Small Heat Shock Protein 20 Interacts With Protein Phosphatase-1 and Enhances Sarcoplasmic Reticulum Calcium Cycling

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**Background:** Heat shock proteins (Hsp) are known to enhance cell survival under various stress conditions. In the heart, the small Hsp20 has emerged as a key mediator of protection against apoptosis, remodeling, and ischemia/reperfusion injury. Moreover, Hsp20 has been implicated in modulation of cardiac contractility ex vivo. The objective of this study was to determine the in vivo role of Hsp20 in the heart and the mechanisms underlying its regulatory effects in calcium (Ca) cycling.

**Methods and Results:** Hsp20 overexpression in intact animals resulted in significant enhancement of cardiac function, coupled with augmented Ca cycling and sarcoplasmic reticulum Ca load in isolated cardiomyocytes. This was associated with specific increases in phosphorylation of phospholamban (PLN) at both Ser16 and Thr17, relieving its inhibition of the apparent Ca affinity of SERCA2a. Accordingly, the inotropic effects of Hsp20 were abrogated in cardiomyocytes expressing nonphosphorylatable PLN (S16A/T17A). Interestingly, the activity of type 1 protein phosphatase (PP1), a known regulator of PLN signaling, was significantly reduced by Hsp20 overexpression, suggesting that the Hsp20 stimulatory effects are partially mediated through the PP1–PLN axis. This hypothesis was supported by cell fractionation, coimmunoprecipitation, and coimmunolocalization studies, which revealed an association between Hsp20, PP1, and PLN. Furthermore, recombinant protein studies confirmed a physical interaction between AA 73 to 160 in Hsp20 and AA 163 to 330 in PP1.

**Conclusions:** Hsp20 is a novel regulator of sarcoplasmic reticulum Ca cycling by targeting the PP1–PLN axis. These findings, coupled with the well-recognized cardioprotective role of Hsp20, suggest a dual benefit of targeting Hsp20 in heart disease. (*Circ Res. 2011;108:1429-1438.*)

**Key Words:** contractility ■ phosphatase ■ phospholamban ■ sarcoplasmic reticulum

The 17-kDa chaperone protein heat shock protein 20 (Hsp20) belongs to a family of at least 10 different small Hsp, which transiently increase to enhance cell survival in stress conditions.1 Hsp20 is expressed in multiple tissues, but it is most abundant in muscle.1 Of note, the levels of Hsp20 are increased in animal or human hearts under ischemic conditions.2 Exercise training.3 rapid right ventricular pacing,4 pharmacological treatment by doxorubicin,5 and chronic β-adrenergic stimulation.6 Currently, the functional significance of Hsp20 has been extensively studied in smooth and cardiac muscles.1,7 In the heart, Hsp20 protects against ischemia/reperfusion-induced injury.9 β-agonist-mediated remodeling,6 and apoptosis.6,9 Thus, the increased Hsp20 expression in failing hearts may constitute an important compensatory mechanism. Interestingly, acute increases of Hsp20 levels or activity in isolated cardiomyocytes are also associated with enhanced contractile parameters, suggesting an additional role of Hsp20 function in the heart.10,11 However, the mechanisms and subcellular pathways underlying the Hsp20-mediated effects on contractility have not been identified.

Cardiac contractility and calcium (Ca) cycling are regulated by a fine equilibrium of protein phosphorylation, which is enacted by the balance of protein kinase and phosphatase activities.12 Reversible Ser/Thr protein phosphorylation represents the cellular basis for integration of

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key signaling pathways and the responses of the heart to increased demands during sympathetic stimulation.12 The majority of Ser/Thr phosphatase activity is attributed to type 1 protein phosphatase (PP1) and type 2 phosphatases.13–16 Among these phosphatases, PP1 is of particular importance because it has been implicated as an important negative regulator of cardiac function.13,17,18 In fact, increases in its levels and activity have been suggested to play a major role in the aberrant sarcoplasmic reticulum (SR) Ca cycling and pathogenesis of heart failure.12,19 Accordingly, overexpression of the PP1 catalytic subunit in the mouse heart, at levels similar to those observed in human failing hearts, strongly suppressed contractility and induced heart failure.18 Current evidence indicates that substrate specificity for this versatile enzyme is achieved through its auxiliary proteins, which target it to subcellular compartments.12 In cardiac muscle, PP1 is present in the sarcoplasmic reticulum and it is the main phosphatase that dephosphorylates phospholamban (PLN), impacting SR Ca transport, and Ca load, leading to augmented function. The present findings, coupled with previous observations on the cardioprotective function of Hsp20 in vivo, suggest that Hsp20 may have a dual beneficial role in the heart, increasing both cell survival and SR Ca cycling.

Materials and Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org and includes detailed information regarding the generation of Hsp20 transgenic mice, in vivo catheterization, ex vivo Langendorff perfusion, mouse left ventricular myocyte isolation and measurements of mechanics, Ca kinetics and electrophysiology, quantitative immunoblotting, SR Ca uptake, adult rat and mouse ventricular myocyte isolation and adenovirus-mediated gene transfer, isolation of cardiac microsomes enriched in SR membranes, PP1 activity assay, coimmunoprecipitations, immunofluorescence studies, generation of recombinant proteins, blot overlay assay, and statistical analyses.

