Blockade of Hsp20 Phosphorylation Exacerbates Cardiac Ischemia/Reperfusion Injury by Suppressed Autophagy and Increased Cell Death

Jiang Qian, Xiaoping Ren, Xiaohong Wang, Pengyuan Zhang, W. Keith Jones, Jeffery D. Molkentin, Guo-Chang Fan,* Evangelia G. Kranias*

Rationale: The levels of a small heat shock protein (Hsp20) and its phosphorylation are increased on ischemic insults, and overexpression of Hsp20 protects the heart against ischemia/reperfusion injury. However, the mechanism underlying cardioprotection of Hsp20 and especially the role of its phosphorylation in regulating ischemia/reperfusion–induced autophagy, apoptosis, and necrosis remain to be clarified.

Objective: Herein, we generated a cardiac-specific overexpression model, carrying nonphosphorylatable Hsp20, where serine 16 was substituted with alanine (Hsp20S16A). By subjecting this model to ischemia/reperfusion, we addressed whether: (1) the cardioprotective effects of Hsp20 are associated with serine 16 phosphorylation; (2) blockade of Hsp20 phosphorylation influences the balance between autophagy and cell death; and (3) the aggregation pattern of Hsp20 is altered by its phosphorylation.

Methods and Results: Our results demonstrated that Hsp20S16A hearts were more sensitive to ischemia/reperfusion injury, evidenced by lower recovery of contractile function and increased necrosis and apoptosis, compared with non-TG hearts. Interestingly, autophagy was activated in non-TG hearts but significantly inhibited in Hsp20S16A hearts following ischemia/reperfusion. Accordingly, pretreatment of Hsp20S16A hearts with rapamycin, an activator of autophagy, resulted in improvement of functional recovery, compared with saline-treated Hsp20S16A hearts. Furthermore, on ischemia/reperfusion, the oligomerization pattern of Hsp20 appeared to shift to higher aggregates in Hsp20S16A hearts.

Conclusions: Collectively, these data indicate that blockade of Ser16-Hsp20 phosphorylation attenuates the cardioprotective effects of Hsp20 against ischemia/reperfusion injury, which may be attributable to suppressed autophagy and increased cell death. Therefore, phosphorylation of Hsp20 at serine 16 may represent a potential therapeutic target in ischemic heart disease. (Circ Res. 2009;105:1223-1231.)

Key Words: apoptosis • myocardial infarction • autophagy • heat shock protein

Coronary reperfusion is the primary therapeutic strategy in ischemic heart disease, nevertheless, it may at first exacerbate cellular damage sustained during the ischemic period (ischemia/reperfusion injury).1 As a consequence of such injury, adaptive stress responses occur immediately, some of which are involved with upregulation of heat shock proteins (Hsps).2 In fact, Hsp synthesis arises transiently under a wide spectrum of stressful stimuli as a protective mechanism.3 Within the superfamily of Hsps, the small (s)Hsps with molecular masses ranging from 12 to ≈43 kDa have received particular attention.4–6 Recently, several members of sHsps have been identified as protective mediators during myocardial ischemia, including αB-crystallin, Hsp27, and Hsp20.4–6 Hsp20, sharing a conserved domain with αB-crystallin and Hsp27,7 is the only member within the sHsps family that contains a consensus peptide motif (RRAS) for protein kinase A (PKA)/PKG–dependent phosphorylation at Ser16.8 We and others have demonstrated that the levels of cardiac Hsp20 and its phosphorylation were significantly increased, compared with Hsp27 and αB-crystallin, in animal hearts on ischemic conditions, exercise training, rapid right ventricular pacing, and pharmacological treatment by doxorubicin and chronic β-adrenergic stimulation.6,9–11 More recently, we have identified a P20L substitution in human Hsp20, which was associated with diminished phosphorylation at Ser16 and complete abrogation of the protective effects of Hsp20, suggesting an instrumental role of phosphorylated Hsp20 in...
cardioprotection. Indeed, the constitutively phosphorylated mutant of Hsp20 (Hsp20\textsuperscript{S16D}) conferred protection against β-agonist–induced apoptosis in cultured myocytes; conversely, the constitutively dephosphorylated mutant, namely Hsp20\textsuperscript{S16A}, displayed no antiapoptotic properties, implying a mechanistic link between phosphorylated Hsp20 and its protection.

