Myocardial Hypertrophic Preconditioning Attenuates Cardiomyocyte Hypertrophy and Slows Progression to Heart Failure Through Upregulation of S100A8/A9

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Background—Transient preceding brief ischemia provides potent cardioprotection against subsequent long ischemia, termed ischemic preconditioning. Here, we hypothesized that transient short-term hypertrophic stimulation would induce the expression of hypertrophy regression genes and render the heart resistant to subsequent hypertrophic stress, and slow the progression to heart failure, as well.

Methods and Results—Cardiomyocyte hypertrophy was induced in mice by either transverse aortic constriction or an infusion of phenylephrine, and in neonatal rat ventricular cardiomyocytes by norepinephrine exposures. In the preconditioning groups, hypertrophic stimulation was provided for 1 to 7 days and then withdrawn for several days by either aortic debanding or discontinuing phenylephrine or norepinephrine treatment, followed by subsequent reexposure to the hypertrophic stimulus for the same period as in the control group. One or 6 weeks after transverse aortic constriction, the heart weight/body weight ratio was lower in the preconditioning group than in the control group, whereas the lung weight/body weight ratio was significantly decreased 6 weeks after transverse aortic constriction. Similar results were obtained in mice receiving phenylephrine infusion and neonatal rat ventricular cardiomyocytes stimulated with norepinephrine. Both mRNA and protein expression of S100A8 and S100A9 showed significant upregulation after the removal of hypertrophic stimulation and persisted for 6 weeks in response to reimposition of transverse aortic constriction. The treatment with recombiant S100A8/A9 inhibited norepinephrine-induced myocyte hypertrophy and reduced the expression of calcineurin and NFATc3, but the silencing of S100A8/A9 prevented such changes.

Conclusions—Preconditioning with prohypertrophic factors exerts an antihypertrophic effect and slows the progression of heart failure, indicating the existence of the phenomenon for hypertrophic preconditioning. doi: 10.1161/CIRCULATIONAHA.114.013789.

Key Words: cardiomegaly ■ heart failure ■ myocardial preconditioning ■ S100A8 protein ■ S100A9 protein

Myocardial hypertrophy is characterized by an increase of cardiomyocyte protein synthesis and cell volume, and it is crucial for the transition from adaptive to maladaptive cardiac function with the progression to irreversible changes. Although some extent of cardiac hypertrophy serves to reduce wall stress and compensate for an increased load on the myocardium,1 the effect of sustained prohypertrophic signaling on cardiomyocytes is detrimental and makes a major contribution to eventual progression to heart failure.2,3 Clinical and experimental studies have shown that the withdrawal of pressure overload, such as aortic debanding in animals and aortic valve replacement in patients with aortic stenosis, leads to the regression of myocardial hypertrophy4-6 and various beneficial molecular changes.4,7 It has been reported that intermittent systolic overload promotes the improvement of myocardial performance in adult animals,8 producing both a mild hypertrophic response and a favorable fetal gene expression profile.9 However, it is completely unknown whether the removal of short-term or long-term pressure overload renders

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the heart resistant to subsequent prolonged prohypertrophic stimulation.

The phenomenon of ischemic preconditioning whereby brief episodes of ischemia increase cardiac resistance to subsequent prolonged ischemia has received considerable attention since it was first reported by Murry et al in 1986. In addition to ischemia, pretreatment with hypoxia, hyperbaric oxygen, or certain drugs can induce this protective effect of preconditioning. Similarly, it would be plausible that short-term hypertrophic stimulation makes the heart resistant to subsequent hypertrophic stress. Indeed, an animal study has shown that a short-term antihypertensive therapy has a prolonged antihypertrophic effect on the myocardium and can protect the heart. In addition, it was reported that relief from cardiac pressure overload significantly alters the gene expression profile, including some of the known antihypertrophic genes. Thus, it appears that antihypertensive therapy or short-term hypertrophic stimulation creates an antihypertrophic memory, but it is unclear how long such an effect persists.

It is well known that a similar level of pressure overload (e.g., hypertension) can cause different degrees of myocardial hypertrophy. Also, the prevalence of myocardial hypertrophy is <50% in patients with essential hypertension, suggesting that factors that resist prohypertrophic stimulation exist in many patients. Experimental studies have demonstrated that some factors can prevent cardiac hypertrophy independent of an antihypertensive effect, but it remains unclear how to induce such antihypertrophic factors for therapeutic purposes. Based on the points mentioned here, we propose a new concept termed myocardial hypertrophic preconditioning. Our hypothesis is that short-term hypertrophic stimulation can render the heart resistant to subsequent hypertrophic stress and slow the progression to heart failure. In this study, we attempted to demonstrate the phenomenon of hypertrophic preconditioning and investigate the mechanisms involved.

Methods

All procedures were performed in accordance with our Institutional Guidelines for Animal Research and the investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised in 1996).

Cell Culture

The neonatal rats were euthanized by 2% isoflurane inhalation and cervical dislocation. The isolation and culturing of neonatal rat ventricular cardiomyocytes (NRVCs) and fibroblasts were performed as described previously. Three groups were designed: (1) the nor-epinephrine (NE) group: 1 μmol/L NE (dissolved in Dulbecco’s modification of Eagle’s medium) for 48 hours; (2) the preconditioning (Pre)+NE group: after stimulation for 12 hours, NE was removed for another 12 hours, and then NE was added again to stimulate for 48 hours; (3) the control group: Dulbecco’s modification of Eagle’s medium for 48 hours. Cell surface area and the expression of ANP, β-MHC, calcineurin, and nuclear factor of activated T cells (NFAT) in cardiomyocytes, procollagen I, and procollagen III mRNA in fibroblasts were harvested and analyzed.

