to preserve cardiac cell viability. However, reperfusion can trigger further damage to the myocardium (ie, ischemia/reperfusion [IR] injury) via reactive oxygen species–induced oxidative stress, calcium overload, or calpain activation.[2–4] Both ischemic injury and subsequent IR injury after restoration of blood flow represent important therapeutic targets.

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Interventional approaches against IR injury have centered on the study of ischemic preconditioning (IPC), in which nonlethal ischemic stress to the heart (IPC) protects against subsequent lethal IR injury in the heart.[5–7] IPC is the most powerful intrinsic cellular mechanism to protect the heart as well as other organs, such as brain, liver, and kidney, from IR injury.[8–11] A variety of signaling molecules, including survival kinases such as phosphatidylinositol 3 kinase (PI3K), Akt, GSK3β, and ERK1/2 and scaffolding proteins such as caveolin-3 (CaV3), contribute to IPC response.[12–17] Recently, IPC-activated signaling events have been classified into 2

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major pathways: the reperfusion injury salvage kinase (RISK) pathway and the survivor activating factor enhancement (SAFE) pathway. The RISK pathway consists of PI3K-Akt-GSK3β and ERK1/2 signaling events, whereas the SAFE pathway involves activation of tumor necrosis factor-α and the JAK-STAT3 axis.[18–21] It has been suggested that CaV3-dependent clustering of signaling machinery at the caveolae membrane domains is involved in IPC-mediated cell protection,[22,23] but the mechanism enabling this spatial organization of IPC signaling events remains largely elusive.

We recently discovered that MG53, a muscle-specific TRIM-family protein (TRIM72), forms a functional complex with CaV3 in skeletal muscle and contributes to intracellular vesicle trafficking and myogenesis in skeletal muscle cells.[24] Notably, MG53 is expressed exclusively in the heart and skeletal muscle, with highest expression in the myocardium.[25] Although our previous studies have shown an essential role of MG53 in acute membrane repair in skeletal muscle,[25] it is unknown whether and how MG53 elicits cardiac protection in response to various insults, particularly IR injury. In the present study, we demonstrate that MG53 is a primary component of cardiac IPC machinery, marking MG53 as a novel therapeutic target for ischemic heart disease.

Methods

Detailed methods, including myocardial infarct size measurement,[26] are available in the online-only Data Supplement.

Animals

Animals were maintained in the Center for Experimental Animals (an Association for Assessment and Accreditation of Laboratory Animal Care–accredited experimental animal facility) at Peking University, Beijing, China. All procedures involving experimental animals were performed in accordance with protocols approved by the Committee for Animal Research of Peking University, Peking, China, and conformed to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication No. 86-23, revised 1985).

Rat In Vivo Myocardial IR Model

Male Sprague-Dawley rats (with body weight of 200 to 250 g) were anesthetized with pentobarbital (40 mg/kg IP) and ventilated via 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 IR was performed by tightening the snare for 45 minutes and then loosening it for 12 hours (for RNA extraction) or 24 hours (for protein extraction and infarct size measurement). IPC was induced by 4 episodes of 10-minute ischemia followed by 5 minutes of reperfusion before the 45-minute ischemia. Blood samples for lactate dehydrogenase (LDH) measurement were collected 4 hours after reperfusion from rats subjected to IR and centrifuged for 10 minutes at 3000 rpm for serum.

Isolated Mouse Heart Langendorff Perfusion

Adult MG53 knockout and wild-type littermate control mice (20 to 30 g) were anesthetized by intraperitoneal injection of pentobarbital (70 mg/kg). The heart was excised and perfused on a Langendorff apparatus at constant pressure of 55 mm Hg. The buffer was continuously gassed with 95% O_2/5% CO_2 (pH 7.4) and warmed by a heating bath/circulator. The heart temperature was continuously monitored and maintained at 37±0.5°HC. Global ischemia was induced by cessation of perfusion for 30 minutes followed by reperfusion. IPC was achieved by 2 cycles of 5 minutes of ischemia followed by 5 minutes of reperfusion before the more sustained IR that caused myocardial infarction.

Determination of Myocardial Injury by LDH Release

The effluent from the perfused heart was accumulated for every 5 minutes of reperfusion. LDH was spectrophotometrically assayed with the use of a kit from Sigma Chemical Co (St Louis, Mo).

Cell Viability Assays

Cardiomyocyte viability was detected by an ATP assay as described previously.[27] Cell viability was also assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously.[28]

Echocardiographic Evaluation

Echocardiography was performed in conscious mice as described previously.[29,30]

PI3K Activity

PI3K activity was measured with a PI3K enzyme-linked immunosorbent assay kit (Echelon Biosciences Inc) following the manufacturer's instructions.[31]

Adenoviral Infection of Neonatal or Adult Rat Ventricular Myocytes

Culture and adenovirus-mediated gene transfer of neonatal or adult rat cardiomyocytes were implemented by methods described previously.[28,32] Adenoviral vectors expressing either green fluorescent protein (GFP)–MG53 or GFP were described previously.[25]

Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction was performed as described previously.[28]

Western Blot and Confocal Immunocytochemical Imaging

Western blotting[28] and confocal immunocytochemical imaging[33] were performed as described previously.

Gene Silencing Through RNA Interference

For gene silencing assay, small hairpin RNAs (shRNAs) comprising a 19-bp stem and 4-bp loop structure were designed against a unique region of mouse MG53 or rat CaV3 and subcloned into the pAd/BLOCK-iTTMDEST vector (Invitrogen, Carlsbad, Calif).

Statistical Analysis

Data are expressed as mean±SEM. Statistical comparisons used 1-way ANOVA, followed by the Bonferroni procedure for multiple-group comparisons. $P<0.05$ was considered statistically significant unless otherwise noted for a specific experiment.

Results

IR-, Hypoxia-, or Oxidative Stress–Induced Myocardial Damage Is Associated With MG53 Downregulation

To investigate whether there is a causative relationship between MG53 expression and cardiomyocyte integrity, we challenged cultured neonatal ventricular myocytes (NVMC) with various insults. Hypoxia led to a progressive decline in MG53 expression in a time-dependent manner with the steady state level of 0.55±0.09-fold of the control value at 24 hours (n=6; $P<0.01$ versus 0 hour) (Figure 1A). The reduction in MG53 abundance correlated with the decrease in myocyte viability over the same time course (Figure 1B).