Notably, studies have shown that ischemia/reperfusion–induced cardiomyocyte necrosis and apoptosis contribute to ventricular dysfunction and end-stage failure. However, there is an increasing awareness that necrosis and apoptosis are not the only mechanisms for cell death. Macroautophagy (commonly referred to as autophagy), which involves the bulk degradation of cytoplasmic contents, may provide alternative mechanisms that determine cell fate in ischemia/reperfusion. In the present study, we sought to address the effects of blocking Hsp20 phosphorylation at Ser16 on ischemia/reperfusion–induced cell injury. Our findings herein demonstrate a detrimental role of nonphosphorylated Hsp20\textsuperscript{S16A} in ischemia/reperfusion injury through suppression of autophagy and increased cell death, which further implicate phosphorylation of Hsp20 as a potential therapeutic strategy for ischemic heart disease.

### Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

### Acquisition of Failing and Donor Human Heart Samples

Human tissues from the anterior wall of 4 nonfailing (donor) and 5 failing left ventricles were obtained from the laboratory of Roger Hajjar (Mount Sinai School of Medicine). The failing hearts were obtained at the time of cardiac transplantation. Information about each patient was collected and linked to the subject number (but not the patient name) and documented in a spreadsheet. Once in the laboratory, the tissue was cut into pieces, frozen in liquid nitrogen, and stored at −80°C. All investigation conforms with the principles outlined in the Declaration of Helsinki.

### Generation of Transgenic Mice

We generated transgenic (TG) mice in the FVB/N background that carry the mouse cardiac Hsp20\textsuperscript{S16A} cDNA under the control of the α-myosin heavy chain mouse promoter, as described. A 0.5-kb mutant mouse Hsp20\textsuperscript{S16A} cDNA was ligated with the murine cardiac α-myosin heavy chain gene promoter (5.5 kb). In all studies, male Hsp20\textsuperscript{S16A} transgenic and non-TG control littermate mice, between 12 to 16 weeks of age, were used. The care of all animals used in the present study was in accordance with the University of Cincinnati animal care guidelines.

### Results

#### Increased Level of Phosphorylated Ser16-Hsp20 in Ischemia/Reperfused and Failing Hearts

It has been shown that the levels of sHsps and their phosphorylation were increased in response to stress signals. Thus, in initial studies, we assessed the levels of total Hsp20 and phospho–Ser16-Hsp20 at Ser16 on ischemia/reperfusion–induced cell injury. Our findings herein demonstrate a detrimental role of nonphosphorylated Hsp20\textsuperscript{S16A} in ischemia/reperfusion injury through suppression of autophagy and increased cell death, which further implicate phosphorylation of Hsp20 as a potential therapeutic strategy for ischemic heart disease.

#### Non-standard Abbreviations and Acronyms

- **Hsp**: heat shock protein
- **LC3**: microtubule-associated protein light chain 3
- **pI**: isoelectric point
- **PK**: protein kinase
- **sHsp**: small heat shock protein

### Figure 1.

Phosphorylation of Hsp20 in ex vivo ischemia/reperfusion (IR) injured wild-type mouse hearts and failing human hearts. The ratio of phospho–Ser16-Hsp20/total Hsp20 was increased in post–ischemia/reperfusion myocardium (n=6; \(P<0.01\), post vs pre) (A) and in failing human hearts (n=3 for heart failure [HF], n=4 for donor; \(P<0.01\), heart failure vs donor) (B). CSQ indicates calsequestrin (loading control).
Sympathetic overactivity is closely connected with cell injury and contractile dysfunction during myocardial ischemia/reperfusion. Thus, regulation of the β-adrenergic receptor–activated cAMP-PKA signaling pathway and its downstream targets might be important to the improvement of cardiac performance after reperfusion. Our findings above and previous studies have suggested a potential role of cAMP-dependent phosphorylation of Hsp20 under ischemic conditions. To examine whether blocking Hsp20 phosphorylation at Ser16 confers any in vivo effects on ischemia/reperfusion injury, we generated transgenic mice that carried the mouse cardiac mutant Hsp20 cDNA, in which serine 16 encoded by codon TCA was mutated into GCA (encoding alanine), was driven by the α-myosin heavy chain promoter (α-MHCp). DNA sequencing of PCR products from Hsp20S16A mice genome confirmed the TCA to GCA mutation. Western blotting analysis showed that in Hsp20S16A TG hearts there was a 7-fold increase in total Hsp20 levels relative to non-TG hearts (NTG). The expression of nonphosphorylatable Hsp20S16A was further confirmed by proteomics. As shown in Figure 2D, only one spot of Hsp20 protein with an isoelectric point (pI) value of 5.5 was detected by 2D electrophoresis in non-TG hearts, and 2 protein spots of Hsp20 (pI=5.5 and pI=5.2) were observed in Hsp20S16A hearts (Figure 2D). Mass spectrometry revealed that the spot with pI value of 5.2 in TG hearts contained the mutant protein of genomic DNA followed by DNA sequencing. This confirmed that the TCA codon, encoding Ser16, was mutated to GCA, which encrypted the alanine residue.
Hsp20S16A peptide (Figure 2E). It is of note that Hsp20S16A hearts contained endogenous wild-type Hsp20 (spot of pI 5.5). However, the phosphorylated Hsp20 levels in these hearts were significantly lower than non-TG hearts, either at basal conditions or after ex vivo ischemia/reperfusion injury (Online Figure II), suggesting that overexpression of mutant Hsp20 prevented the phosphorylation of endogenous wild-type Hsp20.