The effects of recombinant murine S100A8 (also called myeloid-related protein [MRP] 8, Abcam), S100A9 (mouse MRP-14, Abcam) on NRVCs were examined. Five groups were designed as follows: (1) NE group: 1 μmol/L NE treatment for 48 hours; (2) NE+S100A8 group: treatment with 1 μmol/L NE and S100A8 (1 μg/mL) for 48 hours; (3) NE+S100A9 group: treatment with 1 μmol/L NE and S100A9 (1 μg/mL) for 48 hours; and (4) control group: treatment with Dulbecco’s modification of Eagle’s medium for 48 hours. After the culture, cell surface area and the expression of atrial natriuretic peptide (ANP) and β-myosin heavy chains (β-MHCs).

Animal Models and Experimental Protocols

Creation of Drug-Induced Myocardial Hypertrophy Model

C57BL/6 male mice (8–10 weeks, 20–25 g) were anesthetized with a mixture of xylazine (5 mg/kg IP) and ketamine (100 mg/kg IP). After anesthesia, the mice were subjected to subcutaneous pump implantation in the back with an osmotic minipump (Alzet) filled with phenylephrine (PE, 30 mg·kg⁻¹·d⁻¹, dissolved in 0.5 mmol/L ascorbic acid) or vehicle (0.5 mmol/L ascorbic acid). The incision was closed with 2 wound clips. For the preconditioning group, changing from PE to vehicle or vice versa was performed by removing the old pump and implanting a new one. Animals in the control group and PE group received a similar procedure for replacing of pump (filled with vehicle alone) during the preconditioning time window. These mice were euthanized by overdose anesthesia (pentobarbital sodium 150 mg/kg IP) and cervical dislocation at 4 to 8 days to obtain heart and calculate heart weight/body weight ratio. The mice were divided into 3 groups, with 6 to 7 mice in each group, as follows: (1) PE group: PE infusion (in ascorbic acid) for 4 days; (2) Pre+PE group: PE infusion for 1 day and then stop for 1 day, followed by PE infusion for 2 days and stop for 2 days, and finally recovered PE infusion for 4 days; (3) control group: ascorbic acid infusion for 4 days (Figure 1B).
Transverse Aortic Constriction Model
C57BL/6 male mice (8–10 weeks, 18–25 g) were subjected to transverse aortic constriction (TAC) or debanding or sham operation as described elsewhere. In brief, after a left-sided thoracotomy in the second intercostal space, a 7-0 silk ligature was tied around the transverse aorta and a 27-gauge blunted needle that was subsequently removed (Movie I in the online-only Data Supplement), whereas, in sham-operated animals, the ligature was tied loosely around the aorta. At the indicated time, a debanding operation was performed by carefully removing the ligature (Movie II in the online-only Data Supplement). To avoid possible confounding effects of the repeated surgical injury, sham or nonpreconditioning animals were also subjected to a similar open-chest operation. Two experimental protocols were designed for this model (Figure I in the online-only Data Supplement), and found that some animals displayed antihypertrophic phenomena even under a similar high-pressure overload, suggesting that antihypertrophic factors are inducible to render the heart resistant to the persistent pressure overload. Then we used hypertrophic Pre treatments that were designed with modification according to the ischemic preconditioning protocol to test whether hypertrophic preconditioning affords cardiac protection. In the cultured cardiomyocytes, we noted that NRVs showed a significant increase of cell size in response to NE stimulation, whereas preconditioning treatment suppressed this increase (Figure 2A). Meanwhile, the increased expression of fetal genes (ANP and β-MHC) in the preconditioning group was significantly attenuated (Figure 2B). These results indicate that hypertrophic preconditioning renders an antihypertrophic role in cardiomyocytes.

Antihypertrophic Effect of Hypertrophic Preconditioning In Vivo
In mice with induction of myocardial hypertrophy by persistent infusion of PE for a short term of 4 days, the heart weight/body weight ratio and expression levels of hypertrophic markers ANP and β-MHC were significantly smaller in the preconditioning group than in the PE group (Figure 2C and 2D, P<0.05), but no detectable difference was noted on myocardial fibrosis assessed with Masson trichrome staining (Figure 2E).

Using mouse TAC model, we noted that 1 week after TAC, the heart weight/body weight ratio was smaller in the preconditioning group than in the TAC group (5.35±0.17 mg/g versus 5.99±0.22 mg/g, P=0.014; Figure 3A). Our previous study showed that TAC mice may die of acute heart failure; thus, we here examined whether hypertrophic preconditioning exerts influence on survival. As shown in Figure 3B, the survival rate for the first 10 days after TAC was significantly lower in mice receiving preconditioning for 3 days than in mice with TAC alone, suggesting the acute cardioprotection of hypertrophic preconditioning. We further investigated the long-term effect of hypertrophic preconditioning on hypertrophy. At 6 weeks after TAC, heart weight/body weight ratio was significantly smaller in the 2 preconditioning groups than in the TAC group (7.16±0.33 mg/g for TAC, 5.32±0.14 mg/g for Pre1+TAC, and 5.43±0.11 mg/g for Pre2+TAC, P<0.01; Figure 3C and 3D), whereas the cardiomyocyte area was significantly smaller in the Pre1+TAC and Pre2+TAC groups than in the TAC group (Figure 3E). In addition, the increase of fetal gene expression (ANP and β-MHC) was significantly attenuated in the 2 preconditioning groups (Figure 3F). The above findings indicate that hypertrophic preconditioning in vivo improves acute-phase survival and attenuates myocardial hypertrophy.