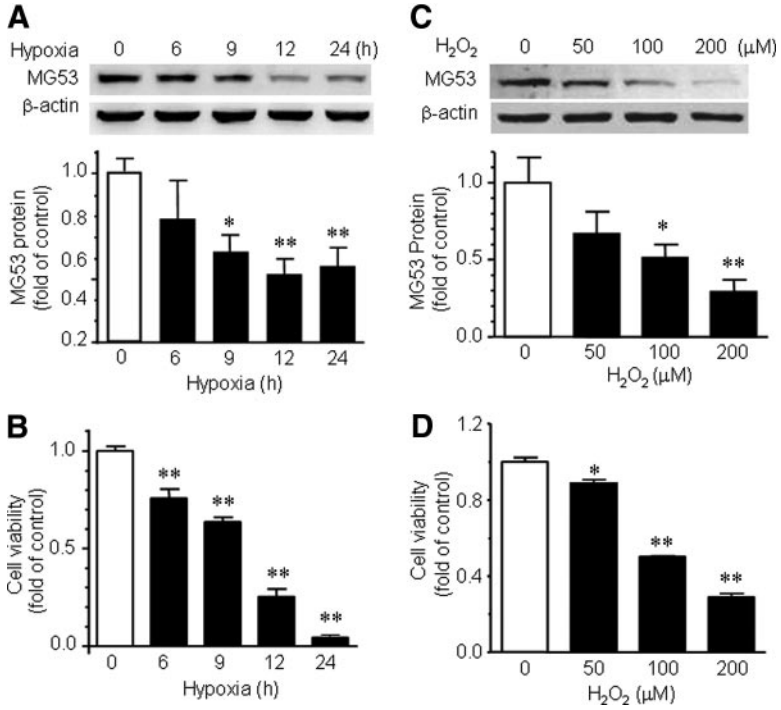

Figure 1. MG53 protein abundance correlates with cardiomyocyte integrity. A, Representative immunoblots and statistical data of MG53 protein levels in NVMC subjected to hypoxia for various times (n=6). B, Cell viability indexed by cellular ATP content in NVMC subjected to hypoxia (n=12). C, Representative immunoblots and statistical data of MG53 protein levels in NVMC subjected to different concentrations of H_2O_2 treatment (n=9). D, Cell viability in NVMC subjected to different doses of H_2O_2 treatment (n=12). For all panels, data are presented as mean±SEM. *P<0.05, **P<0.01 vs 0 hour or 0 μmol/L.

Similarly, oxidative stress with H_2O_2 suppressed MG53 expression in a concentration-dependent manner with a minimal level of 0.29±0.08-fold of the control value at the highest concentration (200 μmol/L) (n=9; P<0.01 versus 0 μmol/L) (Figure 1C). H_2O_2 treatment also triggered cell death in a concentration-dependent manner (Figure 1D). These data indicate that hypoxia- and oxidative stress–induced cardiomyocyte death are closely accompanied by downregulation of MG53.

To explore the potential clinical relevance of hypoxia- and oxidative stress–induced downregulation of MG53 in cardiomyocytes, we used a rat in vivo myocardial IR model (45-minute ischemia followed by reperfusion) with or without 4 episodes of IPC (10-minute ischemia followed by

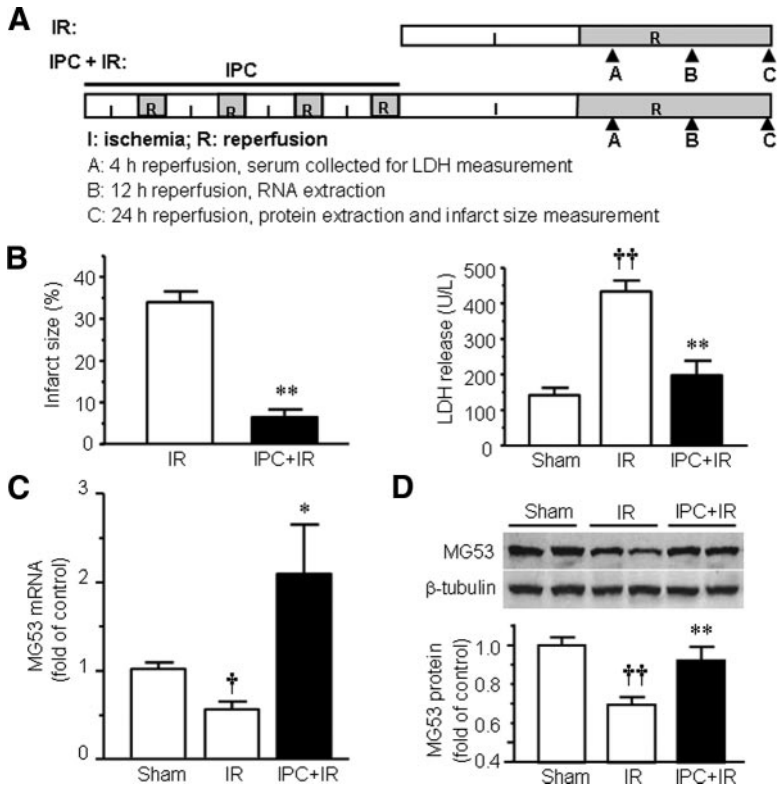

Figure 2. Expression of MG53 correlates with rat heart IR injury. A, Schematic illustration of the protocol used for rat in vivo IR (45-minute ischemia followed by reperfusion) without or with 4 episodes of IPC (10-minute ischemia followed by 5-minute reperfusion). B, Left panel illustrates infarct size in rat hearts subjected to ischemia and 24-hour reperfusion with or without IPC (33.95±2.60% and 6.53±1.81% for IR and IPC+IR, respectively) (n=8; **P<0.01 vs IR); right panel shows serum LDH levels in sham-operated rats or those subjected to ischemia and 4-hour reperfusion with or without IPC (141.51±21.00, 434.19±31.28, and 195.33±37.21 U/L for sham, IR, IPC+IR, respectively; n=8 for each group; **P<0.01 vs IR, ††P<0.01 vs sham). C, MG53 messenger RNA levels in cardiac tissues in the ischemic area from rats subjected to ischemia and 12-hour reperfusion with or without IPC (n=8; *P<0.05 vs IR and sham, †P<0.05 vs sham). D, Representative immunoblots and average data of MG53 protein levels in myocardial tissues of the ischemic area from rats subjected to ischemia and 24-hour reperfusion with or without IPC (0.69±0.04- and 0.92±0.07-fold of sham for IR and IPC+IR, respectively; n=8; **P<0.01 vs IR; ††P<0.01 vs sham).

