Published in final edited form as:

J Mol Cell Cardiol. 2010 February ; 48(2): 360. doi:10.1016/j.yjmcc.2009.11.009.

Regulation of heat shock protein 60 and 72 expression in the failing heart

Y. Wang^{a,d,1}, L. Chen^{a,1}, N. Hagiwara^{a,c}, and A.A. Knowlton^{a,b,*}

^aMolecular and Cellular Cardiology, Cardiovascular Division, University of California, Davis, CA, USA

^bSacramento VA Medical Center, Sacramento, CA, USA

^cRowe Program in Genetics, University of California, Davis, CA, USA

^dDepartment of Human Anatomy, Base Medical College, Center of Scientific Technology, Ningxia Medical University, Yinchuan 750004, PR China

Abstract

Heart failure, a progressive, fatal disease of the heart muscle, is a state of chronic inflammation and injury. Heat shock protein (HSP) 72, a ubiquitous protective protein that is well-established as cardioprotective, is not increased in heart failure. In contrast, HSP60 levels are doubled in the failing heart. We hypothesized that HSF-1 is not activated in heart failure and that the increased expression of HSP60 was driven by NFκB activation. To test this hypothesis, we measured levels of heat shock factor (HSF) -1 and -2, the transcription factors controlling HSP expression, which were increased in heart failure. There was no increased phosphorylation of serine 230 or serine 303/307 in HSF-1, which are thought to regulate its activity; EMSA showed no increase in HSF binding activity with heart failure. Nonetheless, mRNA was increased for HSP60, but not HSP72. In contrast to HSF, NFκB activity was increased in heart failure. HSP60, but not HSP72, contained NFκB binding elements. ChIP assay demonstrated increased binding of NFκB to both of the NFκB binding elements in the heart failure HSP60 gene. TNFα treatment was used to test the role of NFκB activation in HSP60 expression in a cardiac cell line. TNFα increased HSP60 expression, and this could be prevented by pretreatment with siRNA inhibiting p65 expression. In conclusion, HSP72 is not increased in heart failure because HSF activity is not changed; increased expression of HSP60 may be driven by NFκB activation.

Keywords

HSP60; HSP72; Heart failure; NFκB; ERK 1/2; GSK

*Corresponding author. Molecular and Cellular Cardiology, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA. Tel.: +1 530 752 5461; fax: +1 530 754 7167. aaknowlton@ucdavis.edu..

¹Contributed equally to work.

Disclosures: None.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Congestive heart failure (CHF) represents an inflammatory state of chronic stress and injury for cardiac myocytes [1]. The heat shock proteins are a group of protective proteins that are upregulated in response to stress and injury [2,3]. These proteins stabilize macromolecules, fold and unfold proteins, and facilitate removal of irreversibly denatured proteins from the cell. Furthermore, HSP72 and HSC70, a closely related constitutively (HSC) expressed protein, have important roles in protein synthesis [4-6]. HSPs also regulate the activity of key enzymes, such as eNOS [7]. We and others have found that heat shock protein (HSP) 60 is increased in CHF, but the very protective HSP72 is not [8-10]. Increased expression of HSP60 in the failing heart is associated with membrane localization of the protein. We have identified HSP60 on the surface of cardiac myocytes from failing hearts and this was associated with apoptosis, suggesting that the increase in HSP60 may be deleterious [8]. To understand why there is this difference in the expression of these two related proteins, we investigated regulation of expression of these proteins in the failing heart. HSP expression is controlled by the heat shock transcription factors (HSF) 1 and 2. Analysis of the expression of HSF-1 and HSF-2, as well as their phosphorylation state, which regulates activity, and DNA binding was performed.

The HSP60 promoter has been reported to have only a single heat shock element, the binding site for the HSFs that regulate HSP expression [11]. This is in contrast to the three heat shock elements found in the promoter for HSP72 in the human [12]. This suggested that some other factor was driving the increase in HSP60 expression that was observed in heart failure. We hypothesized that NF κ B activation, resulting from the inflammatory state of heart failure, would potentially drive HSP60 expression.

We identified likely NF κ B binding sites in the HSP60 gene. There were no predicted NF κ B binding sites in the HSP72 promoter. We investigated the role of NF κ B in the upregulation of HSP60 expression in the failing heart. The results of these studies are presented here.