Echocardiography, Western Blot, Polymerase Chain Reaction Immunofluorescence, Construction of Lentivirus Carrying Overexpressed or Short-Hairpin RNA for S100A8 or A9, Cell Viability Assay, and Histological Examinations
See details in Materials in the online-only Data Supplement. Infection efficiency and the expression levels of targeted genes S100A8 or A9 are shown in Figure II in the online-only Data Supplement. Sequences of primers for routine polymerase chain reaction, quantitative real-time polymerase chain reaction, and synthesis of S100A8/A9 cDNA are shown in Tables I through III in the online-only Data Supplement, respectively.

Statistical Analysis
Quantitative data are expressed as mean±standard error of the mean. For all statistical tests, multiple comparisons were performed by 1-way analysis of variance with the Bonferroni multiple comparison test (SPSS 16.0). The least-squares method was used to assess linear correlations between selected variables. The overall survival of TAC mice for 10 days was evaluated by using Kaplan-Meier survival analysis, and groups were compared by the log-rank test. P values of <0.05 were considered to be statistically significant.

Results
Antihypertrophic Effect of Hypertrophic Preconditioning In Vitro
Using our database of TAC or sham mice, we analyzed cardiac hypertrophy, pulmonary congestion, and left ventricle hemodynamics in 74 C57 male mice subjected to either TAC or sham operation for 4 to 8 weeks (Figure IIIA through IIIC in the online-only Data Supplement), and found that some animals displayed antihypertrophic phenomena even under a similar high-pressure overload, suggesting that antihypertrophic factors are inducible to render the heart resistant to the persistent pressure overload. Then we used hypertrophic Pre treatments that were designed with modification according to the ischemic preconditioning protocol to test whether hypertrophic preconditioning affords cardiac protection. In the cultured cardiomyocytes, we noted that NRVs showed a significant increase of cell size in response to NE stimulation, whereas preconditioning treatment suppressed this increase (Figure 2A). Meanwhile, the increased expression of fetal genes (ANP and β-MHC) in the preconditioning group was significantly attenuated (Figure 2B). These results indicate that hypertrophic preconditioning renders an antihypertrophic role in cardiomyocytes.

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Hypertrophic Preconditioning Slows Progression of Cardiac Remodeling
In TAC mice, serial echocardiography showed a time-dependent increase of LV end-diastolic dimension and LV end-systolic dimension (Figure 4A and 4B), and of diastolic and systolic LV wall thickness (Figure 4C and 4D), as well, whereas LV fractional shortening decreased over time (Figure 4E). In contrast, hypertrophic preconditioning significantly slowed the increase of LV wall thickness (Figure 4C and 4D), the enlargement of LV dimensions (Figure 4A and 4B), and the decline of LV fractional shortening (Figure 4E). No significant differences were noted between the 2 preconditioning groups (Figure 4).
Hypertrophic Preconditioning Improves the Pathophysiology of Heart Failure

TAC induced congestive heart failure (HF) with an increase of the LW/BW. Six weeks after TAC, the LW/BW was markedly smaller in the Pre1+TAC and Pre2+TAC groups than in the TAC group (9.88±1.00 mg/g for TAC, 5.98±0.12 mg/g for Pre1+TAC (P=0.008), and 6.15±0.11 mg/g for Pre2+TAC (P=0.046); Figure 1A). In addition, histological examination showed that myocardial fibrosis and perivascular fibrosis were significantly attenuated in both preconditioning groups in comparison with the TAC group (Figure 1B). Echocardiographic LV dimensions (Figure 1C) were smaller, LV fractional shortening was larger (Figure 1D), LV end-diastolic pressure was lower (Figure 1E), and LV contractility (Figure 1F) was higher in the preconditioning groups than in TAC alone group (all P<0.05). No significant differences were noted between them on LV posterior wall thickness (LV cavity enlargement in TAC group would decrease wall thickness), LV systolic pressure (suggesting similar pressure overload), heart rate, and LV pressure change rate (Figure IV in the online-only Data Supplement). These findings indicated that hypertrophic preconditioning has an inhibitory effect on cardiac hypertrophy and HF. We subsequently investigated the possible mechanisms involved.

Upregulation of S100A8/A9 After Withdrawal of Prohypertrophic Stimulation

S100A9 was reported to be one of the genes that is specifically induced during the regression of cardiac hypertrophy, so we examined the expression of S100A8 and S100A9 after the removal of stimulation. We found that expression of S100A8 and S100A9 mRNA and their corresponding proteins in cultured NRVCs was similar between control cells and NE-stimulated cells, but was markedly upregulated at 12 hours after the withdrawal of NE (Figure 2A). Consistent with these findings, myocardial gene and protein expression
of S100A8 and S100A9 was also significantly increased in mice 1 day after debanding that had been preceded by 3 days or 1 week of TAC (Figure 6C through 6E).

We further checked how long the upregulation of S100A8/A9 would persist after reimposition of pressure overload followed by debanding for 4 days. As shown in Figure 6D and 6E, S100A8 or A9 was significantly increased in response to debanding, which was continued until 1 week and 6 weeks after reimposition of hypertrophic stimuli.