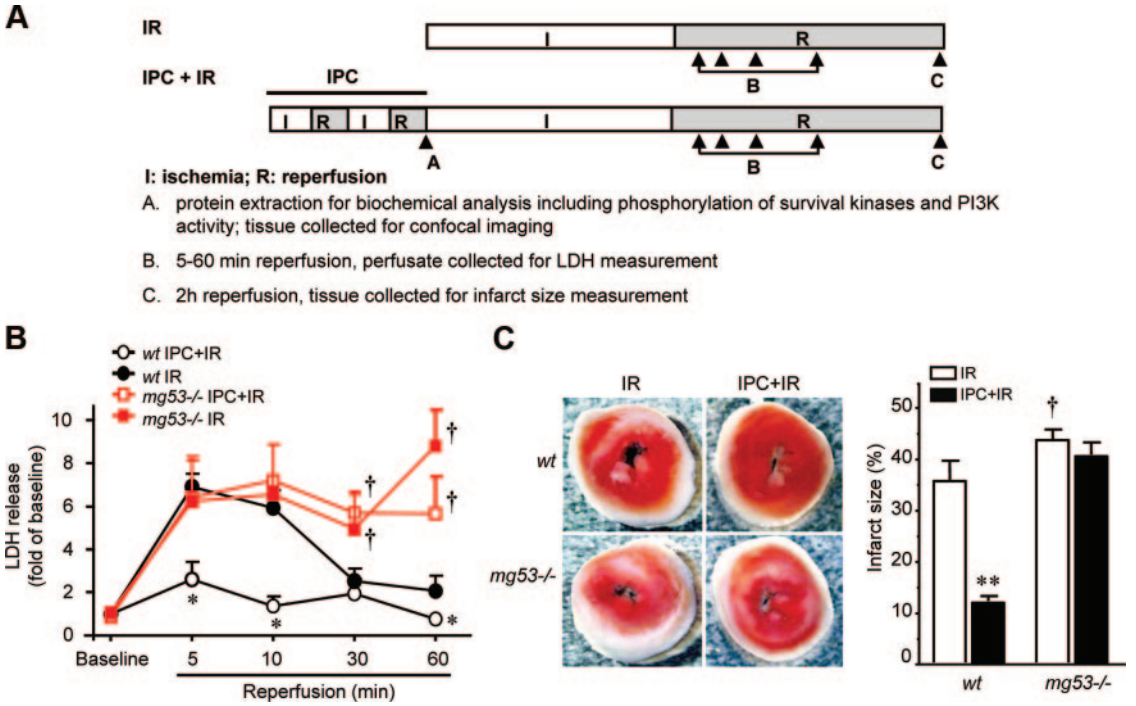

Figure 3. MG53 knockout hearts are vulnerable to IR injury and resistant to IPC protection. A, Schematic illustration of the protocol used for mouse ex vivo IR (30-minute ischemia followed by reperfusion) without or with 2 episodes of IPC (5-minute ischemia followed by 5-minute reperfusion). B, Change of LDH concentration in the efflux of perfused hearts from *wt* and *mg53*$^{-/-}$ mice subjected to 30-minute ischemia and various periods of reperfusion with or without IPC (n=8; **P*<0.05 vs all of the other 3 groups; †*P*<0.05 vs wt IR and *wt* IPC+IR). C, Representative photographs and statistical data of infarct size in perfused *wt* and *mg53*$^{-/-}$ mouse hearts subjected to IR with or without IPC (34.5±3.6% and 12.8±1.1% for *wt* IR and *wt* IPC+IR, respectively; 43.5±2.1% and 40.7±2.4% for *mg53*$^{-/-}$ IR and *mg53*$^{-/-}$ IPC+IR, respectively; n=8 to 9 for each group; ***P*<0.01 vs all of the other 3 groups; †*P*<0.05 vs *wt* IR).

5-minute reperfusion) (Figure 2A). In vivo IR caused myocardial infarction and LDH release (Figure 2B). IPC markedly attenuated IR-induced myocardial infarct size from 34.0±2.6% to 6.5±1.8% (n=8; *P*<0.01) and LDH release from 434.19±31.28 to 195.33±37.21 U/L. (n=8; *P*<0.01). In addition, IR significantly reduced MG53 expression at both messenger RNA and protein levels (Figure 2C and 2D). Importantly, IPC fully prevented IR-induced downregulation of MG53 (Figure 2C and 2D). These in vivo data suggest that IR-induced cardiac injury is likely related to IR-mediated downregulation of MG53 and that IPC-maintained MG53 expression may contribute to IPC-induced cardioprotection.

MG53 Ablation Exaggerates IR Injury and Renders the Heart Resistant to IPC Protection

To test the aforementioned hypothesis, we examined myocardial integrity and morphology in response to IR injury in the presence or absence of IPC (Figure 3A) in wild-type (*wt*) or gene-targeted MG53 knockout (*mg53*$^{-/-}$) mice.[25] Western blotting confirmed the lack of MG53 protein in myocardium from MG53-deficient mice (Figure IA in the online-only Data Supplement). Under physiological conditions, there were no morphological or functional differences between *wt* and *mg53*$^{-/-}$ mice at the age of 2 to 3 months (Figure IB and Table I in the online-only Data Supplement). However, IR-induced myocardial damage during Langendorff perfusion was markedly exaggerated in the *mg53*$^{-/-}$ heart (Figure 3B). The appearance of LDH in the perforate after IR injury provides a direct index of damage to the sarcolemmal membranes of cardiomyocytes in the injured heart. Although the *wt* heart showed a transient LDH increase after IR that was markedly reduced by IPC, the *mg53*$^{-/-}$ heart showed a sustained elevation of LDH release (Figure 3B), suggesting a reduced tolerance of the *mg53*$^{-/-}$ heart to IR injury. For instance, after 30- or 60-minute reperfusion, the values of LDH release from *wt* mouse hearts in the presence and absence of IPC treatment were not significantly different from those of the control, whereas LDH release from *mg53*$^{-/-}$ mouse hearts was sustained at the plateau level in the presence or absence of IPC treatment (Figure 3B). Most importantly, MG53 deficiency completely abolished IPC-mediated cardioprotection, as manifested by the failure of IPC to reduce IR-induced myocardial infarct size, whereas IPC profoundly suppressed IR-induced infarction in *wt* hearts (Figure 3C). Together, our results indicate that MG53 is obligatory to cardioprotection by IPC.