2. Methods

2.1. Heart failure model

A rat coronary ligation model of heart failure was used as previously described [8]. The animal protocol was approved by the University of California, Davis, Animal Research Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals. A high LAD ligation was performed in 5- to 6-month-old male Sprague–Dawley rats as previously reported. A cardiac echo was done 9–12 weeks after ligation to verify the presence of heart failure, as previously detailed [8]. Rats were then deeply anesthetized, and the left ventricle was collected for analysis. A combination of sham-operated and normal hearts were used as controls. Results did not differ between shams and normals, so these groups were combined for analysis.

2.2. Western blotting

Was performed as previously described [13]. Phosphatase inhibitors, 1 mM activated sodium orthovanadate and 1 nM sodium fluoride, were added to samples where indicated. Anti-HSF-1 (LabVision) and anti-HSF-2 (Sigma) were used at 1:200 and 1:2000, respectively. Anti-phospho-HSF-1 antibodies to phospho-serine 230(1:6000) and phospho-serine 303/307 (1:1000) were the generous gift of Lea Sistonen (Abo Akademi University, Turku, Finland). ERK 1/2 (BD Biosciences, 1:5000), anti-phospho-ERK (BD Biosciences, 1:1000), anti-glycogen synthase kinase-3 (GSK-3, StressGen, 1:1000), and anti-phospho-GSK-3 (Affinity BioReagents, 1:500) were used to detect changes in ERK 1/2 and GSK-3 activation. Beta actin (Novus Biologics, Littleton, CO) at 1:10,000 was used as a loading control. Affinity purified anti-mouse IgG-HRP (GE) was used at 1:1000.

2.3. ELISA

HSP60 and HSP72 levels were quantified by ELISA as previously described using commercial kits (StressGen) [8].

2.4. EMSA

EMSA was performed as previously reported [14,15]. In addition, a microtiter plate based assay (Pierce) that quantifies the binding of p65 from nuclear fractions was used to analyze NFκB activation as previously detailed [14,15].

2.5. Chromatin immunoprecipitation (ChIP) assays

Chromatin immunoprecipitation experiments were performed using the ChIP IT™ kit (Active Motif). As the heart has a high protein/DNA ratio, 2.5 times the recommended tissue amount was used. Normal and CHF heart tissue was cut into small pieces and cross-linked by addition of formaldehyde (1% final concentration) for 10 min. Cross-linking was terminated by the addition of glycine (125 mM final concentration). The chromatin was sonicated and the DNA was sheared into 200–1000 bp fragments by sonication five times for 30 s. The chromatin was pre-cleared by adding Protein G beads to reduce non-specific background. Then 10 µl of the pre-cleared chromatin was aliquoted as input. The rest of pre-cleared chromatin were separated into three groups by adding the specific antibody (NFκB, Active Motif, HSF-1, Stressgen, SPA-901E), positive control RNA polII mAb or negative control IgG. The immunoprecipitated DNA were eluted with ChIP elution buffer. The cross-linking was reversed with 5 M NaCl, and RNA was removed with RNase A, followed by digestion with proteinase K. The DNA was purified and PCR analysis was performed, and the products separated on a 3% agarose gel.

2.6. Immunoprecipitation

The heart tissues were cut into small pieces and homogenized in RIPA buffer with protease inhibitors. Then the samples were pre-cleared with protein AG-agarose beads (Santa Cruz) for 2 h. The supernatant was then incubated with an antibody to HSF-1 (Stressgen) overnight at 4 °C on a rocker panel. The immune complexes were precipitated using protein AG-agarose beads. Western blot analysis was performed using antibodies to phosphorylated serine 230 or 303/307, as above. Blots were stripped and then developed for total HSF-1.

2.7. Real-time PCR

RNA was extracted from left ventricular samples ($n=4-6$ rats per group) and processed by a “two-step” RT-PCR. Real-time quantitative PCR was performed using an ABI Prism 7000 Sequence Detection System and SYBR Green as double-strand DNA binding dye (Applied Biosystems, CA). Results were expressed as the ratio relative to an internal control (GAPDH).

2.8. Cell culture

H9c2 cells (ATCC) were used as a cardiac model. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in a humidified atmosphere of 95% O₂/5% CO₂ at 37 °C. Cells were transfected at 60–70% confluence with 25 nM of p65 siRNA (Sigma-Aldrich, St. Louis, MO) using HiPerfect transfection reagent (Qiagen, Valencia, CA) for 48 h according to the manufacturer's instructions. Control transfections were performed with 25 nM of fluorescein conjugated negative control siRNA (Qiagen).

2.9. Statistical analysis

Results are expressed as the mean $\pm$ SEM. Data was analyzed by either the Student's *t* test or by a Mann–Whitney rank sum test, where appropriate. A $p < 0.05$ was considered significant.