Results

Overexpression of Hsp20 Enhances Global Cardiac Contractile Function

Previous ex vivo studies suggested an effect of Hsp20 on cardiac contractility.10,11,24 To delineate the functional effects of Hsp20 in vivo, left ventricular contractile indices of Hsp20 transgenic mice were measured using cardiac catheterization. As shown in the Table, the systolic function, determined by the maximal rate of pressure increase, was significantly higher in Hsp20 hearts, compared with wild types (WT). The arterial blood pressure and maximal pressure were not different between the two groups. Diastolic parameters were also increased in Hsp20 hearts, as evidenced by restoration of the maximal rate of decline of left ventricular systolic pressure (Table). In addition, the minimum pressure (a surrogate of ventricular diastolic suction) and relaxation constant (Tau, a measure of active relaxation) were decreased in Hsp20 hearts. Consistently, Langendorff perfused Hsp20 transgenic (TG) hearts with a fixed pressure of 70 cm H₂O showed increased contractile parameters compared with WT hearts (Online Figure I). Taken together, these findings indicate that increased Hsp20 expression in the adult heart enhances cardiac performance.

Table. Baseline Functional Parameters in Wild-Type and Heat Shock Protein 20 Trangenic Hearts

<table>
<thead>
<tr>
<th></th>
<th>HR (Beats/min)</th>
<th>BP (mm Hg)</th>
<th>Pₘₐₓ (mm Hg)</th>
<th>Pₘᵢₙ (mm Hg)</th>
<th>+dP/dt (mm Hg/s)</th>
<th>−dP/dt (mm Hg/s)</th>
<th>Tau (Weiss)</th>
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<tbody>
<tr>
<td>WT (n=8)</td>
<td>350±11</td>
<td>110/78</td>
<td>99±2</td>
<td>7.9±1.7</td>
<td>7215±135</td>
<td>6167±275</td>
<td>12.6±0.8</td>
</tr>
<tr>
<td>Hsp20 TG (n=5)</td>
<td>368±14</td>
<td>110/75</td>
<td>104±9.2*</td>
<td>5.7±1.1*</td>
<td>9335±216*</td>
<td>8321±179*</td>
<td>8.8±0.6*</td>
</tr>
</tbody>
</table>

BP indicates blood pressure; HR, heart rate; Pₘₐₓ, maximum pressure; Pₘᵢₙ, minimum pressure; +dP/dt, rate of contraction; −dP/dt, rate of relaxation; Tau, relaxation constant; TG, transgenic; WT, wild-type.

*P<0.05 vs corresponding WT group.

Interestingly, the present study identified the small chaperone Hsp20 as a novel regulator of PP1 activity in the heart. Regulation appears to involve a direct physical interaction of Hsp20 with PP1 and inhibition of its enzymatic activity. This is associated with specific increases in PLN phosphorylation, SR Ca transport, and Ca load, leading to augmented function. The present findings, coupled with previous observations on the cardioprotective function of Hsp20 in vivo, suggest that Hsp20 may have a dual beneficial role in the heart, increasing both cell survival and SR Ca cycling.
Hsp20 Enhances Contractility and SR Ca Cycling in Cardiomyocytes

To determine the mechanisms associated with the enhanced cardiac contractility by Hsp20, we evaluated the mechanical parameters and Ca transients in isolated cardiomyocytes, which represent a load-independent system. Overexpression of Hsp20 resulted in significant increases in fractional shortening (FS%), rates of contraction (+dL/dt), and rates of relaxation (−dL/dt), compared to WT (P<0.05; Figure 1A through 1D). Accordingly, the peak of the Ca transient was significantly increased and the Ca decay rate (50% time constant of Ca transient decay) was abbreviated in TG myocytes relative to controls (P<0.01; Figure 1E through 1G). Furthermore, the caffeine-induced Ca transient peak was elevated compared with WT myocytes (P<0.01; Figure 1H and 1I), indicating a higher SR Ca content. However, the caffeine-induced 50% time constant of Ca transient decay, which mainly reflects the Ca extrusion by sodium/calcium exchanger (NCX), was not different between TG and WT myocytes (Figure 1J). Collectively, these data indicate that overexpression of Hsp20 increases cardiomyocyte Ca cycling and contractile performance.

Hsp20 Does Not Alter the Levels of Major Ca Handling Proteins

Given the central role of SR Ca cycling proteins in cardiac contractility,25 it was important to examine whether overexpression of Hsp20 poses any effects on their expression levels. Quantitative immunoblotting analysis did not reveal any significant changes in total levels of SERCA2a, phospholamban, ryanodine receptor 2, calsequestrin, NCX, and L-type Ca channel (Figure 2A and 2B). The activities of the L-type Ca channel and NCX were further investigated using whole-cell voltage clamps. Neither Ca currents in response to a series of depolarizing steps from a holding potential of −50 mV (Figure 2C) nor the average Ca current–voltage relationship (Figure 2D) showed differences between TG and WT ventricular cells. Furthermore, the Ni²⁺-sensitive I_{NCX} current (Figure 2E) and the I_{NCX} density (Figure 2F), which were calculated by normalizing total current to cell membrane...
capacitance, were similar between Hsp20 TG and Hsp20 WT myocytes. Together, these data indicate that the enhanced cardiac function by Hsp20 is not associated with alterations in SR Ca handling proteins or the activities of L-type Ca channel and NCX.