Overexpression of MG53 Is Sufficient to Protect Cardiomyocytes Against Hypoxia- and Oxidative Stress–Induced Cell Death

To further establish the causative relationship between MG53 expression and cardiomyocyte viability, we next acutely upregulated or downregulated MG53 expression in NVMC. Infection of cardiomyocytes with Adv-MG53 resulted in a titer-dependent expression of GFP-MG53 fusion protein (Figure II in the online-only Data Supplement). Overexpression of GFP-MG53 fusion protein profoundly reduced hypoxia- or H_2O_2-induced cell death indexed by 2 independent readouts,

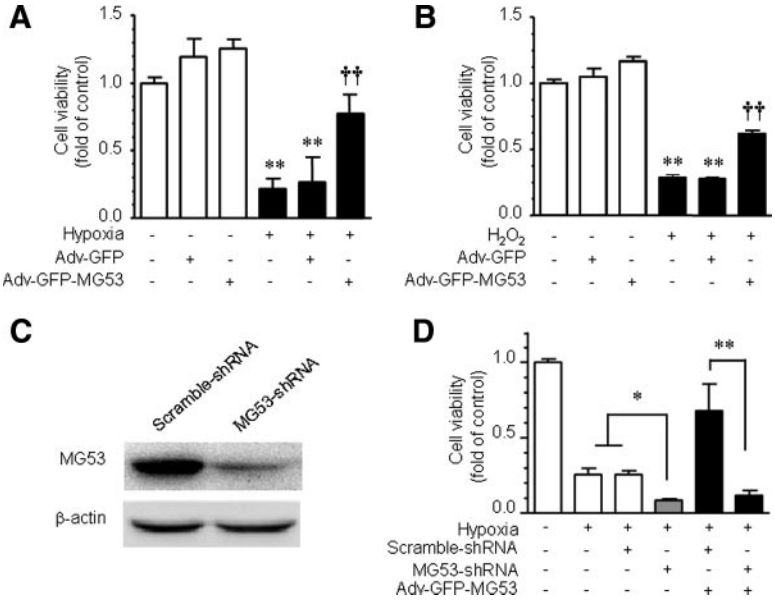

Figure 4. Overexpression of MG53 protects cardiomyocytes against hypoxia- and oxidative stress–induced cell death, whereas knockdown of MG53 acerbates cell death. All experiments were performed in cells infected with Adv-GFP or Adv-GFP-MG53 (30 multiplicity of infection) for 24 hours, and cell viability was indexed by an ATP assay. A, Overexpression of MG53 protects cardiomyocytes against hypoxia-induced cell death. Cell viability values were 0.21±0.07-, 0.27±0.16-, and 0.82±0.12-fold of control for uninfected, Adv-GFP–infected, and Adv-Mg53–infected cells subjected to hypoxia for 12 hours, respectively (n=12 for each group; $^{**}P<0.01$ vs groups in the absence of hypoxia or Adv-GFP-MG53; $\dagger\dagger P<0.01$ vs hypoxia and hypoxia+Adv-GFP). B, Overexpression of MG53 protects cardiomyocytes against H_2O_2-induced cell death. Cell viability values were 0.29±0.02-, 0.27±0.01-, and 0.62±0.02-fold of control for uninfected, Adv-GFP–infected, and Adv-MG53–infected cells subjected to H_2O_2 (200 μmol/L) for 24 hours (n=12 for each group; $^{**}P<0.01$ vs groups in the absence of H_2O_2 or Adv-GFP-MG53, $\dagger\dagger P<0.01$ vs H_2O_2 and H_2O_2+Adv-GFP). C, Representative blot of MG53 protein in lysates of NVMC infected with an adenovirus expressing MG53-shRNA or a scramble-shRNA. D, Cell viability of NVMC in the presence or absence of Adv-MG53, MG53-shRNA, or scramble-shRNA, assayed by ATP content (n=12; $^{*}P<0.05$, $^{**}P<0.01$ as indicated). Note that infection cells with MG53-shRNA fully blocked the protective effect of Adv-MG53 infection.

cellular ATP content and MTT assay (Figure 4A and 4B and Figure IIIA and IIIB in the online-only Data Supplement). In contrast, infection of cells with an adenovirus containing shRNA, which effectively reduced MG53 expression (Figure 4C), exacerbated hypoxia-induced cell death and also eliminated the protective effect of GFP-MG53 overexpression revealed by ATP content (Figure 4D) and MTT assays (Figure IIIC in the online-only Data Supplement). The direct correlation of MG53 expression level with cardiomyocyte viability during hypoxia or oxidative stress supports the conclusion that MG53 plays a crucial role in protection of cardiomyocytes from hypoxia- or oxidative stress–induced damage.

Overexpression of MG53, Mimicking IPC, Activates PI3K-Akt-GSK3β and ERK1/2 Signaling Cascades

To delineate the mechanism underlying MG53-mediated cardioprotection, we next determined whether MG53 affects the RISK pathway in cardiomyocytes. Overexpression of MG53 significantly elevated the phosphorylation levels of several key prosurvival kinases including Akt,[34–36] GSK3β,[15] and ERK1/2 1.45±0.12-, 1.38±0.04-, and 1.30±0.06-fold over their respective control (n=9; $P<0.05$) (Figure 5A). These kinases were also abundantly activated by IPC in *wt* mouse heart (Figure 5B). However, IPC failed to increase the phosphorylation levels of Akt, GSK3β, or ERK1/2 in the MG53-deficient heart (Figure 5B). In addition, the basal phosphorylation levels of these kinases were 50% to 60% lower in the $mg53^{-/-}$ heart than those in their *wt* counterparts. The reduced basal phosphorylation of those kinases may explain the reduced tolerance of the MG53-deficient heart to IR injury.