Recombinant S100A8/A9 Attenuates Hypertrophy and Fibrosis In Vitro

We further investigated whether recombinant S100A8 and S100A9 proteins had antihypertrophic effects in cultured NRVCs and fibroblasts. As shown in Figure 7A and 7B, the treatment with either S100A8 or S100A9 (or both proteins) significantly suppressed the NE-induced increase in the surface area of cardiomyocytes. In comparison with control cells, exposure to NE for 48 hours increased the expression of ANP and β-MHC mRNA in NRVCs (Figure 7C and 7D), and the expression of procollagen I and III mRNA in fibroblasts, as well (Figure 7E), whereas treatment with S100A8, S100A9, or both of these proteins prevented such changes (Figure 7C through E). These findings suggested that S100A8 and S100A9 could attenuate NE-induced hypertrophy and fibrosis in cultured cardiac cells.

The exposure of NRVCs to NE resulted in increased expression of calcineurin, but this was abrogated by the treatment with either S100A8 or S100A9, or both of these proteins (Figure 7F and 7H). When the subcellular localization of NFATc3 was assessed by Western blotting, it was primarily localized in the cytoplasm of control cells and underwent...
translocation to the nucleus in response to NE stimulation, whereas treatment with S100A8 or S100A9, or both of these proteins, inhibited NE-induced nuclear translocation of NFATc3 (Figure 7G and 7H).

Silencing of S100A8/A9 Attenuates the Anti hypertrophic Effects of Hypertrophic Preconditioning

The aforementioned results suggest the important role of S100A8/A9 in myocardial hypertrophy. We then used approaches for gain and loss of function to further address this issue. Infection of cardiomyocytes with lentivirus-S100A8 or A9 upregulated S100A8 or A9 by >200-fold (Figure II in the online-only Data Supplement), which was much higher than the upregulation amplitude in response to debanding (about 6-fold). The cell viability test showed that high-dose overexpression of endogenous S100A8 or A9 and a high dose of exogenous S100A8 or A9 increased cardiomyocyte death (Figure V in the online-only Data Supplement), suggesting that the role of S100A8/A9 is dose dependent. Accordingly, we chose the S100A8/A9-silencing approach. Lentivirus (Lv) carrying short-hairpin (Sh) RNA for S100A8/A9 led to a significant silencing effect (Figure 8A). In comparison with the preconditioning group, both Lv-Sh-S100A8 and A9 significantly reduced the hypertrophic preconditioning effects manifested by an increase of cardiomyocyte cell surface area (Figure 8B), the upregulation of ANP and β-MHC in cardiomyocytes (Figure 8C), and the upregulation of procollagen5s in fibroblasts (Figure 8D), increase of calcineurin protein levels (Figure 8E), and nuclear translocation of NFAT3 (Figure 8F).

In cardiomyocytes infected with both Lv-Sh-S100A8 and A9, a low dose of exogenous S100A8 or A9 still significantly inhibited NE-induced upregulation of calcineurin protein (Figure 8G), suggesting that exogenous S100A8 or A9 can work as nonheterodimer.

Discussion

This study provided evidence for a new concept termed myocardial hypertrophic preconditioning. We demonstrated that preconditioning by prohypertrophic factors increases the resistance of the heart to subsequent hypertrophic stress and delays the progression from hypertrophy to HF, indicating the existence of the hypertrophic preconditioning phenomenon. We further showed that upregulation of S100A8/A9 following removal of transient hypertrophic stimulus contributes to the anti hypertrophic and anti-HF effect of hypertrophic preconditioning, at least in part by suppressing the calcineurin/NFAT pathway.

Cardiac protection by hypertrophic preconditioning has already been reported in the setting of congenital heart disease. In patients with transposition of the great vessels, the LV does not develop properly because it is pumping against low resistance and needs to be strengthened by applying a pulmonary artery band in preparation for corrective surgery. Traditional banding procedures quickly reach the target level of stenosis for ventricular retraining, but cause the abrupt onset of fixed systolic overload that can result in LV failure. Experimental studies have shown that myocardial edema and necrosis occur in hearts with abrupt systolic overload, usually followed by the development of ventricular failure. In contrast, Sekarski et
al demonstrated that ventricular retraining with an adjustable banding device (the target stenosis was gradually reached by the telemetric control system) led to better survival. In addition, Miana et al. reported that intermittent systolic overload by pulmonary banding promoted better myocardial performance in goats, mimicking the physiological hypertrophy achieved by exercise in athletes. These results support our finding that hypertrophic preconditioning improves HF.

In patients with aortic stenosis, the regression of cardiac hypertrophy occurs after aortic valve replacement. Mechanical unloading can cause the regression of hypertrophy and functional improvement, during which process certain genes may be specifically upregulated to either block hypertrophic signaling pathways or trigger atrophic signaling pathways. Yang et al. identified a set of genes specifically induced during the regression of hypertrophy, and confirmed that eyes absent homolog (eya2) blocks the development of cardiomyocyte hypertrophy. Among those genes induced during the regression process, S100A9 was upregulated by ≈6-fold, but its role in myocardial hypertrophy and HF remains elusive. S100A8 (calgranulin A or migration inhibitory factor–related protein 8 [MRP-8]) and its binding partner S100A9 (calgranulin B, or MRP-14) are members of the S100 calcium-binding family of proteins, which have anti-inflammatory and immunoregulatory actions. Although the expression of both S100A8 and S100A9 was reported to be increased in acute coronary syndromes, atherosclerosis, and endotoxin-induced cardiac dysfunction, their exact roles in hypertrophy and HF remain to be elucidated.