**Hsp20 Increases Phosphorylation of PLN and Enhances SR Ca Transport**

Because there were no differences in the expression levels of SR Ca cycling proteins or the activities of L-type Ca channel and NCX, we further investigated the phosphorylation status of key Ca cycling proteins in TG and WT hearts, which constitutes an important regulatory mechanism governing cardiac contractility.25 Interestingly, phosphorylation of PLN at Ser16 and Thr17 in TG hearts was significantly increased compared to WT (Figure 3A and 3B; \( P < 0.01 \)). However, the phosphorylation of ryanodine receptor 2 at Ser2809 or Ser2815 was not altered (Figure 3A and 3B). Furthermore, there was no increase in the phosphorylation of myofibrillar proteins, troponin I, or myosin-binding protein-C (Figure 3A and 3B). These results suggest that Hsp20 shows apparent specificity for PLN phosphorylation.

Because the degree of PLN phosphorylation profoundly affects the activation state of SERCA2a and contractility,26 we next assessed the initial rates of ATP-dependent, oxalate-facilitated SR Ca uptake in cardiac homogenates over a wide range of Ca concentrations, similar to those present in the cardiomyocyte during relaxation and contraction. The incubation conditions in cardiac homogenates, which restrict Ca uptake to SR vesicles, have been defined previously.27 Our results revealed that Hsp20 overexpression significantly enhanced the affinity of SERCA2a for Ca (EC50, Figure 3C inset). Furthermore, no differences in the maximal velocity of SR Ca uptake were noted between the two groups (Figure 3C). These findings indicate that overexpression of Hsp20 in the heart alleviates the PLN inhibition on SERCA2a by enhancing the phosphorylation status of PLN.

Further support for the apparent specificity of Hsp20 for PLN was provided by studies in a transgenic mouse model, which expresses nonphosphorylatable PLN (S16A, T17A double-mutant in the null background27 without any alterations in endogenous Hsp20 levels; Online Figure II). Isolated adult cardiomyocytes from double-mutant and WT mice were infected with Ad.GFP or Ad.Hsp20 for 24 hours before assessment of their contractile parameters. As expected, overexpression of Hsp20 in WT cardiomyocytes significantly increased fractional shortening percentage, \(+\text{dL/dt}\), and \(-\text{dL/dt}\), which were accompanied by en-
enhanced Ca kinetics compared with Ad.GFP controls (Figure 4A–D). However, there were no significant stimulatory effects of Hsp20 in double-mutant PLN cardiomyocytes (Figure 4A through 4D). Furthermore, infection of adult mouse cardiomyocytes from the PLN–KO model with Ad.GFP or Ad.Hsp20 showed no differences in either contractile parameters or Ca transients between these two groups (Online Figure III). Collectively, these data further support the role of PLN phosphorylation in the inotropic effects of Hsp20 in the heart.

**Overexpression of Hsp20 Regulates PP1 Activity**

The phosphorylation status of PLN is largely determined by the balance between protein kinases and phosphatases, including PP1 and PP2A. To investigate whether Hsp20 may tilt this fine-tuned balance, resulting in PLN hyperphosphorylation, we determined the protein phosphatase activities in Hsp20 TG and WT hearts. Our results showed that PP1 activity was significantly decreased in Hsp20-hearts compared with WT (Figure 5A). However, there was no difference in PP2A activity between Hsp20 and WT hearts (Figure 5A). To further verify the effects of Hsp20 on PP1 activity, adult rat cardiomyocytes were infected with either Ad.Hsp20 or Ad.GFP. Consistent with our in vivo findings, acute Hsp20 overexpression was associated with decreases in PP1 activity (Figure 5B). Taken together, these results suggest that Hsp20 may specifically regulate PP1 activity.

**Hsp20 Interacts With PP1 in Cardiomyocytes**

Early studies indicated that the substrate specificity of PP1 is dictated by its subcellular localization, and this enzyme appears to be the main SR phosphatase responsible for dephosphorylating PLN. To determine whether the regulatory effect of Hsp20 on PP1 is attributable to its subcellular localization in SR, microsomal preparations enriched in SR membranes and their respective cytosolic fractions were isolated from TG and WT hearts (Figure 6A). Both PLN and PP1 were abundantly present in the SR-enriched membrane fraction. Hsp20 was mainly present in the cytosolic fraction.
and overexpression increased its abundance in cytosolic and microsomal fractions of TG hearts.

Considering that Hsp20 was present in the SR membrane fraction, we hypothesized that this protein may colocalize with PLN and PP1. Therefore, its subcellular localization was assessed using coimmunostaining with PLN and PP1 antibodies in WT and TG cardiomyocytes. PLN staining revealed a striated pattern, corresponding to the SR membrane system (Figure 6B). Interestingly, immunofluorescence staining of Hsp20 also produced a partially striated pattern, overlapping with PLN (Figure 6B). Furthermore, double staining with PP1 and Hsp20 antibodies revealed colocalization of these two proteins in a similar pattern (Figure 6C). These results suggest that Hsp20 colocalizes with PP1 and PLN in the heart.

The potential association between Hsp20 and PP1 was further confirmed by reciprocal coimmunoprecipitations using antibodies against Hsp20 and PP1 in cardiac homogenates. PP1 was detected by Western blot analysis of anti-Hsp20 immunoprecipitates (Figure 6D, upper panel). Accordingly, the reverse immunoprecipitation indicated that Hsp20 was associated with PP1 (Figure 6D, lower panel).