IPC-Induced Activation of PI3K Is Dependent on MG53

Although these biochemical studies indicate a crucial role of MG53 in the activation of some survival kinases such as Akt, GSK3β, and ERK1/2, we next determined whether MG53 is essential for IPC-induced activation of PI3K. We demonstrated that MG53 deficiency fully abolished IPC-induced activation of PI3K (Figure 6A), indicating that MG53 is obligatory for IPC-induced activation of PI3K, a key component of the prosurvival RISK pathway. It is also noteworthy that the basal PI3K activity was significantly lower in $mg53^{-/-}$ hearts relative to that in *wt* hearts (Figure 6A), as was the case for other survival kinases (Akt, GSK3β, and ERK1/2) (Figure 5B). Together, these results indicate that MG53 is essentially involved in IPC-induced activation of the RISK pathway. In contrast, MG53 is not involved in the SAFE pathway because IPC-induced phosphorylation of STAT-3 remains intact in MG53-deficient hearts (Figure 5A and 5B). In addition, the expression of protein kinase Cε, mitochondrial connexin 43, protein kinase G, and mitochondrial K_{ATP} channels (Kir6.2) was unaltered in $mg53^{-/-}$ mouse hearts relative to *wt* controls (Figure IV in the online-only Data Supplement).

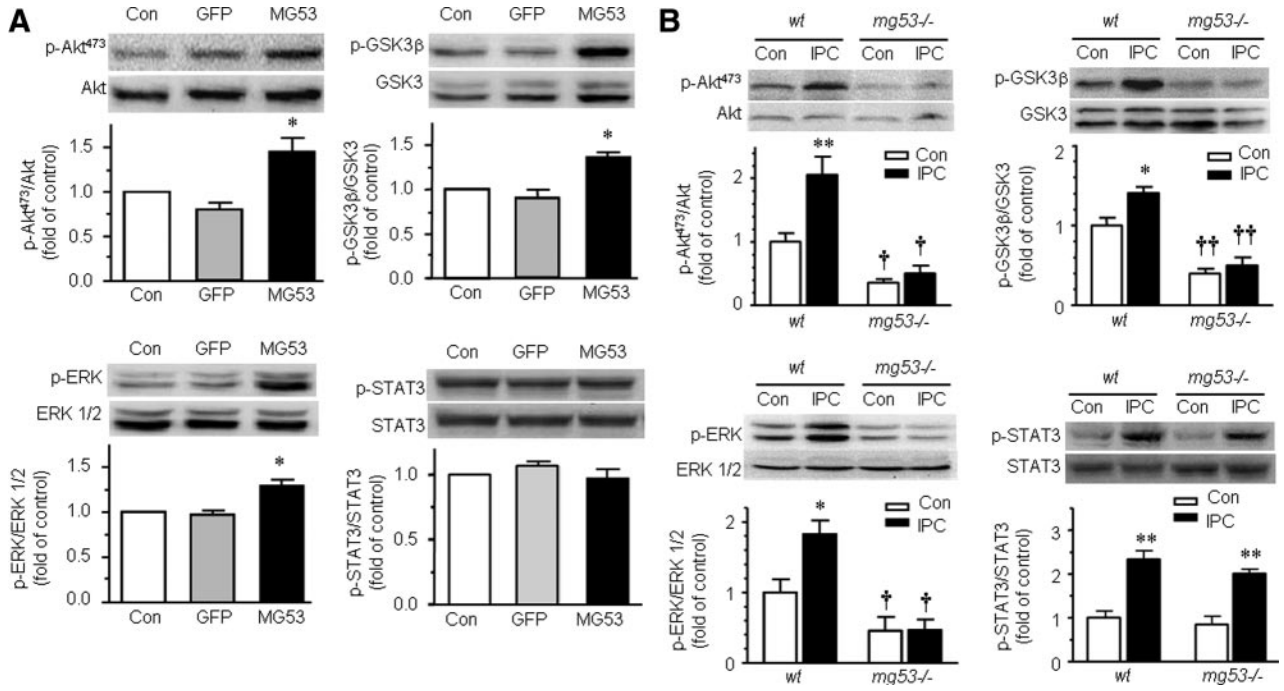

Figure 5. MG53 is essential for IPC-induced activation of the RISK pathway. A, Representative immunoblots and statistical data of phosphorylated and total Akt, GSK3β, ERK1/2, and STAT3 in lysates from NVMC with or without Adv-GFP or Adv-GFP-MG53 infection (n=9; *P<0.05 vs control [Con] and GFP). B, Representative immunoblots and statistical data of phosphorylated and total Akt, GSK3β, ERK1/2, and STAT3 in perfused *wt* and *mg53*$^{-/-}$ mouse hearts with or without IPC (n=8; *P<0.05, **P<0.01 vs *wt* control group; †P<0.05, ††P<0.01 vs the 2 *wt* groups).

Activation of the RISK Pathway Is Required for MG53- and IPC-Mediated Cardiac Protection

The lack of IPC protection in the *mg53*$^{-/-}$ heart may be attributable to the failure of IPC to activate the prosurvival PI3K-Akt-GSK3β and ERK1/2 signaling pathways. Indeed, in the *wt* mouse heart, inhibition of either the PI3K-Akt axis by a PI3K inhibitor, LY294002, or ERK1/2 by PD98059 completely abolished IPC-mediated reduction in infarct size (top panel) and decrease in LDH release (bottom panel) (Figure 6B). Furthermore, cardioprotection by MG53 overexpression was also abrogated by blockade of the PI3K-Akt axis with PI3K inhibitors (LY294002 and wortmannin) or an Akt inhibitor or ERK1/2 activity with PD98059 (Figure 6C). These results indicate that IPC-mediated cardioprotection requires MG53-dependent activation of the RISK pathway.