Figure 5. Effects of hypertrophic preconditioning (Pre or P) on cardiac function and fibrosis. A, Representative macroscopic appearance of the lungs in each group. Scale bar, 2 mm. B, Lung weight/body weight (LW/BW) ratio at 6 weeks after TAC, n=6 or 7 in each group. C, Representative Masson-stain pictures of perivascular and myocardial fibrosis from each group. Scale bar, 50 μm. D, Quantitative analysis of perivascular fibrosis, n=4 per group. E, Quantitative analysis of myocardial fibrosis, n=4 per group. For B, D and E, *P<0.05, **P<0.01 vs TAC group. Before euthanization, left ventricular dimensions (LVD; F) and LV fractional shortening (LVFS; G) were measured by using echocardiography, whereas LV end-diastolic pressure (LVEDP; H) and LV contractility (I) were measured by using a Millar catheter. For F through I, n=5 to 7, *P<0.05, **P<0.01 vs TAC group. LV indicates left ventricular; LVEDd, left ventricular end-diastolic dimension; and TAC, transverse aortic constriction.
have not been clarified. However, cardiac overexpression of S100A8 and S100A9 was reported to decrease calcium flux\(^{39}\), suggesting that these genes may exert an antihypertrophic effect. In the present study, we demonstrated that treatment with S100A8 and S100A9 inhibited NE-induced cardiomyocyte hypertrophy. Fibrosis is well known to play a critical role in chronic HF. Degradation and accumulation of extracellular matrix are important in the process of LV remodeling, and it has been proposed that matrix metalloproteinases can be used as markers of inflammation and fibrosis.\(^{40}\) In agreement with a recent report\(^{41}\) that stimulation of chondrocytes with S100A8 reduces the viability of cardiomyocytes, whereas a low dose of S100A8 exerted no harmful effect, which is in agreement with previous studies showing that a high dose of S100A8 (30 μg/mouse) aggravates lung injury,\(^{42}\) whereas its low dose (10 μg/mouse) protected from acute lung injury.\(^{43}\) Several lines of recent evidence support the idea that S100A8/A9 may exert cell-protective roles. S100A8 was reported to promote angiogenesis,\(^{44}\) which should be beneficial for the improvement of cardiac dysfunction by alleviating the relative ischemia of hypertrophied myocardium. S100A9 knockout mice increased renal damage and fibrosis in response to ischemia/reperfusion,\(^{46}\) and S100A8/A9 also showed the ability to inhibit the cell growth of cancer,\(^{47}\) lending support to our finding that hypertrophic Pre attenuates myocardial fibrosis and myocyte hypertrophy, in part, by the upregulation of S100A9. Similar to ischemia preconditioning, it is plausible that the mechanisms of hypertrophic preconditioning may be far more complicated than the involvement of S100A8/A9, which needs to be clarified in future studies.

Ca\(^{+2}\) is essential for transcriptional activation during cardiac hypertrophy. Among the Ca\(^{+2}\)-dependent signaling pathways implicated in cardiac hypertrophy, the activation of calcineurin and subsequent nuclear translocation of NFAT are particularly...
As members of the S100 calcium-binding family of proteins, S100A8 and S100A9 are likely to exert their intracellular regulatory activities by interacting with specific targets in a Ca\(^{2+}\)-dependent manner. Using cultured cardiomyocytes, we showed that NE increased the expression of calcineurin and nuclear translocation of NFAT, whereas treatment with S100A8 and S100A9 prevented these changes, indicating that S100A8 and S100A9 attenuate cardiac hypertrophy by inhibiting the calcineurin/NFAT–signaling pathway.

It is likely that other factors induced after withdrawal or attenuation of hypertrophic stimulation may contribute to protection of the heart in addition to S100A8/A9. A clinical investigation has shown that periodic intravenous infusion (5 consecutive days every 6 weeks) of iloprost (a prostacyclin analog) protects against the onset or exacerbation of pulmonary artery hypertension and decreases the serum level of N-terminal pro B-type natriuretic peptide, effects similar to those of hypertrophic Pre. Because iloprost has a short half-life of 20 to 30 minutes, its pharmacological antihypertensive effect would not persist for as long as 6 weeks, suggesting that 5 days of treatment with iloprost induces the production of antihypertensive factors that prevent the development of pulmonary hypertension after its withdrawal. Similarly, several experimental studies have demonstrated that antihypertensive

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**Figure 7.** Effect of S100A8/A9 treatment on myocyte hypertrophy in cultured neonatal rat ventricular cardiomyocytes (NRVCs) and fibroblasts. A, Cardiomyocytes stained with α-actin and DAPI. B, Semiquantitative analysis of cardiomyocyte area. C, PCR for ANP and β-MHC expression in NRVCs. Real-time quantitative PCR for expression of ANP and β-MHC in NRVCs (D) and for expression of procollagen I and procollagen III in fibroblasts (E). F, Representative Western blot of calcineurin in NRVCs treated with recombinant S100A8/A9. G, Detection of nuclear factor of activated T cells (NFAT) in subcellular fractions by Western blotting. Histone 3 was the loading control for nuclear extracts, and β-actin was the loading control for cytosolic extracts. H, Semiquantitation of calcineurin and NFATc3 Western blotting results in NRVCs (n = nuclear, c = cytosolic). *P<0.05, #P<0.01 vs NE group, experiments were repeated for 3 to 5 times. ANP indicates atrial natriuretic peptide; DAPI, 4′,6-diamidino-2-phenylindole; β-MHC, β-myosin heavy chain; NE, norepinephrine; and PCR, polymerase chain reaction; A8, S100A8; A9, S100A9; A8/9, S100A8/9.
Inhibition of the compensatory hypertrophy is traditionally believed to be detrimental for cardiac function. However substantial evidence from experimental studies, especially from gene-targeted animals, call into question the necessity of hypertrophic growth of the heart as a compensatory response to hemodynamic stress.\(^{56}\) In the present study, it is plausible to postulate that preconditioning stimuli (short-term TAC) per se should induce a compensatory hypertrophy and then inhibit the progression of hypertrophy (maybe pathological or decompensatory phase) in response to reimposition of hypertrophic stimuli.