Characterization of non-TG and Hsp20S16A mice showed no alterations in body weight (28.9 ± 0.74 versus 28.2 ± 0.56g, n = 10, P = 0.05) or tibia length (1.79 ± 0.09 versus 1.80 ± 0.10 cm). Furthermore, there were no differences in heart weight/body weight and heart weight/tibia length ratio (Online Figure III, A) between these 2 groups. In addition, histological analysis revealed no signs of fibrosis, inflammation, cardiomyocyte hypertrophy, or dystrophy in Hsp20S16A hearts, compared with non-TG controls (Online Figure III, B). Of importance, 7-fold overexpression of Hsp20S16A did not alter the expression of other small heat shock proteins, such as Hsp25 or B-crystallin in the heart (Online Figure III, C).

**Impaired Functional Recovery in Hsp20S16A Hearts During Ischemia/Reperfusion Injury**

As shown in Figure 1, the levels of phosphorylated Hsp20 were increased in the heart on ischemia/reperfusion injury. However, it is unclear whether this phosphorylation is essential to the cardioprotective effects of Hsp20 against ischemic stress. Thus, we subjected the Hsp20S16A hearts to ex vivo 45 minutes of no-flow global ischemia, followed by 2 hours of reperfusion. Non-TG hearts were used as controls. There were no differences in ±dP/dt, left ventricular developed pressure, and end diastolic pressure between the 2 groups under basal conditions (Figure 3A through 3D). However, under ischemia/reperfusion, functional recovery of Hsp20S16A hearts was significantly depressed, as determined by the parameters of +dP/dt (43.1 ± 4.7% versus non-TG control: 81.6 ± 5.0%; Figure 3A), −dP/dt (42.8 ± 4.4% versus 78.2 ± 3.1%; Figure 3B) and left ventricular developed pressure (LVDP) recovery (53.7 ± 4.3% versus 75.5 ± 2.2%; Figure 3C) (P < 0.01). In addition, end diastolic pressure was significantly greater in Hsp20S16A hearts after global, no-flow ischemia/reperfusion compared with non-TG controls (Figure 3D; P < 0.01). Taken together, these data suggest that blockade of Hsp20 phosphorylation in Hsp20S16A hearts is associated with impaired functional recovery on ischemia/reperfusion.

**Increased Necrosis and Apoptosis in Hsp20S16A Hearts on Ex Vivo Ischemia/Reperfusion**

It is recognized that maintaining adequate numbers of myocyte is critical to the overall preservation of structural integrity and cardiac function following ischemia/reperfusion.1 Thus, strategies to maximize postischemic salvage have aimed at preventing 2 forms of cell death, necrosis1,14 and apoptosis.1,15 To delineate the detrimental effects conferred by Hsp20S16A in postischemic cellular damage, the extent of necrotic and apoptotic cell death was examined after ex vivo 45 minutes ischemia followed by 2 hours reperfusion. Under basal conditions, lactate dehydrogenase release, a biochemical marker of necrotic cell death, did not differ between the Hsp20S16A and non-TG hearts (Figure 4A). However, on ischemia/reperfusion, lactate dehydrogenase release was significantly increased by 2-fold in Hsp20S16A hearts, compared to non-TG hearts (Figure 4A; P < 0.05). These results indicate that overexpression of Hsp20S16A promote ischemia/reperfusion–initiated cellular disruption in the myocardium.

Furthermore, we examined whether the functional deterioration of the Hsp20S16A TG hearts was related to increased apoptosis. Heart lysates from a subset of experimental animals were assayed for DNA fragmentation by a quantitative nucleosome assay. Hsp20S16A hearts exhibited a 1.5-fold
Hsp20S16A hearts, subjected to no-flow ischemia followed by reperfusion, exhibited significantly increased total lactate dehydrogenase (LDH) release (A), DNA fragmentation (B), TUNEL-positive nuclei (C), and caspase-3 activity (D) compared to non-TGs (non-TGs: n = 6, Hsp20S16A:n = 6; *P < 0.01 vs non-TG).