3. Results

3.1. Heart function

Hearts used for these studies had a fractional shortening of $17.7 \pm 1.1\%$ for CHF vs. $45.4 \pm 2.6\%$ for controls (sham-operated, $p < 0.001$, $n = 6-8/\text{group}$). LVDD was 1.22 ± 0.03 cm for CHF vs. 0.99 ± 0.02 cm for controls ($p < 0.001$, $n = 6-8/\text{group}$). For three additional normal control hearts, functional data were not collected.

3.2. HSP expression

Previously we have reported that HSP60 is increased in heart failure, but HSP72 is not [8,9]. HSP60 levels by ELISA were 384 ± 22 ng/mg protein in failing rat left ventricle vs. 229 ± 16 in sham left ventricles ($p < 0.005$). HSP72 levels by ELISA in controls and CHF hearts were 31.9 ± 10.7 ng/mg protein and 19.7 ± 3.5 , respectively ($p = \text{n.s.}$). To determine if this difference in HSP expression was secondary to differences in transcription, HSP60 and HSP72 mRNA levels were examined by real-time PCR. HSP60 mRNA from failing hearts was three times that of sham controls, as shown in Fig. 1A ($p < 0.001$). In contrast, HSP72 mRNA was unchanged, consistent with the findings at the protein level (Fig. 1B).

3.3. HSF-1 and HSF-2 expression in CHF

The HSFs regulate the expression of the heat shock proteins. HSF-1 is the primary activator of HSPs in response to stress/injury, though there is now some evidence that HSF-2 interacts with HSF-1 in this setting [16]. HSF-1 levels were more than doubled in CHF, as shown in Fig. 1C. Similarly, HSF-2 levels were almost doubled (Fig. 1D). Differential phosphorylation of HSF-1 is thought to control its activity. Studies suggest that phosphorylation of serine 230 increases HSF-1 activity and phosphorylation at serine 303/307 inhibits HSF-1 activity [17-19]. To investigate this, HSF was immunoprecipitated from cardiac extracts. Serine 230 phosphorylation was somewhat increased in HSF-1 from failing hearts, but this was not significant (Figs. 2A and B). Serine 303/307 did not differ between CHF and control hearts (Figs. 2A and B).

GSK and ERK 1/2 have been implicated in the inhibition of HSF-1 activity. The inhibition of HSF-1 by phosphorylation at serine 303/307 is mediated by sequential phosphorylation by ERK 1/2 and GSK [19]. ERK 1/2 has been reported to change in heart failure, but the results of the limited studies have been conflicting with one group finding an increase in ERK and the other no increase, but an increase in ERK activation [20,21]. To further understand their possible role in the regulation of HSF-1, the expression and activation of GSK and ERK 1/2 were compared in samples of CHF and control hearts. Total ERK 1/2 did not differ between CHF and control hearts (Figs. 3A and B). However, phospho-ERK, the active form, was decreased by approximately 20% in CHF ($p < 0.05$, Figs. 3C and D). Total GSK-3 levels were unchanged in the failing heart compared to controls (Figs. 4A and B). Phospho-GSK-3 levels were decreased in CHF, but this did not reach statistical significance (Figs. 4C and D).

3.4. EMSA

HSF-1 and HSF-2 levels were increased in the failing hearts, but there was no change in activation controlling phosphorylation. Gel shift assays were done to look for activation of HSF-1 and HSF-2. As shown in Fig. 5A, there was no increase in binding of HSF, which would be either HSF-1 or -2, by EMSA over low basal levels. Supershift studies indicated that the

low level of binding seen was for HSF-1 in both groups (Fig. 5B). There was no supershift for HSF-2 in either normal or failing heart samples (not shown).

3.5. NFκB

Has been reported to be increased in heart failure, and we investigated whether activation of this transcription factor occurred, and whether it might contribute to increased HSP60 expression in heart failure. As shown in Fig. 5C, there was significant increase in binding of NFκB ($p<0.05$) in heart failure using an ELISA for p65 activation. The less sensitive and less quantitative EMSA showed overall greater activation of NFκB in CHF hearts vs. controls (Fig. 5D).