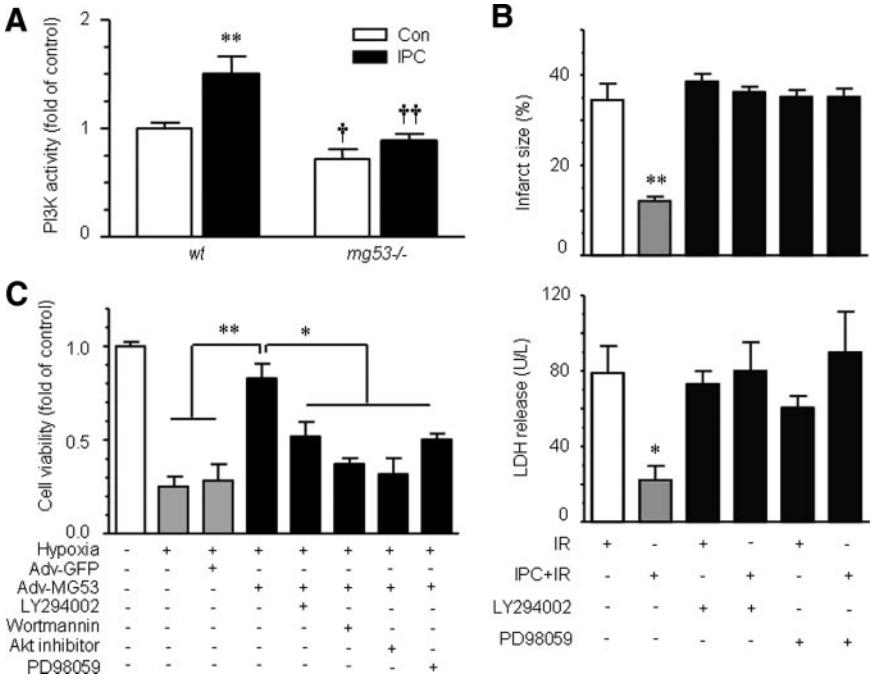

Figure 6. Activation of the RISK pathway is necessary for IPC-induced, MG53-dependent cardiac protection. A, PI3K activity in perfused hearts from *wt* and *mg53*$^{-/-}$ mice with or without IPC (n=4; **P<0.01 vs all of the other 3 groups; †P<0.05, ††P<0.01 vs the corresponding *wt* groups). Con indicates control. B, Statistical data of infarct size (upper) and LDH release (lower) of perfused *wt* mouse hearts subjected to 30-minute ischemia and 2-hour reperfusion with or without LY294002 (5 μmol/L) or PD98059 (10 μmol/L) pretreatment 10 minutes before IR or IPC+IR (n=8; *P<0.05, **P<0.01 vs all of the other groups). C, Protective effect of MG53 overexpression is abolished by inhibition of PI3K-Akt axis or ERK1/2 activity. Cell viability was assayed by cellular ATP content in NVMC infected with Adv-GFP or Adv-GFP-MG53 with or without 1-hour pretreatment with LY294002 (10 μmol/L), wortmannin (1 μmol/L), an Akt inhibitor (1 μmol/L), or PD98059 (10 μmol/L) (n=15; *P<0.05, **P<0.01 as indicated).

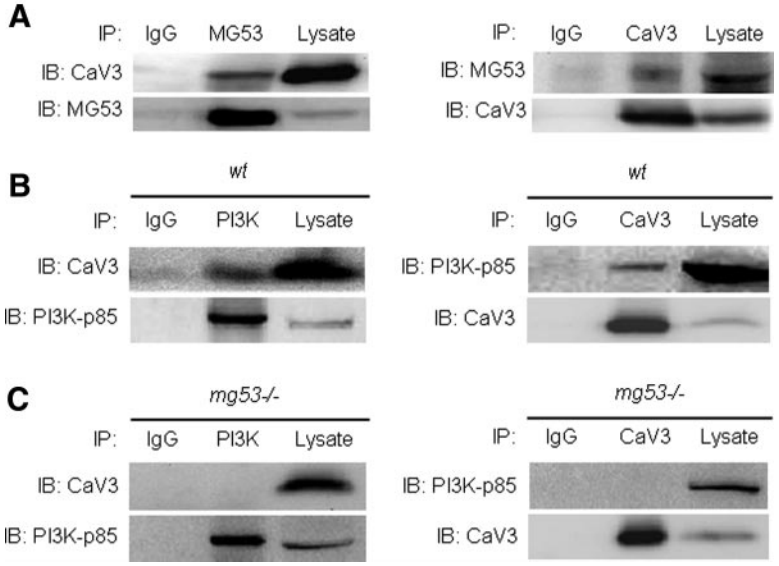

Figure 7. MG53 is required for CaV3 interaction with PI3K. A, Coimmunoprecipitation of endogenous MG53 and CaV3 in lysates of *wt* mouse hearts (n=4). B and C, Representative blots of lysates of *wt* (B) and *mg53*$^{-/-}$ hearts (C) for the coimmunoprecipitation of the p85 subunit of PI3K and CaV3 (n=6). Note that the intermolecular interaction between the p85 subunit of PI3K and CaV3 was disrupted in the *mg53*$^{-/-}$ heart. IB indicates immunoblots; IP, immunoprecipitation.

Interaction of MG53 With CaV3 Is Required for MG53-Mediated Activation of Prosurvival Kinases and Cardioprotection

The next question regards the manner in which MG53 participates in IPC-induced activation of the RISK pathway. Previous studies have shown that IPC-mediated cardioprotection involves the action of CaV3 at caveolae structures on the cell membrane[37,38] and that, when overexpressed, MG53 forms a protein complex with CaV3 in skeletal muscle to regulate the membrane trafficking and remodeling process.[24,39] In native myocardium, the physical interaction between MG53 and CaV3 was revealed by a coimmunoprecipitation assay (Figure 7A). In addition, in isolated adult cardiomyocytes, immunofluorescent signals of MG53 and CaV3 displayed a similar intracellular distribution at the light microscopic resolution (Figure V in the online-only Data Supplement).