To determine whether the observed association between Hsp20 and PP1 is direct, and to narrow the regions on these 2 proteins responsible for their physical interaction, various recombinant PP1 and Hsp20 constructs were generated. Recombinant full-length (PP1(aa1–330)) and 2 deletion constructs of PP1 (PP1(aa1–187) and PP1(aa163–330)) were generated in fusion with the maltose-binding protein (MBP; Figure 6E and 6G). In addition, an Hsp20 construct containing residues 1 to 160 (Hsp20(aa1–160)) and 2 deletion constructs (Hsp20(aa1–82) and Hsp20(aa73–160)) were expressed as glutathione-S-transferase (GST)-fusion proteins in Escherichia coli (Figure 6F and 6I). Using blot overlay assays, we evaluated binding of GST-Hsp20(aa1–160) (≈40 kDa) to the full-length and the 2 truncated MBP-PP1 proteins. Full-length MBP-PP1(aa1–330) and MBP-PP1(aa163–330) showed strong binding to GST-Hsp20(aa1–160), therefore mapping the region of PP1 required for its association with Hsp20 to residues 163 to 330 (Figure 6H). Similarly, on reciprocal blot overlay experiments, we evaluated binding of MBP-PP1(aa1–330) (≈80 kDa) to the full-length GST-Hsp20(aa1–160) and the 2 Hsp20 deletion constructs GST-Hsp20(aa1–82) and GST-Hsp20(aa73–160). We found that GST-Hsp20(aa1–160) and GST-Hsp20(aa73–160) interacted with MBP-PP1(aa1–330) (Figure 6J). Taken together, these data suggest that Hsp20 and PP1 directly interact with each other and that the region involved in their binding is within their C-terminal domains.

**Discussion**

This study presents the first evidence on inhibition of PP1 by the small Hsp20. PP1 is a major Ser/Thr protein phosphatase in the cardiomyocytes, where its activity is regulated by the endogenous inhibitor-1 and inhibitor-2 proteins. Herein we identified an additional control switch for PP1 enzymatic activity provided by the cardioprotective protein Hsp20. Regulation of PP1 by Hsp20 involves a physical direct interaction between these two proteins, leading to amplified SR Ca cycling and augmented cardiac inotropy (Figure 7).

Previous studies have shown that inhibition of PP1 activity in genetic models through overexpression/activation of inhibitor-1 or inhibitor-2 results in a hypercontractile cardiac phenotype. These findings are compatible with the idea that suppression of PP1 activity increases the phosphorylation status of PLN with subsequent disinhibition of SERCA2a, enhanced SR Ca uptake, and SR Ca load. The apparent specificity of Hsp20 toward PLN was further confirmed in acutely infected cardiomyocytes from: (1) PLN double-mutant mice, which abrogated the stimulatory effects of Hsp20 on contractile and Ca kinetic parameters in the absence of the Ser16 and Thr17 phosphorylation sites (Figure 4A through 4D); and (2) PLN-KO mice, which prevented the Hsp20 stimulatory effects in the absence of PLN (Online Figure III). Although these studies in isolated cardiomyocytes represent an ex vivo system with limited functional analysis, they offer an advantage over cross models, which may develop compensatory mechanisms contributing to the observed phenotype. This unique preference of Hsp20 for PLN may be important from a therapeutic point of view because increased ryanodine receptor phosphorylation may poten-
In failing hearts, it should be noted that additional modulators of SERCA activity exist, such as the membrane-associated antiapoptotic protein Bcl-2, which has been shown to underlie one of the Hsp20 mechanisms, preventing cardiac ischemia/reperfusion injury. However, Hsp20 did not alter the expression level of Bcl-2 and coimmunoprecipitation studies excluded a direct association between these two proteins. Thus, the Hsp20 regulatory effects on cardiac contractility do not appear to be mediated by the Bcl-2/SERCA pathway.
indicated, but also with inhibitor-1 (Online Figure IV), suggesting the presence of a multiprotein regulatory complex. However, it is not currently known whether Hsp20 associates with inhibitor-1 directly or indirectly via PP1.

The mechanisms underlying the apparent specificity of Hsp20 for hyperphosphorylation of PLN may involve the location of PP1 within the proper SERCA/PLN functional domain of the SR. It has been recognized that PP1 associates with specific subunits or regulatory proteins, which target it to different subcellular locations/compartments, modulating its activity toward unique substrates. In the heart, it is suggested that PP1 associates with SR through its glycogen-binding subunit, which contains a hydrophobic SR anchoring region. This would allow for PP1 compartmentalization and amplification of the Hsp20 effects on phospholamban phosphorylation through the PP1/PLN axis. Interestingly, Hsp20 is also present in the SR-enriched fraction and there appears to be an association between PP1 and Hsp20. Recombinant protein assays confirmed a physical interaction between Hsp20 and PP1. Thus, Hsp20, PP1, and PLN may comprise a supramolecular complex, facilitating the targeting of PP1 toward the SR-associated PLN.

Recently, two distinct peptide motifs, which are responsible for the interaction of regulatory proteins with PP1, have been documented: (1) [R/K]-x-(0,1)-V-x-F, a well-characterized and conventional consensus motif, which binds to the C-terminal region of PP1 catalytic subunit; and (2) F-x-x-[R/K]-x-[R/K], which was initially identified in Bcl-xL, Bcl-w, and Bad proteins, but its binding site on PP1 catalytic subunit is still unknown. By sequence screening, we found that Hsp20 does not contain the [R/K]-x-(0,1)-V-x-F motif. However, the region between residues 117 and 122 (FHRRYR), which locates within a predicted C-terminal protein–protein interaction domain, possesses criteria for a putative F-x-x-[R/K]-x-[R/K] motif binding to PP1 (Online Figure V). Our GST pull-down assay confirmed that the C-terminal region of Hsp20 (aa73–160) binds to PP1 catalytic subunit (Figure 6I and 6J).