Figure 8. Silencing of S100A8/A9 antagonizes the effect of hypertrophic preconditioning in neonatal rat ventricular cardiomyocytes (NRVCs) and fibroblasts. S100A8 and S100A9 were knocked down by using short hairpin of RNA for S100A8 (ShA8) and S100A9 (ShA9) and then constructed into lentivirus. A, Silencing effect of ShA8 and ShA9. *P<0.05 vs vector, n=3. B, Representative pictures of cardiomyocytes stained with α-actin and DAPI and semiquantitative analysis of cardiomyocyte area. Real-time quantitative PCR for expression of ANP and β-MHC in NRVCs (C) and for expression of procollagen I and procollagen III in fibroblasts (D). E, Western blot analysis of calcineurin expression in cardiomyocytes. *P<0.05 vs P+NE group. F, Western blot analysis of nuclear factor of activated T cells (NFAT) in subcellular fractions by Western blotting in cardiomyocytes. Histone 3 and β-actin were the loading control for nuclear (n) extracts and cytosolic (c) extracts, respectively. *P<0.05 vs P+NE group. G, Western blotting of calcineurin in cardiomyocytes with S100A8/A9 silencing and NE stimulation (1 μmol/L) in the presence/absence of exogenous S100A8 or S100A9 (1 μg/mL). *P<0.05 vs ShA8/9+NE group. All experiments were repeated 3 to 5 times. ShA8/9 = short hairpin RNA of S100A8/9, multiplicity of infection (MOI)=5 for ShA8 or ShA9. ANP indicates atrial natriuretic peptide; β-MHC, β-myosin heavy chain; NE, norepinephrine; P, preconditioning; and PCR, polymerase chain reaction.
In conclusion, this study provided the first evidence for the phenomenon of myocardial hypertrophy preconditioning. We demonstrated that preconditioning by prohypertrophic factors exerts an antihypertrophic effect and slows the progression of HF, indicating the existence of hypertrophic preconditioning. Suppression of the calcineurin/NFAT pathway by S100A8/A9 partially explains the cardiac protection of hypertrophic preconditioning.

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Disclosures
None.

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Myocardial Hypertrophic Preconditioning Attenuates Cardiomyocyte Hypertrophy and Slows Progression to Heart Failure Through Upregulation of S100A8/A9
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Supplemental Methods

**Echocardiography**

Both cardiac function and remodeling were dynamically evaluated in mice by echocardiography using a Sequoia 512 system with a 17L-5 probe(Siemens, Germany). Two-dimensional parasternal short-axis images of the left ventricle (LV) were obtained at the level of the papillary muscles. From M-mode tracings, the LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), LV diastolic posterior wall thickness (LVPWd), LV posterior wall systolic thickness (LVPWs) and LV fractional shortening (LVFS) were measured.

**Western Blot**

Total proteins were obtained from whole heart homogenates or cultured cells. Nuclear and cytoplasmic protein extracts were made using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific Pierce) according to the manufacturer’s instructions. Samples were loaded onto 8-15% SDS-polyacrylamide gels and the proteins were transferred to polyvinyl difluoride membranes. The membranes were blocked with 5% skim milk at room temperature for 2 h, and then incubated overnight at 4°C with the primary antibodies. The following antibodies were used for the Western blotting analysis: Anti-S100A8 (BM4029B, Acris) and anti-S100A9 antibodies (ab75478, Abcam), Anti-calcineurin A antibody (ab3673, Abcam), anti-NFATc3 (#sc-8321, Santa Cruz), Anti-Histone H3 (#4499, CST). The blots were detected using a SuperSignal ECL kit (Invitrogen, Carlsbad, CA) in a Western blotting detection system (Kodak Digital Science, Rochester, NY) and quantified by densitometry using the Image J Analysis software (National Institutes of Health, Bethesda, MD)

**Polymerase Chain Reaction**

Total RNA was extracted from cultured cells and murine hearts with a total RNA isolation system (Omega, USA). The sequences of the primers for ANP, β-MHC, and GAPDH are detailed in Supplementary material online (Table S1). Products were quantified by using Image J Analysis Software.

**Real-time PCR**

Real-time PCR system (7500 Real time PCR system, Applied Biosystems; USA) and Quantitect SYBR Green real-time PCR method were used for detection and quantification. The sequences of the primers for ANP, β-MHC, S100A8, S100A9, procollagen I and procollagen III and GAPDH are detailed in Supplementary material online (Table S2)

**Immunofluorescence**

The cells were fixed using 4% paraformaldehyde for 15 min. After washing and permeabilized with 0.1% Triton X-100 for 5 min, the cells were blocked with a 5% solution of BSA (1 h, room temperature). Primary antibodies, anti-NFATc3 (#sc-8321, Santa Cruz) and anti-α-actin (Santa Cruz) were applied overnight at 4°C. The corresponding secondary antibodies were added for incubation for 30 min at room temperature. To visualize nuclei, fixed cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes.
Construction of recombinant lentivirus carrying short hairpin RNA (shRNA) for S100A8 or A9
The shRNA target sequences of S100A8 and A9 genes were selected using RNAi Target Sequence Selector and the shRNA oligonucleotides were designed using shRNA Sequence Designer (Clontech web) and then synthesized. After annealing, the shRNA oligonucleotides were cloned into the Lentiviral vector pLVX-shRNA2 which contains the ZsGreen1 marker (Clontech, 632179, Mountain view, CA) to construct pLVX-shRNA2-S100A8 or A9 and empty vector (pLVX-shRNA2-NC, negative control). The recombinant pLVX-shRNA2-S100A8 or A9 vector and the Lenti-X HTX Packaging System were used to produce high-titer lentivirus from 293T packaging cells.