3.6. ChIP assay

Three NFκB binding sites were identified in the human HSP60 gene by sequence analysis with MatInspector (version 7.7.3, Genomatix, <http://www.genomatix.de/>). One of these was in the promoter and two in introns (Table 1). There were two predicted NFκB binding sites in the rat HSP60 gene, which is linked head to head with HSP10 (accession # U68562) by a short bi-directional promoter [11]. Analysis of the intronless rat HSP72 (inducible, accession number X75357) promoter predicted no NFκB binding sites, though two heat shock elements (HSE, HSF binding sites) were predicted, as previously reported [22]. Similarly, analysis of the inducible human HSP72 (accession number M11717) predicted 2 HSE in the promoter, but no NFκB binding sites [23]. A ChIP assay was done to look for binding of p65 to the HSP60 gene. The two predicted binding sites for NFκB for rat HSP60 were located in introns. These predicted NFκB binding sites were located at 7084–7096 and 7207–7219 and are referred to here as 1 and 2, respectively. A ChIP assay was used to analyze binding of NFκB at these two sites. The primers used for these sites are summarized in Table 2. As shown in Fig. 5E, we found binding of NFκB at both sites in the heart failure samples. Low level binding was found in the sham controls. Thus, in the CHF samples increased NFκB activation and binding was evident along with specific evidence of binding to NFκB elements in the HSP60 gene.

To further investigate the role of NFκB in the upregulation of HSP60 in heart failure, H9c2 cells were used as a cardiac model. Cells were treated with siRNA, which took 48 h to reduce p65 expression (Fig. 6A). Cells were pretreated with either an siRNA control or with p65 siRNA for 48 h and then treated with 10 ng/ml TNFα for 24 h. As shown in Figs. 6A–C, TNFα increased HSP60 expression by almost 70%. Reduction in p65 with siRNA prevented the TNFα mediated increase in HSP60.

4. Discussion

Heart failure is a chronic state of inflammation and stress on the heart tissue. In this setting, an increase in HSP72 would be beneficial, and yet we and others have found that HSP72 is not increased and that the induction of HSP72 following heat shock is blunted in the failing heart [9,10]. In contrast, HSP60 is increased in heart failure; we investigated the mechanism underlying this disparity in expression of HSP60 and HSP72 [8-10,24]. One would anticipate that the failing heart would benefit from increased expression of HSP72, which can refold denatured proteins, accelerate removal of irreparably denatured proteins via the ubiquitin–proteasome system and facilitate synthesis of new proteins by protecting newly synthesized peptides. However, in heart failure there may be sustained stimulation for activation of HSF, and this may result in a loss of response.

The HSP72 promoter has been found to have somewhat more HSE than HSP60, but this difference is not striking [12]. There were a number of predicted HSE in introns in the HSP60 gene, and the significance of these remains to be understood. Understanding the differential

regulation of HSP60 and HSP72 can provide new insight into heart failure. We found that mRNA for HSP60 is increased, while that for HSP72 is not. To further substantiate a role for NF κ B in the upregulation of HSP60 in heart failure, the effect of TNF α on HSP60 was investigated in H9c2 cells, an embryonic cardiac cell line. TNF α increased HSP60 expression by almost 70%, and this was prevented by downregulation of p65 expression by RNAi. These results suggest that TNF α mediates the increased expression of HSP60 in heart failure through NF κ B activation.

4.1. Regulation of HSF activation

A number of different phosphorylation sites are predicted for HSF-1, the primary transcription factor for the heat shock (stress) response. There is consensus that phosphorylation of serines 303/307 by ERK 1/2 and GSK inhibits activation of HSF [17,19]. There was no difference in phosphorylation of serines 303/307 between the control and CHF hearts. Phosphorylation at serine 230 has been found to increase activation of HSF-1, and we found that failing hearts had no significant change in phosphorylation at this site [18]. Not all agree that phosphorylation at serine 230 contributes to HSF-1 activation [25]. Other phosphorylation sites are likely involved in the regulation of HSF-1 activation [25]. It is also likely that additional inhibitory sites remain to be identified. It is also possible that other modifications play a role. Sumoylation has been found to inhibit DNA binding by HSFs, but we were unable to clearly identify sumoylation of cardiac HSF (data not shown) [26,27].

4.2. ERK and GSK in heart failure

There have been mixed reports about the expression and activation of ERK 1/2 and GSK in the failing heart [20,21]. In the rat heart with CHF, we found no difference in total ERK 1/2 or GSK; however, we did find less phospho-ERK 1/2 in the failing heart samples. There was considerable variation in phosphorylation of GSK, and though phosphorylation of the CHF samples was less, this did not reach statistical significance. Although phospho-ERK1/2 was decreased and phospho-GSK tended to be lower, there was no difference in phosphorylation at serines 303/307. These results suggest that another kinase may be involved in phosphorylation of these sites.