A previous study showed that incubation of transiently permeabilized myocytes with phospho-Hsp20 peptide analogues, which contain the N-terminal 13 amino acids of Hsp20, increased contractility associated with an abbreviated Ca transient decay. However, the nonphosphorylated peptide with similar length had no effects on contractility. The apparent discrepancy between the effects of nonphosphorylated peptide analogues and our results may be attributable to differences in peptide length, techniques used, experimental conditions, and models. Consistent with our current findings, another chaperone protein, Hsp70, has been identified as an SR Ca regulatory protein. Deletion of Hsp70 resulted in a delayed decline of Ca transients, decreased SR Ca content, and decreased rates of contraction and relaxation, which were related to decreases in SERCA2a expression. However, we did not observe any alterations in SERCA2a expression levels by either acute or chronic overexpression of Hsp20. Collectively, these findings underscore the need for further studies on the interactions between chaperones and SR Ca regulatory proteins.

In heart failure, reduced output evokes an increase in catecholamines and other neuroendocrine factors, which negatively impact function by downregulation of β-adrenergic receptors and decreased protein kinase A activity, resulting in dephosphorylation of key phosphoproteins. Overexpression of Hsp20 may have benefits in improving ventricular performance of failing hearts through targeting the PP1–PLN signaling axis. This is especially important in light of the elevated PP1 enzyme activity in SR. However, it should be pointed out that disturbed Ca handling is not the only cause of heart failure. Loss of cardiomyocytes in the infarcted heart, increased load work on the surviving myocardium by the scar tissue, limited perfusion distal to coronary stenosis, suboptimal preload attributable to stiffness of the hypertrophic ventricle, and matrix remodeling are but a few additional factors that can contribute to global cardiac dysfunction.

Interestingly, Hsp20 also possesses cardioprotective effects by preserving the viability of injured myocardium. Thus, our findings lead to the suggestion that Hsp20 may have a double benefit in the failing myocardium by a combination of contractile-dependent and cardioprotection-dependent effects.

Taken together, this is the first study to our knowledge that defines a role for Hsp20 in enhancing cardiac contractility and Ca handling. In addition to its recognized cardioprotective effects against myocardial injuries, our results strongly support the hypothesis that Hsp20 may represent a “refined” target, augmenting contractility and providing cardiac protection in heart failure.

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Disclosures
E.G. Kranias is a scientific founder of Nanocor.

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Hsp20 Regulates Cardiac Protein Phosphatase 1

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Novelty and Significance

What Is Known?

- Heat shock proteins (Hsp) are important mediators of cell survival under stress conditions.
- Hsp20 is a small Hsp that protects the heart against ischemic injury, \(\beta\)-agonist remodeling, and apoptosis.
- Acute expression of Hsp20 in cardiomyocytes stimulates contractility, but the underlying in vivo mechanisms are not currently known.

What New Information Does This Article Contribute?

- Hsp20 enhances cardiac function accompanied by increased sarcoplasmic reticulum (SR) calcium (Ca) cycling.
- The enhanced contractility is associated with specific increase in phospholamban (PLN) phosphorylation by Hsp20.
- The stimulatory effects of Hsp20 are ascribed to inhibition of protein phosphatase 1 (PP1) activity by its direct physical interaction, indicating that Hsp20 represents a novel regulator of PP1.

Hsp20 is constitutively expressed in multiple tissues, but it is most abundant in muscle. Cardiac overexpression of Hsp20 is associated with protection against ischemia/reperfusion-induced injury, \(\beta\)-agonist-mediated remodeling, and apoptosis. Here, we identified Hsp20 as a novel regulator of PP1 activity in the heart. Regulation appears to involve a direct physical interaction of Hsp20 with PP1 and inhibition of its enzymatic activity. The reduced PP1 activity by Hsp20 results in specific increases of PLN phosphorylation, SR Ca transport, and Ca load, which lead to augmented function. These findings have major implications in heart failure, in which decreased SR Ca handling is attributed partially to increased PP1 activity. Hsp20 may target the PP1–PLN signaling axis and improve ventricular performance as well as increase cell survival, thus having a dual benefit in heart disease.
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Methods

Generation of Hsp20 Transgenic Mice
Hsp20 transgenic mice (FVB/N) were generated by standard procedures. Animals used in this study were littermates at 12–16 weeks of age. Both males and females were used, as there were no differences between genders. All the mice were handled and maintained according to protocols approved by the Animal Care & Use Committee of the University of Cincinnati. The investigation conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.

In vivo Catheterization
To assess the effects of Hsp20 expression on left ventricular function, in vivo measurements of the steady-state baseline hemodynamic parameters were performed using a 1.4F micromanometer-tipped pressure volume catheter (SPR-839, Millar Instruments), as described previously. Data were analyzed by Millar's PVAN software (Version 3.5)

Ex vivo Langendorff Perfusion
The effects of Hsp20 expression on left ventricular function were assessed using an isolated perfused heart model as previously described. Hearts were rapidly excised and mounted on a Langendorff apparatus (~70cm H₂O perfusion pressure), perfused with Krebs-Henseleit buffer (noncirculating). A fluid-filled balloon made of plastic film was inserted into the left ventricle and attached to a pressure transducer, which was connected to a Heart Performing Analyzer (Micro-Med). A bipolar electrode (NuMed) was inserted into the right atrium, and atrial pacing was performed at 400bpm with a Grass S-5 stimulator. The continuous left ventricular pressure was measured after 30 minutes of stabilization.