Construction of recombinant lentivirus carrying S100A8 or A9
The full coding sequence of S100A8- or A9-IRES-ZsGreen1 was obtained by whole gene synthesis (primer sequences for cDNA synthesis are shown in Table S3) and then cloned into the Lentivirus vector pLVX-IRES-Neo (Clontech, 632181, Mountain view, CA) to construct pLVX-Mrp8/14-IRES-ZsGreen1-IRES-Neo or empty vector (pLVX-IRES-ZsGreen1-IRES-Neo). The S100A8 or A9 cDNA clones were sequenced completely to confirm the absence of cloning artifacts and mutation. The recombinant lentivirus amplification and titer determination were conducted according to the Lenti-X™ Lentiviral Expression Systems User Manual (Clontech; PT5135-1).

Infection of recombinant lentivirus in neonatal rat cardiomyocytes and fibroblasts
The overexpression or knockdown of S100A8 or A9 was achieved by transfecting cultured neonatal rat cardiomyocytes with the recombinant lentivirus (multiplicity of infection (MOI) =5). After transfection for 24hr, the virus-containing transduction medium was replaced with fresh growth medium. Further incubating the cells for 72 hours to allow the recombinant lentivirus to achieve the maximum effect. Infection efficiency and silencing/overexpression effect were evaluated using a fluorescence microscopy, real-time-PCR (Primers sequences were listed in Table S3) and western blot.

Cell viability assay
After trypsinization, neonatal rat cardiomyocytes were seeded and cultured on 96-well plates at an initial density of 0.2 × 10⁴/well. Cells were then stimulated with lentivirus carrying S100A8/A9 (MOI = 5-15) for 72 hours or recombinant S100A8/A9 (1 and 10 μg/mL) for 48 hours. Cell viability was measured using methyl thiazolyl tetrazolium (MTT) assay. For this, 0.02 mL of MTT solution [5 mg/mL in PBS (Phosphate Buffer Solution)] was added to each well, and incubated for 4 h at 37°C. Thereafter, the medium was replaced by 0.15 mL dimethylsulfoxide for 10 min incubation. The optical density (OD) at 492 nm was measured by Microplate spectrophotometer (Thermo Scientific, Franklin, MA, USA). All experiments were performed in triplicate.

Histological examinations
Hearts were fixed in 10% formalin, then dehydrated and embedded in paraffin, and 4-μm-thick sections were cut and stained with Hematoxylin and Eosin or Masson's trichrome. Cross section area of cardiomyocytes and myocardial fibrosis were calculated using Image J software.
### Supplemental Tables

#### Table S1. Sequences of the primers for routine PCR

| Transcript      | Forward primer (5’–3’) | Reverse primer (5’–3’) | Size (bp) |
|-----------------|------------------------|------------------------|-----------|
| ANP (mouse)     | GGCTCCTTCTCCATCACCAA   | TGTTATCTTCCGTTACCAG    | 420       |
| β-MHC (mouse)   | TCGTGGAGCGGCGCAACAC    | TCAAGGGCTCCAGTGTCAGGCT | 849       |
| GAPDH (mouse)   | ACCAACTGCTTACCCCCC     | GCATGTCAGTCACCAAACCG   | 281       |

The primers for ANP and β-MHC were also used for PCR with rat cardiomyocytes because the good homology.

#### Table S2. Sequences of the primers for real-time PCR

| Transcripts     | Forward primer (5’–3’) | Reverse primer (5’–3’) | Size (bp) |
|-----------------|------------------------|------------------------|-----------|
| ANP (rat)       | CTCCGATAGATCTGCCCTCTGAA| GGTACCAGGAGCTTGCAGCCTA | 216       |
| β-MHC (rat)     | ATCAAGGAAAGCAGGAGAAGGC| CCTGTCTACAGGTGCATCA    | 196       |
| β-actin (rat)   | ATCGTGCGGGACATCAAGG    | CAGGAAGGAGGCTAGAACA    | 180       |
| Procollagen I (rat) | CTCGTCACAGCCTTCAC     | AATCCAGTAGTAATCGCTCTTC | 176       |
| Procollagen III (rat) | CTACACCTGCTCTCTCATT   | CCACCATTCTCCGACT      | 232       |
| GAPDH (mouse)   | ATGTGTCCGTCGTGGATCTGA | TTGCTGTGAAGTCGAGGAG    | 151       |
| S100A8 (rat)    | CTGAGTGCCCTCAGTTTGTCAG| GCTCTGTACTCTTTGTCGTC  | 158       |
| S100A9 (rat)    | AGCTGGAGCGCAGACATAAGC | TTCCGCCTTTCAGGAGGTGTC  | 89        |