4.3. NF κ B, HSP60 expression and heart failure

Full sequence analysis with MatInspector (Genomatix) predicted two NF κ B binding sites in the rat HSP60 gene, which is linked head to head with HSP10 (accession #U68562) [11]. Analysis of the human HSP60 gene predicted three NF κ B binding sites. No potential NF κ B binding sites were identified in the HSP72 promoter. The HSP72 gene is intronless. A number of other investigators have found activation of NF κ B in heart failure [28,29]. Knockout of p50 has been reported to decrease the severity of heart failure [30,31]; We found increased activation of NF κ B in the CHF hearts using a p65 activation assay, but not p50. The ChIP assay was used to analyze binding at the two predicted NF κ B sites in rat HSP60. Both sites showed increased binding of p65 in the CHF hearts, though the difference was greater for site 1, which may be more critical for HSP60 induction in heart failure. As no increase in p50 binding was found, it is likely that an alternate complex, such as p52/p65 may be involved (p65 lacks a DNA binding domain, but has an NLS), as occurs with the alternate pathway for NF κ B activation [32,33]. Activators of the alternate pathway include TNF α , which is elevated in heart failure and in our rat model of heart failure [8]. Thus, TNF α could drive the increase in HSP60 seen in failing hearts. These results support a role for NF κ B in upregulation of HSP60 in heart failure. This was further tested in H9c2 cells. Treatment with TNF α increased HSP60 expression, and this could be blocked by pretreatment with p65 siRNA. A control siRNA had no effect on the TNF α mediated increase in HSP60.

The current findings have implications for cardiac myocyte loss in heart failure. HSP60 in heart failure cardiac myocytes localizes to the plasma membrane and the cell surface, in addition to its normal distribution to the mitochondria and cytosol [8]. HSP60 can also be found in the plasma in heart failure along with TNF α [8]. Others have reported that the release of HSP60 with cardiac injury can temper the immune response to HSP60 acutely [34]. However, extracellular HSP60 is not benign, as suggested by a number of studies and as reviewed elsewhere [35-38]. We have recently reported that extracellular HSP60 activates TLR-4 or a TLR-4 complex, leading to cytokine production and myocyte apoptosis [39]. Release of TNF can then lead to further myocyte apoptosis and increased HSP60 through activation of NF κ B, based on the findings in the current study. Thus there is potentially a vicious cycle of TNF α and HSP60 expression in heart failure leading to greater levels of HSP60, which may then be released from the cell, followed by apoptosis mediated by TLR-4 activation by HSP60 and subsequent TNF α release.

In conclusion, although both HSF-1 and HSF-2 are increased, they were not activated and there was no increase in HSP72 mRNA and protein. This suggests that the lack of increase in HSP72 is regulated at the transcription factor level. It is likely that other modifications of HSF-1, which remain to be identified, are involved. In contrast, both HSP60 mRNA and protein were increased. NF κ B was activated in the failing heart samples, and there was binding of p65 to two predicted NF κ B binding elements in the HSP60 gene in the heart failure samples. These results implicate NF κ B as a mediator in the increased expression of HSP60. NF κ B activation may be driven by the increase in TNF seen in heart failure and present in our model, as previously reported [8]. Further experiments demonstrated that TNF α increased HSP60 expression, and this could be inhibited by down-regulation of p65. These findings support a role for NF κ B in the increase in HSP60 in heart failure.

Acknowledgments

Supported by the Department of Veterans Affairs (A.A.K.), NIH HL077281 (A.A.K.) and HL079071 (A.A.K.).

References

- [1]. Hilfiker-Kleiner D, Landmesser U, Drexler H. Molecular mechanisms in heart failure: focus on cardiac hypertrophy, inflammation, angiogenesis, and apoptosis. *J Am Coll Cardiol* Oct 27;2006 48(9SupplA):A56–66.
- [2]. Beere HM. Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways. *J Clin Invest* Oct 1;2005 115(10):2633–9. [PubMed: 16200196]
- [3]. Jolly C, Morimoto RI. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *JNCI Cancer Spectr* Oct 4;2000 92(19):1564–72.
- [4]. Beckmann RP, Mizzen LA, Welch WJ. Interaction of HSP 70 with newly synthesized proteins: implications for protein folding and assembly. *Science* 1990;248:850–4. [PubMed: 2188360]
- [5]. Beck SC, De Maio A. Stabilization of protein synthesis of thermotolerant cells during heat shock: association of heat shock protein-72 with ribosomal subunits of polysomes. *J Biol Chem* 1994;269:21803–11. [PubMed: 8063823]
- [6]. Voss MR, Gupta S, Stice JP, Baumgarten G, Lu L, Tristan JM, et al. Effect of mutation of amino acids 246-251 (KRKHKK) in HSP72 on protein synthesis and recovery from hypoxic injury. *Am J Physiol Heart Circ Physiol* Aug 12;2005 :H687–92.
- [7]. Miao RQ, Fontana J, Fulton D, Lin MI, Harrison KD, Sessa WC. Dominant-negative Hsp90 reduces VEGF-stimulated nitric oxide release and migration in endothelial cells. *Arterioscler Thromb Vasc Biol* Jan 1;2008 28(1):105–11. [PubMed: 17975117]
- [8]. Lin L, Kim SC, Wang Y, Gupta S, Davis B, Simon S, et al. HSP60 in heart failure: abnormal distribution and role in cardiac myocyte apoptosis. *Am J Physiol* 2007;293:H2238–47.