Mouse Myocyte Isolation and Measurements of Mechanics and Ca- Kinetics
Isolation of mouse left ventricular myocytes was carried out as described previously. Briefly, mouse hearts were excised from anesthetized (pentobarbital sodium, 70 mg/kg, i.p.) adult mice, mounted in a Langendorff perfusion apparatus, and perfused with Ca-free Tyrode solution at 37°C for 3 min. The normal Tyrode solution contained 140 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 10 mM glucose, and 5 mM HEPES, pH 7.4. Perfusion was then switched to the same solution containing 75 units/ml type 1 collagenase (Worthington), and perfusion continued until the heart became flaccid (~10–15 min). The left ventricular tissue was excised, minced, pipette-dissociated, and filtered through a 240-μm screen. The cell suspension was then sequentially washed in 25, 100, 200 μm and 1 mM Ca-Tyrode and resuspended in 1.8 mM Ca-Tyrode for further analysis. To obtain intracellular Ca signals, cells were incubated with the acetoxyethyl ester form of fura-2 (Fura-2/AM; 2 μM) for 30 min and resuspended in 1.8 mM Ca-Tyrode solution. The myocyte suspension was placed in a Plexiglas
chamber, which was positioned on the stage of an inverted epifluorescence microscope (Nikon Diaphot 200), and perfused with 1.8 mM Ca-Tyrode solution. Cell shortening and Ca-transients were measured at room temperature (22–23°C) in separate experiments, as documented below. The room temperature allowed the myocytes to be stable for up to 2 h with constant pacing. Myocytes were field stimulated to contract by a Grass S5 stimulator through platinum electrodes placed alongside the bath (0.5 Hz, bipolar pulses with voltages 50% above myocyte voltage threshold). Contraction of myocytes from random fields were videotaped and digitized on a computer. For Ca signal measurements, cells were loaded with Fura-2 (Fura-2 /AM; 2 μM) and alternately excited at 340 and 380 nm by a Delta Scan dual-beam spectrophotofluorometer (Photon Technology International) at baseline conditions and upon rapid application of 10 mM caffeine. Ca-transients were expressed as the 340/380 nm ratio of the resulting 510 nm emissions. SR Ca load was measured upon rapid application of 10 mM caffeine. Data were analyzed by Felix software (Photon Technology International).

**Electrophysiology of Isolated Mouse Left Ventricular Myocytes**

Isolated mouse left ventricular myocytes were maintained at room temperature (24°C) and perfused with Tyrode’s solution containing (in mM) NaCl 140, KCl 5.4, MgCl₂ 1, CaCl₂ 1.8, HEPES 5, and glucose 10 (pH = 7.4). The L-type Ca-channel and the Na-Ca exchanger (NCX) currents were recorded using the whole-cell patch clamp technique with an Axonpatch-200B amplifier (Axon Instruments, Foster City, CA), as described previously. NCX current was recorded using whole-cell voltage clamp at 30°C with commonly used external solution (in mM): NaCl 140, MgCl₂ 1, Glucose 10, HEPES 5, CaCl₂ 2, BaCl₂ 1, CsCl 4, nifedipine 0.01, ryadonine 0.005 and ouabain 0.02 (pH=7.4); and internal solution (in mM): Aspartic Acid 80, CsOH 80, TEA-Cl 20, MgCl₂ 2.5, HEPES 10, EGTA 11, CaCl₂ 7.5, CsCl 15, NaCl 10 and Na₂-ATP 4 (pH=7.2). The reversal potential of NCX was calculated as $E_{(NCX)} = 3E_{(Na)} - 2E_{(Ca)} = 3 \times 51 - 2 \times 103 = -53$ mV, which is consistent with the reversal potential observed experimentally (around -50 mV). Data collection and analysis were performed using pCLAMP software (Axon Instruments, Foster City, CA).

**Quantitative Immunoblotting**

Alterations in the levels of total proteins or their phosphorylation status were analyzed from whole heart homogenates or microsomal extracts by Western blotting. Briefly, an appropriate amount of heart homogenate or microsomal extract was separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). After blocking with 5 % non-fat milk, membranes were incubated with primary antibodies, followed by appropriate secondary antibodies. Primary antibodies against various proteins of interest used in this study were as follows: pSer16-PLN, pThr17-PLN and pSer2809-RyR2 (Badrilla), pSer2815-RyR2 (gift from Dr. Andrew Marks, Department of Physiology and Cellular Biophysics, Columbia University), PLN (Upstate), RyR2 (Sigma), pSer22/pSer23-TnI and TnI (Research Diagnostics Inc.), pSer282-MyBP-C (Axxora), MyBP-C (Santa Cruz), SERCA2a (custom-made commercially, Affinity...
Bioreagents), LTCC (Alomone Labs), NCX (Affinity Bioreagents), CSQ (Affinity Bioreagents), PP1 (Sigma), and GAPDH (Affinity Bioreagents). The horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:5,000) were from Amersham Biosciences (Piscataway, NJ). The membranes were developed by an enhanced chemiluminescence Western blot analysis detection system (Amersham Biosciences). All the protein levels were quantified using AlphaEaseFC software (Alpha Innotech, San Leandro, CA).