Increased Necrosis and Apoptosis in Hsp20S16A Hearts After In Vivo Ischemia/Reperfusion

To further examine whether overexpression of Hsp20S16A may be detrimental in ischemic/reperfused hearts in vivo, the Hsp20S16A and non-TG animals were subjected to 30 minutes myocardial ischemia, via left anterior descending coronary artery occlusion, followed by 24 hours of reperfusion.24 The area at risk, determined by negative staining after reperfusion with phthalo blue dye and expressed as percent of left ventricle, was not significantly different between Hsp20S16A and non-TG hearts (Figure 5B; Hsp20S16A: 57.8 ± 1.1%; non-TG: 59.9 ± 2.2%; P > 0.05, n = 6), indicating that a comparable degree of ischemic jeopardy existed between these 2 groups after occlusion of the left anterior descending coronary artery. The infarct-to-risk region ratio was 22.0 ± 3.0% in non-TG hearts post–ischemia/reperfusion, similar to previous reports.25,26 whereas it was significantly increased (53.5 ± 0.8%, P < 0.005) in Hsp20S16A hearts (Figure 5C; n = 6). Furthermore, apoptotic TUNEL-positive nuclei27 were also increased in Hsp20S16A hearts after 24 hours of reperfusion (Online Figure IV). Consistent with the ex vivo finding, overexpression of Hsp20S16A aggravates in vivo ischemia/reperfusion injury.

Inactivation of Autophagy in Hsp20S16A Hearts After Ex Vivo Ischemia/Reperfusion

Autophagy is an intracellular bulk degradation process, whereby cytosolic, long-lived proteins and organelles are degraded and recycled.16 Recent studies have shown that autophagy plays an important role in ischemia/reperfusion injury,17 and its activity levels determine its beneficial or detrimental effects in such injury.17 Thus, we assessed the autophagy activity in Hsp20S16A hearts by measurement of the microtubule-associated protein light chain (LC)3 and specifically the ratio of LC3-II/LC3-I, as well as Beclin1 protein level,28 in comparison with non-TG hearts. Conversion of cytosolic LC3-I to membrane-conjugated LC3-II is correlated with the number of autophagosomes, indicative of autophagic activity.28,29 Furthermore, Beclin1, an autophagy related protein, is a critical player in the formation of autophagosome.30 Western blotting analysis showed that the ratio of LC3-II/LC3-I was increased in non-TG hearts after ischemia/reperfusion (Figure 6A and 6B), suggesting that ischemia/reperfusion initiated autophagy. Interestingly, the ratio of LC3-II/LC3-I was elevated in Hsp20S16A hearts under basal conditions but was significantly decreased following ischemia/reperfusion (Figure 6A and 6B). Alterations of another autophagy-related protein, Beclin1 (Figure 6A and 6C), were parallel to the changes of the LC3-II/LC3–I ratio in Hsp20S16A and non-TG hearts. Taken together, these data
indicate that suppression of ischemia/reperfusion–induced autophagy may contribute to the depressed cardiac functional recovery in Hsp20S16A hearts.

**Pretreatment of Hsp20S16A Hearts With Rapamycin Improved Functional Recovery in Response to Ischemia/Reperfusion**

To further examine whether activation of autophagy in Hsp20S16A TG hearts would rescue its postischemic function, we administered rapamycin to Hsp20S16A mice, which induces autophagy by inhibiting mTOR (mammalian target of rapamycin).31 Pretreatment with rapamycin did not alter the preischemic cardiac function (Online Figure V). As shown in Figure 6D, treatment with rapamycin significantly increased the LC3-II/LC3-I ratio (D) and improved recovery of left ventricular developed pressure (LVP) (E) and decreased end diastolic pressure (EDP) (F) following ischemia/reperfusion (I/R), compared with saline-treated controls (non-TG: n=6, Hsp20S16A: n=6). *P<0.01, rapamycin vs saline group.

**Effects of Mutant S16A on Hsp20 Oligomerization Patterns Following Ischemia/Reperfusion**

The phosphorylation status of Hsp20 has been suggested to have a role in its structural organization, manifesting in the aggregation patterns of the protein, which might be associated with its function. As a result, we examined the oligomerization pattern of Hsp20 in non-TG and Hsp20S16A transgenic hearts before and after global ischemia/reperfusion by sucrose gradient ultracentrifugation. This method revealed that non-TG hearts displayed Hsp20 oligomers up to 150 kDa before ischemia/reperfusion, and this pattern slightly shifted to the right (larger complex) after ischemia/reperfusion. In contrast, at basal levels, Hsp20S16A hearts displayed Hsp20 aggregate patterns composed primarily of oligomers between 150 to 250 kDa. After ischemia/reperfusion, Hsp20S16A hearts displayed Hsp20 oligomers that were shifted to a larger complex profile (>250 kDa; Figure 7), suggesting that phosphorylation of Ser16 may alter the ability of Hsp20 to aggregate.