- [9]. Knowlton AA, Kapadia S, Torre-Amione G, Durand JB, Bies R, Young J, et al. Differential expression of heat shock proteins in normal and failing human hearts. *J Mol Cell Cardiol* 1998;30:811–8. [PubMed: 9602430]
- [10]. Tanonaka K, Furuhashi KI, Yoshida H, Kakuta K, Miyamoto Y, Toga W, et al. Protective effect of heat shock protein 72 on contractile function in the perfused failing heart. *Am J Physiol* 2001;281:H215–22.
- [11]. Hansen J, Bross P, Westergaard M, Nielsen M, Eiberg H, Borglum A, et al. Genomic structure of human mitochondrial chaperonin genes: HSP60 and HSP10 are localised head to head on chromosome 2 separated by a bidirectional promoter. *Hum Genet* 2003;112:71–7. [PubMed: 12483302]
- [12]. Fiszer-Kierzkowska A, Wysocka A, Jarzab M, Lisowska K, Krawczyk Z. Structure of gene flanking regions and functional analysis of sequences upstream of the rat hsp70.1 stress gene*1. *Biochim Biophys Acta (BBA) — Gene Struct Expr* Jan 3;2003 1625(1):77–87.
- [13]. Nakano M, Mann DL, Knowlton AA. Blocking the endogenous increase in HSP72 increases susceptibility to hypoxia and reoxygenation in isolated adult feline cardiocytes. *Circulation* 1997;95:1523–31. [PubMed: 9118521]
- [14]. Hamilton KL, Gupta S, Knowlton AA. Estrogen and regulation of heat shock protein expression in female cardiomyocytes: cross-talk with NF κ B signaling. *J Mol Cell Cardiol* Apr;2004 36(4):577–84. [PubMed: 15081317]
- [15]. Hamilton KL, Mbai FN, Gupta S, Knowlton AA. Estrogen, heat shock proteins, and NF κ B in human vascular endothelium. *Arterioscler Thromb Vasc Biol* Sep 1;2004 24(9):1628–33. [PubMed: 15231513]
- [16]. Chen FC, Brozovich FV. Gene expression profiles of vascular smooth muscle show differential expression of mitogen-activated protein kinase pathways during captopril therapy of heart failure. *J Vasc Res* 2008;45:445–54. [PubMed: 18418003]
- [17]. Kline MP, Morimoto RI. Repression of the heat shock factor 1 transcriptional activation domain is modulated by constitutive phosphorylation. *Mol Cell Biol* Apr 1;1997 17(4):2107–15. [PubMed: 9121459]
- [18]. Holmberg CI, Hietakangas V, Mikhailov A, Rantanen JO, Kallio M, Meinander A, et al. Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1. *Embo* 2001;20:3800–10.
- [19]. Chu B, Soncin F, Price BD, Stevenson MA, Calderwood SK. Sequential phosphorylation by mitogen-activated protein kinase and glycogen synthase kinase 3 represses transcriptional activation by heat shock factor-1. *J Biol Chem* Nov 29;1996 271(48):30847–57. [PubMed: 8940068]
- [20]. Communal C, Colucci WS, Remondino A, Sawyer DB, Port JD, Wichman SE, et al. Reciprocal modulation of mitogen-activated protein kinases and mitogen-activated protein kinase phosphatase 1 and 2 in failing human myocardium*1. *J Card Fail* Apr;2002 8(2):86–92. [PubMed: 12016632]
- [21]. Takeishi Y, Huang Q, Abe Ji, Che W, Lee JD, Kawakatsu H, et al. Activation of mitogen-activated protein kinases and p90 ribosomal S6 kinase in failing human hearts with dilated cardiomyopathy. *Cardiovasc Res* Jan;2002 53(1):131–7. [PubMed: 11744021]
- [22]. Mestrl R, Chi SH, Sayen MR, Dillmann WH. Isolation of a novel inducible rat heat-shock protein (HSP 70) gene and its expression during ischaemia/hypoxia and heat shock. *Biochem J* 1994;298:561–9. [PubMed: 8141767]
- [23]. Hunt C, Morimoto RI. Conserved features of eukaryotic HSP 70 genes revealed by comparison with the nucleotide sequence of human HSP 70. *Proc Natl Acad Sci U S A* 1985;82:6455–9. [PubMed: 3931075]
- [24]. Latif N, Taylor PM, Khan MA, Yacoub MH, Dunn MJ. The expression of heat shock protein 60 in patients with dilated cardiomyopathy. *Basic Res Cardiol* 1999;94:112–9. [PubMed: 10326659]
- [25]. Guettouche T, Boellmann F, Lane W, Voellmy R. Analysis of phosphorylation of human heat shock factor 1 in cells experiencing a stress. *BMC Biochemistry* 2005;6(4):1–14. [PubMed: 15715904]
- [26]. Hietakangas V, Ahlskog JK, Jakobsson AM, Hellesuo M, Sahlberg NM, Holmberg CI, et al. Phosphorylation of serine 303 is a prerequisite for the stress-inducible sumo modification of heat shock factor 1. *Mol Cell Biol* Apr 15;2003 23(8):2953–68. [PubMed: 12665592]