**SR Ca-Uptake**

Initial SR Ca-uptake rates were determined in cardiac homogenates, using oxalate to restrict uptake to SR vesicles and $^{45}$CaCl$_2$, as previously described.$^4$ Briefly, 100-250 μg of cardiac homogenate were incubated at 37°C in a reaction buffer containing: 40 mmol/L imidazole, pH=7.0, 95 mmol/L KCl, 5 mmol/L NaN$_3$, 5 mmol/L MgCl$_2$, 0.5 mmol/L EGTA, and 5 mmol/L potassium oxalate. The initial uptake rates were determined over a wide range of Ca-concentrations (pCa 8 to 5). Ca-uptake into SR vesicles was initiated by addition of 5 mmol/L ATP, and aliquots were filtered through a 0.45 μm Millipore filter after 0, 30, 60 and 90 seconds. The specific $^{45}$Ca-uptake values were analyzed by non-linear regression, using the OriginLab 5.1 program to obtain the Ca-affinity (EC$_{50}$) and the Ca-uptake rates.

**Adult Rat Ventricular Myocyte Isolation and Adenovirus-Mediated Gene Transfer**

Rat ventricular myocytes were isolated from adult male Sprague–Dawley rats (6–8 weeks old) and plated on laminin-coated glass coverslips or dishes, as described previously.$^5$ Recombinant adenoviruses Ad.GFP encoding GFP, Ad.Hsp20 containing both Hsp20 and GFP were generated by using the AdEasy-1 expression system, as described previously.$^6, 7$ Cardiomyocytes seeded on coverslips or dishes were infected with adenovirus in diluted media, at a multiplicity of infection (MOI) of 500, for 24 h.

**Adult Mouse Cardiac Myocytes Isolation, Culture and Adenovirus-Mediated Gene Transfer**

Adult mouse cardiomyocytes were isolated according to the protocols developed by the Alliance for Cellular Signaling$^8$ and modified by us. Briefly, adult WT and double mutant PLN mice$^6$ (8-10 weeks old) were anesthetized with Avertin (100 mg/kg i.p., Sigma) and hearts rapidly removed. Hearts were perfused with Krebs Henseleit Bicarbonate (KHB) buffer followed by digestion with liberase blendzyme I (0.25 mg/ml, Roche). Myocytes were dissociated by teasing the ventricles with forceps, followed by addition of 10 % serum and 12.5 μM CaCl$_2$. The concentration of CaCl$_2$ was gradually increased to 1 mM and cells were suspended in plating medium containing 10 mM of 3-butanedione monoxime (BDM). Myocytes were plated on laminin-coated (10 μg/mL) dishes for 2 hour at 5% CO$_2$ and 95% air at 37°C. After 2 hour of attachment, cardiac myocytes were infected with adenoviruses containing the cDNA sequence of wild type Hsp 20 (Ad.Hsp20), or green fluorescent protein (Ad.GFP) at a multiplicity of infection.
(MOI) of 500 in 1 ml of 10 µM blebbistatin (Toronto research Chemicals, Canada) culture media. Experiments were performed 24 hours after infection.

**Isolation of Cardiac Microsomes Enriched in SR Membranes**

Briefly, hearts were homogenized at 4°C in Buffer A composed of imidazole (10 mM, pH 7.0), sucrose (300 mM), dithiothreitol (1 mM), sodium metabisulphite (1 mM), and phenylmethylsulfonyl fluoride (0.3 mM). Isolation of microsomal fractions enriched in SR membranes was achieved by differential centrifugation of the cardiac homogenate. Homogenates were centrifuged at 8,000g (20 min) and the pellets rehomogenized in buffer A and centrifuged as above. The supernatants from the two spins were combined, 4 M NaCl was added to a final concentration of 0.6 M, and centrifuged at 100,000g (60 min). The supernatant after 100,000g centrifugation was used as the cytosolic fraction. The resulting pellet was washed in Buffer A and recentrifuged at 100,000g (60 min). The final pellet was resuspended in Buffer A. The yield of protein in the final pellet was similar between wild type and transgenic hearts.

**PP1 Activity Assay**

Protein phosphatase activity was assessed in cardiac homogenates and cell lysates (1 µg total protein) with the Protein Serine/Threonine Phosphatase Assay System (New England Biolabs) according to the manufacturer's instructions and as described previously. Okadaic acid (10 nM) was used to discern between PP1 and protein phosphatase-2A (PP2A) activities.9

**Co-Immunoprecipitations and Immunofluorescence Studies**

Association of Hsp20 with PP1 was studied by co-immunoprecipitation, as previously described. A homemade anti-Hsp20 antibody and anti-PP1 antibody (Sigma) were used. The immunofluorescence studies of isolated cardiomyocytes were carried out as previously described. Homemade anti-Hsp20, anti-PLN (Sigma), and anti-PP1β (Thermo Scientific) antibodies were used for staining, followed by appropriate secondary antibodies (Alexa Fluor anti-rabbit 594 or Alexa Fluor anti-mouse 488, Invitrogen) with 1:500 (vol/vol) dilution in blocking buffer. Immunofluorescence images were generated from serial sections of isolated cardiomyocytes using Zeiss confocal microscopy.