The primers for S100A8/A9 were also used for PCR with mouse myocardial tissue because the good homology.
Table S3  Primer sequences for synthesis of S100A8 and S100A9 cDNA

| Primers        | sequences(5’-3’)                                           |
|----------------|-------------------------------------------------------------|
| S100a8-rat-F   | gatccGCTCATAAAGACAGCCACATTCAAGAGATGATGGCTGTCTTTATGAGCTTTTTACGCGTg |
| S100a8-rat-R   | aattcACGCGTAAAAAAAGCTCATAAAAGACAGCCACATCTCTTTTATGAGCTTTTTATGAGCg |
| S100a9-rat-F   | gatccGCTCTAGGAAGTATGGACATTTCAAGAGATGGCTGTCTTTATGAGCTTTTTACGCGTg |
| S100a9-rat-R   | aattcACGCGTAAAAAAACTCTAGGAAGTATGGACATTTCAAGAGATGGCTGTCTTTATGAGCg |
Supplemental Figures

Figure S1

Figure S2
Figure S3
**Figure S4**

A) LVPWd (mm) vs Sham, TAC, TAC+P1, TAC+P2

B) LVSP (mmHg) vs Sham, TAC, TAC+P1, TAC+P2

C) Heart rate (bpm) vs Sham, TAC, TAC+P1, TAC+P2

D) LVdp/dt (mmHg/s) vs dp/dt max, dp/dt min

**Figure S5**

Lv-A8(MOI): - 5 10 15 - - - - - - - - -
Lv-A9(MOI): - - - - - - - 5 10 15 - - - -
r-A8(μmol): - - - - - - - 1 0 10 - - - -
r-A9(μmol): - - - - - - - 0 1 0 10 - - - -
Supplemental Figure Legends

Figure S1 Cardiac hypertrophy induced by transverse aortic constriction (TAC) for 3 days or 1 week. Heart weight/body weight ratio (HW/BW) was significantly increased in response to TAC for either 3 days or 1 weeks. *$P < 0.01$ vs. Sham group. Diamonds: mean ± SE.

Figure S2 Infection efficiency of lentivirus carrying S100A8/A9 or hairpin RNA for S100A8 and the corresponding expression levels of S100A8 or A9 in neonatal rat cardiomyocytes. Satisfactory efficiency of lentivirus (Lv) was reached when multiple of infection (MOI) = 5. Overexpression of S100A8 (Lv-A8) or S100A9 (Lv-A9) increased the gene expression levels by 258 and 698 folds, respectively, while silencing them using short hairpin RNA (ShRNA) led to downregulation of S100A8 and S100A9 by more than 95%. Lv-shA8: lentivirus carrying short hairpin RNA for S100A8; Lv-shA9: lentivirus carrying short hairpin RNA for S100A9. *$P < 0.001$ vs. Control.

Figure S3. Antihypertrophic factors exist in pressure overload mice. C57 mice (male) were subjected to transverse aortic constriction (TAC) or sham operation for 4-8 weeks. Left ventricular hemodynamic and morphology of heart and lung were evaluated immediately before and after the sacrifice, respectively. (A) Correlations between heart weight to body weight ratio (HW/BW) and lung weight to BW ratio (LW/BW), left ventricular systolic pressure (LVSP) and HW/BW, as well as LVSP and LW/BW, n = 74. (B) Representative LVSP curve recording from sham and TAC mice. (C) Mice with LVSP ≥200 mmHg in panel A were selected and then were divided into two groups according to the value of HW/BW (mice with HW/BW <8.5 mg/g were assigned into antihypertrophic group (Anti-Hyp), while mice with HW/BW ≥8.5 mg/g were included into hypertrophic group). No statistical difference on LVSP was noted between the two groups (left panel), HW/BW was 7.1 ± 0.16 in antihypertrophic group and 10.3 ± 0.4 mg/g in hypertrophic group (middle panel), while LW/BW in antihypertrophic group was significantly lower than in hypertrophic group (right panel), n = 13 and 15 in antihypertrophic and hypertrophic group, respectively, *$P<0.01$.

Figure S4 Results of echocardiography and invasive left ventricular (LV) hemodynamics at 6 weeks after operation. (A) LV posterior wall thickness (LVPWd). (B) LV systolic pressure (LVSP). (C) Heart rate (beats/minute). (D) LV pressure change rate. TAC: transverse aortic constriction, P1: hypertrophic precondition protocol 1, P2: hypertrophic precondition protocol 2. *$P < 0.05$ vs. sham.

Figure S5 Cardiomyocyte viability in response to endogenous and extraneous S100A8 or S100A9. Cultured neonatal rat cardiomyocytes were stimulated with lentivirus carrying S100A8/A9 (Lv-A8 or Lv-A9) for 72 hours or recombinant S100A8/A9 (r-A8 or r-A9) for 48 hours, then cell viability was evaluated using MTT assay. MOI: multiple of infection. *$P < 0.05$ vs. control (1st bar).
Video Legends

The supplementary video 1: This video shows the procedure of transverse aortic constriction in a mouse. After a left-sided thoracotomy in the 2nd intercostal space, the transverse aorta was isolated between the origin of the right innominate and left common carotid arteries, then a 7-0 silk ligature was tied around the transverse aorta and a 27 G blunted needle, the latter was promptly removed to yield a constriction of 0.4 mm in diameter.

The supplementary video 2: This video shows the procedure of transverse aortic debanding in a mouse. At the designated time points, the aortic band was removed, referred to as Debanding. After reopening the chest in the 2nd intercostal space, the ligated aortic arch is found, and then the narrowing suture was cut and removed from the aortic arch.