To test whether the MG53-CaV3 complex directly interacts with components of the prosurvival PI3K-Akt-GSK3β signaling pathway, we performed coimmunoprecipitation assays and found a physical interaction between the p85 subunit of PI3K and CaV3 (Figure 7B). Interestingly, this interaction was disrupted in the *mg53*$^{-/-}$ heart (Figure 7C), although MG53 deficiency did not alter the expression of the p85 subunit of PI3K or CaV3 (Figure VI in the online-only Data Supplement). These results indicate that MG53 is required for CaV3 interaction with PI3K.

Using adenoviral-mediated delivery of shRNA against CaV3, we defined whether CaV3 is involved in MG53-mediated myocyte protection after hypoxia. In cells infected with Adv-MG53 and subjected to hypoxia, cell viability was reduced from 0.69±0.02- to 0.10±0.01-fold of control by transfecting cells with CaV3-shRNA but not scramble-shRNA (n=9; $P<0.01$), indicating that gene silencing CaV3 fully eliminated the protective effect of MG53 overexpression against hypoxic cell death (Figure 8A and 8B). This effect on cell survival was directly correlated with the degree of phosphorylation of Akt, GSK3β, and ERK1/2, and MG53 overexpression–induced hyperphosphorylation of Akt, GSK3β, and ERK1/2 was abolished after silencing CaV3 expression (Figure 8C and 8D and Figure VII in the online-only Data Supplement). These data suggest that the formation of a functional complex of MG53-CaV3-PI3K is obligatory to MG53-dependent activation of the PI3K-Akt-GSK3β and ERK1/2 signaling pathways and thereby to MG53-mediated cardioprotection.

Discussion

MG53, a novel protein primarily expressed in striated muscles, participates in acute membrane repair in skeletal muscle.[25] In the present study, we have provided multiple lines of evidence to define MG53 as an indispensable component of cardiac IPC machinery. First, hearts lacking MG53 are more vulnerable to IR injury, as manifested by increased infarct size and LDH release. Second, MG53-deficient hearts become resistant to IPC protection. In contrast, overexpression of MG53 protects cardiomyocytes against hypoxia- and oxidative stress–induced cell death. Mechanistically, intermolecular interaction of MG53 with CaV3 is a prerequisite for IPC-mediated activation of the prosurvival RISK pathway (mainly PI3K-Akt-GSK3β and ERK1/2 signaling events) without altering the SAFE pathway. Thus, MG53 is a powerful endogenous cardiac protective factor that plays an indispensable role in IPC-mediated myocardium protection.

PI3K-Akt-GSK3β Axis and ERK1/2 Signaling Cascade Are Primary Pathways Relaying IPC Response

A number of prosurvival kinases, including PI3K, Akt, GSK3β, and ERK1/2, which are known as RISK,[40] have been implicated in IPC-mediated cardioprotection.[13–16,41] Pharmacological and genetic studies have shown that the PI3K-Akt-GSK3β axis is essentially involved in IPC-induced myocardium protection.[12,13,15] However, whether ERK1/2 contributes to cardiac IPC response remains controversial.[41–43] In the present study, we have demonstrated that IPC-induced activation of PI3K-Akt-GSK3β and ERK1/2 pathways is MG53 dependent and that inhibition of either pathway fully blocks IPC-induced,

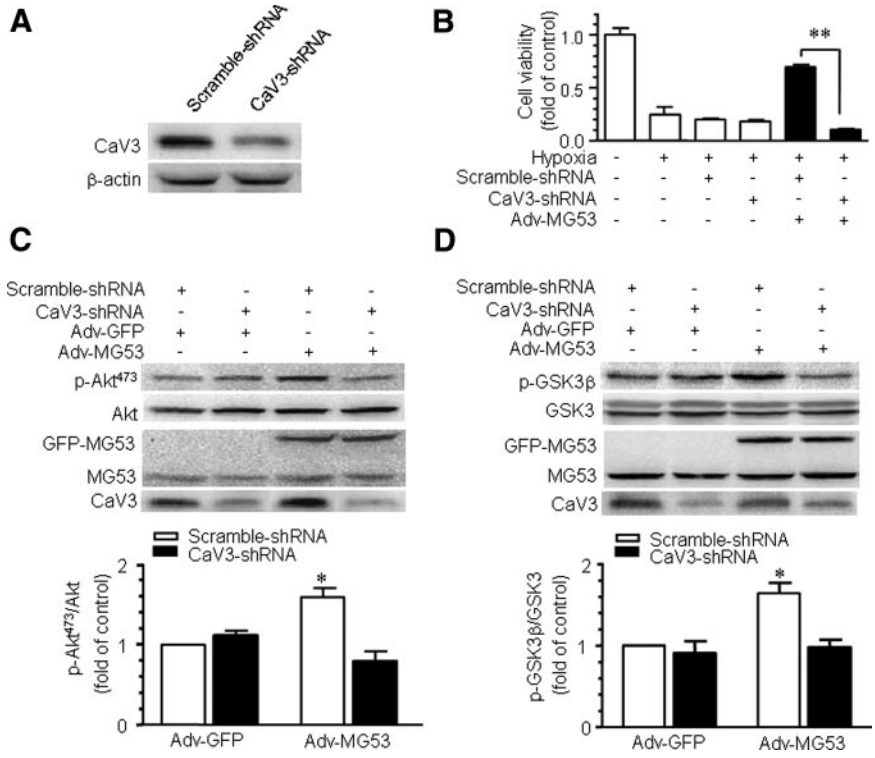

Figure 8. Gene silencing CaV3 blocks MG53 overexpression–mediated cardiomyocyte protection and activation of prosurvival kinases including Akt and GSK3β. A, Representative Western blots of CaV3 in the lysates of NVMC infected with Adv-scramble-shRNA or Adv-CaV3-shRNA (n=5). B, Cell viability of NVMC infected with Adv-MG53 and subjected to hypoxia for 12 hours with or without Adv-CaV3-shRNA or Adv-scramble-shRNA (n=9; $^{**}P<0.01$ as indicated). C and D, Representative immunoblots and statistical data of phosphorylated and total Akt (C) and GSK3β (D) in the lysates of NVMC infected with Adv-GFP or Adv-MG53 (30 multiplicity of infection, 24 hours) with or without Adv-CaV3-shRNA or Adv-scramble-shRNA (n=5; $^{*}P<0.05$ vs all of the other 3 groups).