**Generation of Recombinant Proteins and Blot-Overlay Assay**

To evaluate the interaction between PP1 and Hsp20, full length and deletion constructs of PP1 and Hsp20 were generated by PCR amplification and were subcloned in the EcoRI/Sall sites of the pMAL-c2x vector (New England Biolabs) or pGEX5x-1 vector (Amersham Biosciences), respectively. PCR was performed using primers 5’ ATGTCCGACAGCGAGAAGCT 3’ and 5’ CATGGCACATGATTTCGT 3’ for full length MBP-PP1(aa1−330) construct, primers 5’ ATGTCCGACAGCGAGAAGCT 3’ and 5’ CGGAATCTGCTCCATAGAC 3’ for MBP-PP1(aa1−187), and primers 5’ GCCATAGTGGACGAAAAGA 3’ and 5’ CATGGCACATGATTTCGT 3’ for MBP-PP1(aa163−330). Full length and deletion constructs of Hsp20 were generated by
PCR amplification, using primers 5’ AGCAGGATGGAGATCCCTGT 3’ and 5’ CCAGCCCCCTCCTACTTG 3’ for GST-Hsp20(aa1−160), primers 5’ AGCAGGATGGAGATCCCTGT 3’ and 5’ GTGCTTCACGTCTAGCAGCAC 3’ for GST-Hsp20(aa1−82) and primers 5’ CACTTTTCGGTGCTGCTAGA 3’ and 5’ CCAGCCCCCTCCTACTTG 3’ for GST-Hsp20(aa73−160). The sequence of all generated constructs was verified by sequencing analysis (Macrogen, Seoul, Korea). Expression of GST and MBP fusion proteins was performed, as previously described 11 and recombinant proteins were purified by affinity chromatography on Glutathione Sepharose 4B™ beads (Amersham Biosciences) or amylose resin beads (New England Biolabs), according to manufacturer’s instructions.

The blot-overlay assays were performed as previously described. 11 Briefly, ~2.5 μg of affinity-purified MBP, MBP-PP1(aa1−330), MBP-PP1(aa1−187), and MBP-PP1(aa163−330) recombinant proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific sites on the membrane were blocked by incubation in buffer A (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20, 2 mM dithiothreitol, 0.5% NP-40 and 5% nonfat milk) for 16 h at 4°C. The membrane was then incubated with 3 μg/ml GST-Hsp20(aa1−160) fusion protein in buffer A, in the presence of 1 mM ATP, for 5 h at 25°C. Following five washes in buffer C (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20, 2 mM dithiothreitol, 1% NP-40), the membrane was probed with anti-GST antibody (Amersham Biosciences) and bands were visualized using ECL reagents. In a set of parallel assays, 3 μg of affinity-purified GST, GST-Hsp20(aa1−160), GST-Hsp20(aa1−82) and GST-Hsp20(aa73−160) fusion proteins were subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes, allowed to interact with MBP-PP1(aa1−330) protein as described above and the membrane was probed with anti-MBP antibody (New England Biolabs).

**Statistics**

All results are expressed as mean ± SEM. Comparisons were evaluated by Student’s *t* test for two groups and one-way ANOVA for multiple groups. In all analyses, *P* < 0.05 was considered statistically significant.

**References:**


Sarcoplasmic reticulum calcium overloading in junctin deficiency enhances cardiac contractility but increases ventricular automaticity. *Circulation.* 2007;115:300-309.


Online Figure I

![Bar graph showing +dP/dt and -dP/dt values for WT and TG hearts.](image)

**Figure I.** The left ventricular function is enhanced in Hsp20-TG heart, as measured by ex vivo Langendorf preparations. *, p<0.05 vs. WT, n=5.

Online Figure II

![Western blot images showing Hsp20 and Calsequestrin expression levels in WT and PLN-DM hearts.](image)

**Figure II.** Hsp20 expression levels were measured in the WT and PLN-DM hearts. Calsequestrin was used as a loading control.
Figure III. The inotropic effects of Hsp20 are abrogated in the absence of PLN. Overexpressed Hsp20 in PLN-KO cardiomyocytes had no effects on contractility: (A) fractional shortening (FS%), (B) Maximum rates of contraction (+dL/dt), (C) maximum rates of relaxation (-dL/dt), and (D) twitch Ca amplitude in cultured WT and PLN-KO (10-12 cardiomyocytes/heart, n=3 hearts).
Online Figure IV

**Figure IV**: Immunoprecipitations (IP) using WT cardiac homogenates and the anti-Hsp20 antibody (Lane C). Lane A: Pre-IP cardiac homogenate (positive control); Lane B: agarose beads (negative control).
Online Figure V

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**Figure V. PP1 binding motif and structure prediction of Hsp20.** A putative PP1-binding motif, F-x-x-[RK]-x-[RK], has been identified in the 117 – 122 residues (underlined segment) of mouse Hsp20 amino acid sequence. The secondary structure of Hsp20 was predicted by PROF (divergent profile-based neural network prediction method, B Rost, P Fariselli & R Casadio Prot. Science, 1996;7:1704-1718) and PHD (simple profile-based neural network prediction method, B Rost. Methods in Enzymology, 1996;266:525-539). A predicted protein-protein interaction site was identified on Hsp20 sequence (residue 117-150 in red color), which overlapped the PP1-binding motif in residues 117-122. Based on PHD, there was no identified helical trans-membrane region. PROF_sec: PROF predicted secondary structure, H=helix, E=extended (sheet), blank=other (loop). Rel_sec_1: reliability index for PROF_sec prediction (0=low to 9=high). PHD predicted membrane helix: blank=non-membrane. Rel_sec_2: reliability index for PHD prediction (0=low to 9=high).