**Discussion**

The findings herein indicate that phosphorylation of Hsp20 at Ser16 is essential for protecting the myocardium from ischemia/reperfusion damage. Blockade of this phosphorylation site in a model with cardiac overexpression of Hsp20S16A resulted in loss of cardioprotective effects exhibited by overexpression of wild-type Hsp20 and fostered worse functional recovery from ischemia/reperfusion. More interestingly, this detrimental effect of Hsp20S16A was associated with increased necrosis and apoptosis but decreased autophagy (Figure 8). To our knowledge, this is the first...
It is noteworthy that in vivo or ex vivo myocardial ischemia involves a large and progressive release of catecholamines from adrenergic nerve terminals, and excessive stimulation of myocardial β-adrenergic receptors by catecholamines may further accelerate the ischemia-induced cell damage. Instead, we observed an increased ratio of phospho-Ser16/total Hsp20 in ischemia/reperfusion–injured wild-type mouse hearts, as well as in failing human hearts. This could be interpreted as a compensatory protective response to the accumulated catecholamines in the ischemic myocardial tissue. In fact, our previous study has shown that the protective effect of Hsp20 against ischemia/reperfusion was associated with increased phosphorylation of Hsp20. Our data presented here further support the in vivo significance of Hsp20 phosphorylation in its cardioprotective effects against ischemia/reperfusion injury.

The mechanisms underlying the detrimental effects of Hsp20S16A on ischemia/reperfusion may involve several pathways. Firstly, the increase of apoptosis is likely an important factor responsible for severe injuries in postischemic Hsp20S16A hearts. It is well accepted that cardiomyocyte apoptosis could be a fundamental part of the myocardial process that initiates or aggravates cardiac injury. For example, conditional overexpression of active caspase-8 demonstrated that very low levels of myocyte apoptosis were sufficient to cause lethal, dilated cardiomyopathy. Accordingly, our laboratory also observed that reduced cardiac apoptosis significantly contributed to better functional recovery in Hsp20-overexpressing hearts on ischemia/reperfusion. Hence, blockade of Hsp20 phosphorylation at Ser16 is associated with increased cardiomyocyte apoptosis triggered by ischemia/reperfusion, leading to lower functional recovery.

Secondly, there is an increasing awareness that autophagy plays a critical role in ischemia/reperfusion injury, which is an important addition to the well-known necrosis and apoptosis processes. Under basal conditions, low level of autophagy is necessary for the turnover of long-lived proteins and cytoplasmic organelles in the heart. In response to ischemia, the extent of autophagy depends on the severity and duration of ischemic insults. For example, modest levels of autophagy, induced by mild to moderate hypoxia/ischemia, appear to be protective by degrading and removing damaged mitochondria, therefore preventing activation of apoptosis. On the other hand, high levels of autophagy triggered by severe hypoxia or ischemia/reperfusion may cause self-digestion and eventual cell death. Accordingly, Decker and Wildenthal reported that as early as 40 minutes of ischemia led to upregulation of autophagy, and that subsequent reperfusion of 1 hour induced a drastic enhancement of autophagy in Langendorff perfused rabbit hearts. In their model, induction of modest levels of autophagy was correlated with functional recovery of rabbit hearts after ischemia/reperfusion. Therefore, the level of autophagy may determine whether it is protective or detrimental to the heart in response to ischemia/reperfusion. The beneficial increase of autophagy may be responsible for elimination of damaged, presumably nonfunctional organelles, including mitochondria, along with the restoration of normal cardiac structure and function. Furthermore, autophagy can spill over into regulation of apoptotic and necrotic cell death, specifically, through autophagic proteases. Moreover, the key machinery of autophagy, autophagosome itself, when fused with the lysosome, can also regulate necrotic cell death.

Consistently, we found that a brief period (20 minutes) of ischemia followed by 2 hours of reperfusion induced reversible cardiac injury (complete contractile recovery) in wild-type murine hearts. By contrast, prolonged ischemia (60 minutes) and 2 hours of reperfusion resulted in a large infarct along with minimal recovery of contractility (≈10%) (Online Figure VI). The degree of injury inflicted...
during 45 minutes of ischemia followed by 2 hours of reperfusion was intermediate and correlated with a modest activation of autophagy (1.3-fold increase of the LC3-II/LC3-I ratio). Thus, we selected a 45 minutes/2 hours of ischemia/reperfusion protocol for our present study. As expected, autophagy was increased in our non-TG hearts on ischemia/reperfusion, which corresponded to ∼75% of functional recovery. However, Hsp20S16A hearts displayed inhibition of ischemia/reperfusion–activated autophagy, and this may contribute to deterioration of energy homeostasis, leading to impaired functional recovery (≈54%). Accordingly, activation of the autophagy process in Hsp20S16A hearts by pretreatment with rapamycin restored their functional recovery on ischemia/reperfusion. Furthermore, we have noticed a modest (1.27-fold) increase of autophagy in Hsp20S16A hearts under basal conditions, which did not affect its basal myocardial function, compared with non-TG controls. We also found that pretreatment of Hsp20S16A hearts with rapamycin did not alter basal contractile function (Online Figure V). These observations suggest that a modest increase of autophagy has no effects on basal cardiac function. However, it should be admitted that rapamycin might have some other protective effects beyond activating autophagy, such as inducing potent preconditioning-like effects against myocardial infarction through opening of mitochondrial ATP-sensitive K⁺ channels.