- [27]. Anckar J, Hietakangas V, Denessiouk K, Thiele DJ, Johnson MS, Sistonen L. Inhibition of DNA binding by differential sumoylation of heat shock factors. *Mol Cell Biol* Feb 1;2006 26(3):955–64. [PubMed: 16428449]
- [28]. Wong SCY, Fukuchi M, Melnyk P, Rodger I, Giaid A. Induction of cyclooxygenase-2 and activation of nuclear factor- κ B in myocardium of patients with congestive heart failure. *Circulation* Jul 14;1998 98(2):100–3. [PubMed: 9679714]
- [29]. Frantz S, Fraccarollo D, Wagner H, Behr TM, Jung P, Angermann CE, et al. Sustained activation of nuclear factor κ B and activator protein 1 in chronic heart failure. *Cardiovasc Res* Mar 1;2003 57(3):749–56. [PubMed: 12618236]
- [30]. Frantz S, Hu K, Bayer B, Gerondakis S, Strotmann J, Adamek A, et al. Absence of NF- κ B subunit p50 improves heart failure after myocardial infarction. *FASEB J* Sep 1;2006 20(11):1918–20. [PubMed: 16837548]
- [31]. Kawano S, Kubota T, Monden Y, Tsutsumi T, Inoue T, Kawamura N, et al. Blockade of NF- κ B improves cardiac function and survival after myocardial infarction. *Am J Physiol* 2006;291:H1337–44.
- [32]. Karin M, Lin A. NF- κ B at the crossroads of life and death. *Nat Immunol* 2002;3:221–7. [PubMed: 11875461]
- [33]. Stice JP, Knowlton AA. Estrogen, NF κ B and the heat shock response. *Mol Med* 2008;14:517–8. [PubMed: 18431462]
- [34]. Schett G, Metzler B, Kleindienst R, Amberger A, Recheis H, Xu Q, et al. Myocardial injury leads to a release of heat shock protein (hsp) 60 and a suppression of the anti-hsp65 immune response. *Cardiovasc Res* 1999;42:685–95. [PubMed: 10533609]
- [35]. Knoflach M, Kiechl S, Mayrl B, Kind M, Gaston JSH, Van der Zee R, et al. T-cell reactivity against HSP60 relates to early but not advanced atherosclerosis. *Atherosclerosis* Dec;2007 195(2):333–8. [PubMed: 17070529]
- [36]. Shamaei-Tousi A, Halcox J, Henderson B. Stressing the obvious? Cell stress and stress proteins in cardiovascular disease. *Circ Res* 2007;74:19–28.
- [37]. Xiao Q, Mandal K, Schett G, Mayr M, Wick G, Oberhollenzer F, et al. Association of serum-soluble heat shock protein 60 with carotid atherosclerosis: clinical significance determined in a follow-up study. *Stroke* Dec 1;2005 36(12):2571–6. [PubMed: 16254226]
- [38]. Knowlton AA, Srivatsa U. Heat-shock protein 60 and cardiovascular disease: a paradoxical role. *Futur Cardiol* 2008;4:151–61.
- [39]. Kim SC, Stice JP, Chen L, Jung JS, Gupta S, Wang Y, et al. Extracellular heat shock protein 60, cardiac myocytes and apoptosis. *Circ Res* 2009;105:1186–95. [PubMed: 19875724]

Fig. 1.