MG53-dependent cardioprotection. These findings indicate that MG53 plays a crucial role not only in acute membrane repair but also in the IPC-activated prosurvival RISK pathway. In addition to the RISK pathway, activation of the SAFE pathway is now recognized as a RISK-free pathway that confers protection in IPC.[44,45] Interestingly, our data suggest that MG53 is involved in the RISK but not in the SAFE pathway in mouse myocardium (Figure 5A and 5B).

It is also noteworthy that, in the present study, we have demonstrated that MG53 is essentially involved in the activation of the RISK pathway in response to IPC even before the heart is subjected to index ischemia. In this regard, previous studies have shown that biphasic activation of survival kinases such as Akt and ERK1/2 occurs at IPC and during early reperfusion, respectively.[16] Whether MG53 also participates in the early reperfusion-induced activation of the RISK pathway merits future investigation.

Additionally, in MG53-deficient hearts, basal levels of the activation of PI3K, Akt, GSK3β, and ERK1/2 are significantly suppressed relative to those in *wt* counterparts, suggesting that retaining MG53 at the normal level plays a crucial role in the maintenance of myocardial integrity in addition to its contribution to IPC-induced cardioprotection. Indeed, MG53-deficient hearts display profoundly exaggerated damage in response to IR injury regardless of the presence or absence of IPC. Similarly, MG53 gene silencing acerbates hypoxia-induced cardiomyocyte death, highlighting the importance of MG53 in cardiac protective signaling.

MG53 Facilitates IPC Through Interaction With CaV3 and Activation of Prosurvival Factors

Caveolins have scaffolding domains that anchor and regulate the function of a variety of signaling proteins,[46] thereby providing temporal and spatial regulation of cellular signal transduction. In particular, CaV3 is a member of CaV family expressed mainly in striated muscles.[47] Although previous studies have shown that CaV1 (a CaV family member similar to CaV3) interacts directly with PI3K in tumor cell[48] and fibroblast,[49] there is no direct evidence that CaV3 shares the same ability to physically interact with PI3K. In the present study, we have demonstrated that CaV3 can physically associate with the p85-PI3K in myocardium in an MG53-dependent manner. This finding is supported by the previous notion that CaV3 activates downstream kinases of PI3K including Akt and GSK3β in the heart.[17]

Multiple lines of evidence suggest that the intermolecular interaction between MG53 and CaV3 is obligatory to IPC-induced, MG53-dependent activation of the RISK pathway. First, MG53 can physically interact with CaV3, as manifested by their coimmunoprecipitation. Second, the functional complex of MG53-CaV3 is required for the physical interaction of CaV3 with the p85 subunit of PI3K, an important upstream kinase of both Akt and GSK3β.[13,15] Furthermore, MG53 ablation blocks IPC-induced activation of PI3K. Equally important, CaV3 gene silencing prevents MG53-mediated phosphorylation of Akt, GSK3β, and ERK1/2 and prosurvival effects. Because MG53 ablation fully impairs IPC-induced activation of the RISK pathway and IPC protection, the MG53-CaV3 protein complex is likely a functional unit responsible for activating survival kinases, particularly PI3K-Akt-GSK3β and ERK1/2 cascades, thus resulting in IPC protection.

In summary, the present study has demonstrated that the *mg53*$^{-/-}$ heart has defective RISK signaling with an intact SAFE pathway and does not respond to IPC-mediated cardioprotection and that overexpression of MG53 enhances

Akt, GSK3β, and ERK1/2 phosphorylation and provides cardioprotective benefits. MG53 controls the RISK survival pathway through its interaction with CaV3 to activate the RISK signaling pathway. These present findings define MG53 as a primary component of the cardiac IPC response, thus providing a potentially important novel therapeutic target for the treatment of ischemic heart disease.

To translate our present bench discoveries into clinical medicine, many important issues need to be addressed. First, IPC can be temporally described as 2 phases: the first-window preconditioning (or the classic preconditioning), which occurs within minutes and lasts only a few hours, and the second-window preconditioning, which develops many hours after the early protection and lasts 3 to 4 days.[50] Although our study clearly indicates that MG53 is necessary for the classic preconditioning, it awaits future investigation to determine whether MG53 contributes to the second window of preconditioning. Second, the recently identified ischemic postconditioning, in which brief episodes of IR are applied at the onset of reperfusion, also confers powerful cardioprotection via signaling pathways similar to those in IPC.[21,51,52] Future study is required to define the potential role of MG53 in ischemic postconditioning. Finally, some important species differences exist in IPC and ischemic postconditioning signaling pathways.[53] Caution should be taken when we translate our bench discoveries from rodents into clinical application of MG53 in the treatment of human ischemic heart disease.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Cardiac ischemia is the current leading cause of death in the Western world. Because of the limited regenerative capacity of cardiomyocytes, ameliorating ischemia-induced myocardial damage is an important therapeutic target in the treatment of ischemic heart disease. The deleterious effects of cardiac ischemia are ameliorated by ischemic preconditioning (IPC), in which transient ischemia protects against subsequent severe ischemia/reperfusion. In the present study, we have identified MG53, a muscle-specific tripartite motif family protein (TRIM72), as a primary component of cardiac IPC response. MG53-mediated cardioprotection is, at least in part, independent of its known function in membrane repair because IPC profoundly suppresses apoptotic events, which do not involve breakdown of the sarcolemmal membrane, in an MG53-dependent manner. Because IPC is a powerful intrinsic mechanism against ischemia/reperfusion-induced myocardial damage, the identification of MG53 as a primary component of the cardiac IPC response opens a promising therapeutic avenue for the treatment of ischemic heart disease. To translate our bench discoveries from rodents into clinical medicine, many important issues need to be addressed, including determining the potential role MG53 in ischemic postconditioning, in which brief episodes of ischemia/reperfusion applied at the onset of reperfusion confer cardioprotection, in mammalian species including rodents, large animals, and humans. Relative to IPC, ischemic postconditioning is clinically more attractive because of its therapeutic application at the predictable onset of reperfusion. Because MG53 is a muscle-specific TRIM protein, an intriguing question is whether other members of the TRIM family contribute to organ protection, particularly in organs in which an IPC response can be observed.