Finally, nonphosphorylatable Hsp20 may affect its oligomerization, which offsets the protective effects of Hsp20. It is well-known that the phosphorylative capacity and the resulting aggregation patterns of small heat shock proteins may influence their cytoprotective ability during cellular stress. For example, the phosphorylated form of Hsp27 was concentrated in small and medium-sized oligomers, whereas its nonphosphorylated form was present in larger oligomers. Unexpectedly, nonphosphorylatable mutants of Hsp25, the rodent form of Hsp27, conferred better protection than wild-type Hsp25 in L929 cells subjected to tumor necrosis factor (TNF)–α and H₂O₂-induced cytotoxicity. Furthermore, overexpression of wild-type Hsp27 or a nonphosphorylatable Hsp27 mutant was equally capable of protecting from an ischemic insult in adult rat myocytes and transgenic mouse models. These conflicting results may be partially attributable to the use of different models and variability in Hsp27 proteins between studies. Previous in vitro studies have shown that phosphorylation at Ser16 regulates the aggregation pattern of Hsp20, which is consistent with the observation that the N-terminal residues of sHsps are necessary for complex formation. Interestingly, our pull-down assay indicated that phospho–Ser16–Hsp20 is associated with autophagy-related Beclin1 (Online Figure VII), suggesting that Beclin1 may be a potential target of phosphorylated Hsp20 in regulating autophagy. The blockade of Hsp20 phosphorylation at Ser16 promoted formation of large aggregates and possibly disassociated Beclin1, which may promote the detrimental effects under stress conditions.

In conclusion, the present findings indicate that blockade of Hsp20 phosphorylation at Ser16 is associated with increased cardiac ischemia/reperfusion injury, partially through reduced activation of autophagy and increased apoptosis, as well as necrosis (Figure 8). Thus, Hsp20 and its phosphorylation may constitute an important modality for cardioprotection against myocardial infarction.

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Disclosures

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Supplement Material

Methods and Materials

Morphological Analysis

Mouse hearts were fixed in 10% neutralized formalin and embedded in paraffin. Serial sections (5μm) were routinely stained with hematoxylin–eosin and Masson’s Trichrome, examined under a light microscope (×400) and photographed for morphological analysis. Paraffin-embedded tissue was also examined for sarcolemma staining labeled with wheat germ agglutinin (WGA, Invitrogen), according to manufacturer’s instruction.

2-Dimensional (2-D) Gel Electrophoresis

Mouse left ventricular tissue was isolated and proteins were extracted for 2-D gel electrophoresis by the Proteomics Lab of the Genome Research Institute, University of Cincinnati. 2-D gel images were analyzed using ImageMaster 2D Elite software. The protein spots of interest were excised and their tryptic peptides were subjected to MALDI-TOF or LC-MS/MS for identification.

Perfused Heart Experiments

Isolation and perfusion of mouse hearts by the Langendorff method was carried out, as described previously. Briefly, hearts were retrograde-perfused with a phosphate-free Krebs-Henseleit buffer equilibrated at 37°C with 5% CO₂/95% O₂, pH 7.4. First, hearts were perfused at constant pressure (65cm H₂O) for a 30-minute stabilization period. Global ischemia was applied by eliminating flow for 45min, followed by 2h reperfusion. Contractility was assessed with a fluid-filled intraventricular balloon connected to a pressure transducer (Micro-Med Ltd). The end-diastolic pressure (EDP) was set to 5 to 10 mmHg. A bipolar electrode (NuMed) was inserted into the right atrium, and atrial pacing was performed at 400 bpm with a Grass S-5 stimulator. At the end of the perfusion period, hearts were either frozen rapidly in liquid nitrogen and stored at -80°C or immersed in 10% buffered formalin.

Pretreatment of Mouse Hearts with the Autophagy Inducer

Hsp20^{S16A} or non-TG mice were divided into two groups (n=6): controls and rapamycin treated (autophagy inducer). Rapamycin (5ng total in 0.5ml saline²), or the corresponding volume of saline (controls) was administered intraperitoneally (i.p.), and 60min after injection animals were subjected to ex vivo ischemia/reperfusion, as described above.