(A) Real-time PCR for HSP60 normalized to GAPDH. (B) Real-time PCR for HSP72 normalized to GAPDH. (C) Graph summarizes relative expression of HSF-1 by Western normalized to beta actin. Lower panel, western showing expression of HSF-1 and beta actin on the same blot. (D) Relative expression of HSF-2 by Western normalized to beta actin. Lower panels, Western showing expression of HSF-2 and GAPDH on same blot. * $p < 0.001$ vs. C, + $p < 0.05$ vs. C; $n = 4-6$ /group.

Fig. 2.

Phosphorylation of HSF-1 at serine 230 (thought to be activating) and serine 303/307 (inhibitory) by Western. (A) Graph summarizes relative expression of phospho-serine230 and phospho-serine303/307 each normalized to total HSF-1. (B) Westerns for phospho-serine 230, phospho-serine303/307 and HSF-1 on same blot. HSF-1 was immunoprecipitated for these experiments. C, control; HF, heart failure. $n=6-14$ /group.

Fig. 3.

(A) Graph summarizes relative expression of ERK by Western normalized to beta actin. (B) Blot showing total ERK and beta actin from same blot. (C) Graph summarizes phospho-ERK by western normalized to beta actin. (D) Western for phospho-ERK and total ERK on same blot. * $p < 0.05$ vs. C. $n = 4/\text{group}$.

Fig. 4. (A) Graph summarizes relative expression of GSK by Western normalized to beta actin. (B) Western for total GSK and beta actin on the same blot. (C) Graph summarizes phospho-GSK normalized to total GSK by Western. (D) Western showing expression of phospho-GSK and total GSK done on same blot. $p=ns$. $n=4/\text{group}$.

Fig. 5.

HSF and NFκB activation. (A) EMSA shows HSF activation in both controls and CHF heart samples with loading of a large amount of nuclear extract. CC, cold compete. (B) Supershift for HSF-1, faint supershift seen (arrow) for all lanes. (C) Graph summarizes p65 binding activity in control nuclear extracts vs. CHF. $n=5-7/\text{group}$. $*p<0.05$. (D) Representative EMSA for NFκB. CC (cold compete), sham and CHF samples, respectively. (E) Representative results for ChIP assay for NFκB binding sites 1 (300 bp) and 2 (150 bp). Input is total DNA for each sample (starting material), and IgG control lanes are shown on far right for control and CHF, respectively.

Fig. 6.

NFκB and HSP60 expression. (A) Effect of siRNA on p65 protein levels in H9c2 cells. Control siRNA(siRNA-C) shown on left. (B) Representative western blot showing effect of TNFα on HSP60 expression in H9c2 cells and effect of p65 knockdown on HSP60. (C) Graph summarizes results of multiple experiments. TNFα treatment status shown at bottom. * $p < 0.05$ vs. all.

Table 1

Predicted HSF and NFκB binding sites in HSP60 and HSP72 — sites listed have correct orientation and are either in promoter or in intron. Acc., accession number.

Gene	Predicted NFκB binding site	Location	Predicted HSFs binding site (heat shock element)	Location
Rat HSP60	1.7084-7096	Intron	3678-3697	Promoter
Acc. U68562	2.7207-7219	Intron	6233-6257	Intron
			9293-9317	Intron
			9326-9350	Intron
			11702-11726	Intron
			13330-13351	Intron
Human HSP60	342-354	Promoter	41-65	Promoter
Acc. NC_000002	3274-3286	Intron	182-206	Promoter
	12144-12156	Intron	4256-4280	Intron
			5413-5437	Intron
			12661-12685	Intron
Rat HSP72	None		78-102*	Promoter
Acc. X75357			160-184*	Promoter
Human HSP72	None		64-88	Promoter
Acc. M11717			157-181	Promoter

* Overlap two sites identified by Mestrl [22].

Table 2

Primers used for ChIP assay.

Site 1 6894-7203	Forward	5'CCACTGTTCTGGCACCGTCTATTGCCA3'
309 bp product	Reverse	5'GCCCTGTATTTTCACTTCCATTAAAT3'
Site 2 7203-7279	Forward	5'CTTTCGGTATATCCCTGGCTGTCCT3'
76 bp product	Reverse	5'CAGATCTATTTGAAGCTAACTAGCC3'