In Vivo Ischemia/Reperfusion and Assessment of Myocardial Infarction

Ischemia/reperfusion was produced in Hsp20^{S16A} and non-TG controls by transiently ligating the left anterior descending coronary artery (LAD). The mice underwent 30min of occlusion,
followed by 24 hours of reperfusion. Infarct assessment was performed, as described in our previous studies.\(^3\) The heart was perfused with 1% 2, 3, 5-Triphenyltetrazolium chloride (TTC). Afterwards, the occluder was retied and the heart was perfused with 5% phthalo blue. Hearts were transversely cut into 5 to 6 sections. Infarct size was expressed as the percentage of the infarct area (unstained by TTC) relative to the total area at risk (AAR, unstained by phthalo blue). The total AAR was calculated by adding all sections together, and then presented as percentage of the whole left ventricle in each heart.

**Evaluation of Cardiac Injury and Apoptosis**

Cardiac injury was assessed by measuring lactate dehydrogenase (LDH) release. Perfusion effluent was collected every 10 minutes of pre-ischemia and during reperfusion. Total LDH released from the heart was determined, using an In Vitro Toxicology Assay Kit (Sigma) and expressed as units per gram of wet heart weight. DNA fragmentation was analyzed using a Cell Death Detection ELISA plus kit (Roche), which quantified the cytoplasmic histone-associated DNA fragments (180 base pair nucleotides or multiples) in cardiac lysates. Results were normalized to the standard, provided in the kit, and expressed as a fold increase over control. For terminal dUTP nick end-labeling (TUNEL) assays, hearts were removed from the apparatus after ischemia/reperfusion, and the atrial tissue was dissected away. The ventricles were fixed in 10% buffered formalin and later embedded in paraffin according to standard procedures and 5 \(\mu\text{m}\)-thick sections were obtained to perform TUNEL assays using the DeadEnd™ Fluorometric TUNEL system (Promega, Madison, WI), according to the manufacturer’s instructions. TUNEL-positive nuclei (green) were determined by randomly counting 10 fields of the midventricular section and were expressed as a percentage of the total nuclei population. Cardiomyocytes were stained with \(\alpha\)-sarcomeric actin (1:50 dilution; Sigma) labeling; nuclei were stained by DAPI (Invitrogen). Sections were analyzed with a fluorescence microscope. Changes in the caspase-3 activity were studied using Western blot analysis. The presence of caspase-3 activation was assessed by the observation of a 17-kDa subunit, which was derived from the cleavage of the 32-kDa proenzyme caspase-3.

**Western Blot**

Western blot analysis was performed as previously described.\(^3\) After blocking, the membranes were probed with specific antibodies against Hsp20 (Research Diagnostics, Inc), phospho-Ser16-Hsp20 (customized antibody\(^4\) from Affinity BioReagents, Inc), \(\alpha\)B-crystallin (Affinity BioReagents Inc), Hsp25 (Research Diagnostics, Inc), Beclin 1 (BD Biosciences) and LC3 (Medical & Biological Laboratories). The ECL system (Amersham Biosciences, Inc) was used for detection.

**Sucrose Gradient Electrophoresis**

Frozen heart samples were homogenized in 10 mmol/L HEPES-buffered saline (pH=7.4) containing phosphatase inhibitor and proteinase inhibitor cocktail sets (Calbiochem) and
0.5% Triton X-100. After protein quantification, 100 µg of protein was loaded on a 2mL sucrose gradient (5% to 40%) and spun at 166,180 g for 5 hours with a TLS 55 rotor. Fractions (100 µL) were collected from the top and mixed with Laemmli buffer directly before denaturation and analysis by 12% SDS-PAGE for immunoblot analysis, as described.5

Co-immunoprecipitation

Association of phospho-Ser16-Hsp20 with Beclin -1 was studied by co-immunoprecipitation, as described previously.6, 7 Briefly, protein lysates were extracted from wild type mouse cardiac homogenates with 1 × cell lysis buffer (Cell Signaling, #9803), which was supplemented with 1mM PMSF and protease inhibitor cocktail (Sigma), and centrifuged at 13,000rpm for 30 min at 4°C. The phospho-Ser16-Hsp20 or anti-Beclin 1 (BD Biosciences) antibody (4µg) was added into 1ml diluted cell lysates (1µg/µl) and incubated overnight on a rotary wheel at 4 °C. Protein G PLUS agarose beads (Santa Cruz Bitotech) (1µg antibody/10 µl agarose beads) were added into the above mixture, and incubated for an additional 1-2 h at 4 °C. Beads were sedimented and washed 6 times with the cell lysis buffer. Beads-bound proteins were dissolved in 2xSDS sample buffer, and boiled at 95 °C for 5 min. Finally, the identity of proteins was determined by immunoblotting. Preimmunoprecipitated WT heart homogenate was used as positive control (+), and immunoprecipitate with anti-IgG PLUS agarose was used as negative control (–).

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using a 2-tailed Student t-test for unpaired observations and ANOVA followed by the Bonferroni post hoc test for multiple comparisons (Systat 11). P<0.05 was considered statistically significant.

References


Online Figure I. Basal function of non-transgenic and Hsp20\(^{S16A}\) hearts. ± dP/dt (A, B), LVDP (C) and EDP (D) in Hsp20\(^{S16A}\) TG (7x or 5x overexpressing) hearts were not significantly different than those of non-TGs under basal conditions (non-TGs: n=10, TG: n=8; P>0.05, Hsp20\(^{S16A}\) vs. non-TG). NTG: non-transgenic, TG: transgenic.
Online Figure II. Phosphorylation of Hsp20 in non-transgenic and Hsp20$^{S16A}$ hearts before or after ex vivo ischemia/reperfusion injury. Before I/R, the ratio of phospho-Ser16/total Hsp20 was decreased in Hsp20$^{S16A}$ hearts, compared with non-transgenics (P<0.05). I/R induced higher level of phosphorylation of Hsp20 in non-transgenic, compared with Hsp20$^{S16A}$ hearts myocardium (n=6, *: P<0.01)
Online Figure III. Characterization of Hsp20<sup>SI6A</sup> hearts. (A) There is no difference in body weight or tibia length between non-TGs and Hsp20<sup>SI6A</sup> TGS (P>0.05). Heart weight/body weight (HW/BW) and heart weight/tibia length (HW/TL) ratios were similar between Hsp20<sup>SI6A</sup> TG mice and non-TGs at 12 weeks of age (n=10, P>0.05). (B) Ventricular sections from 12 week-old Hsp20<sup>SI6A</sup> mice stained with HE and Masson’s T indicated no inflammation or cardiac fibrosis. WGA staining of Hsp20<sup>SI6A</sup> hearts showed similar myocyte size with non-TGs (n=6, P>0.05). (C) Hsp25 and αB-crystallin (αBC) were not altered in Hsp20<sup>SI6A</sup> hearts, compared with non-TGs (n=6, P>0.05).
Online Figure IV. Evaluation of apoptosis in non-transgenic and Hsp20^{S16A} transgenic myocardium subjected to \textit{in vivo} 30min ischemia, followed by 24h reperfusion. (A-F) Representative images of TUNEL-positive nuclei according to their apoptosis- and necrosis-like morphology. Arrows indicate TUNEL-positive nuclei with either condensed chromatin (apoptosis, A-C) or diffused chromatin distribution (necrosis, D-F). Blue-DAPI indicates nuclear counterstaining; green indicates TUNEL staining and red indicates actin staining of myocytes by Alexa 594 (Invitrogen). (G) The number of TUNEL- positive nuclei was evaluated in 10 fields for apoptosis-like appearance. Plotted were means±SEM for 3 animals per group.
Online Figure V. Basal function of Hsp20S16A and non-TG hearts was not changed after pretreatment with rapamycin. After 1h of rapamycin administration, mouse hearts were subjected to Langendorff perfusion. After 30min of stabilization, LVDP was measured. There was no significant difference of LVDP between Hsp20S16A and non-TG hearts, whether saline-treated or rapamycin-treated. (n=6, P>0.05)
Online Figure VI. Functional recovery of wild type hearts under multiple ischemia/reperfusion protocols. Wild type mouse hearts were subjected to different time courses of ischemia (20min, 45min and 60min) followed by 2h of reperfusion. Recovery of LVDP (expressed as percentage of pre-ischemic value) upon I20min/R120min, I40min/R120min, and I60min/R120min was 100%, 71.5±2.2%, and 11±1.4%, respectively. (n=6, *: P<0.01, vs. I20min/R120min).
Online Figure VII. Protein interaction of phospho-S16-Hsp20 with Beclin-1.

Co-immunoprecipitation is performed by using cardiac homogenates of wild type mice and the anti-phosho-S16-Hsp20 (A) or anti-Beclin (B) antibodies. The precipitates were analyzed by immunoblotting with anti-p-Hsp20 or anti-Beclin antibodies, as indicated. Preimmunoprecipitated WT heart homogenate was used as positive control (+), and immunoprecipitate with anti-IgG PLUS agarose was used as negative control (−). IP, immunoprecipitation; IB, immunoblotting.