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The Heat Shock Paradox and Cardiac Myocytes: Role of Heat Shock Factor

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Abstract

The induction of the heat shock response is accepted to be a protective response, reducing injury and improving cell survival. However, when inflammation precedes heat shock there is an unexpected increase in injury, known as the heat shock paradox, which is hypothesized to be a mechanism underlying multi-organ dysfunction. We hypothesized that the heat shock paradox would occur in adult cardiac myocytes and that heat shock factor (HSF)1 would contribute to injury. Heat shock (HS) at 42°C and TNF (10 ng/ml) were used as the HS and the inflammatory insult, respectively. The combination of TNF followed by HS (TNF/HS) caused the greatest amount of apoptosis in adult rat cardiac myocytes. TNF/HS resulted in an increase in heat shock protein (HSP) 60, compared to untreated cells, those receiving HS/TNF, or TNF alone. There was no increase in heme oxygenase 1 in any of the groups. HSP72 increased in all the groups, with the greatest levels with TNF/HS. NFκB activation was greatest with TNF/HS. Pretreatment with a DNA binding decoy for HSF1 prevented the increase in HSPs and decreased apoptosis in all groups. However, the increase in iNOS, seen in all treatment groups, was unaffected by the HSF1 binding decoy. We conclude that the heat shock paradox occurs in adult cardiac myocytes, that HSP60 is increased as part of the heat shock paradox, and that HSF1 activation contributes to injury.

Keywords

HSF1; NFκappaB; HSP60; apoptosis; MODS; iNOS

Introduction

Induction of the heat shock response with increased expression of the heat shock proteins is well-established as a protective response.(1–3) However, when an inflammatory stimulus precedes heat shock there is an unexpected increase in injury, known as the heat shock paradox.(4;5) The heat shock paradox has been reported in several different settings

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including endothelial cells and a mouse model of sepsis, and may be a mechanism for multi-organ dysfunction.(4;5) The mechanism of this deleterious response remains elusive. The heat shock paradox is of great interest because of its potential role in the multi-organ dysfunction syndrome (MODS) seen in critically ill patients in the intensive care unit. We investigated the heat shock paradox using isolated rat adult cardiac myocytes. We hypothesized that activation of heat shock factor (HSF) 1, the heat shock protein transcription factor activated in response to stress, was a critical element for injury, despite the overall protective effects of the expression of heat shock proteins (HSPs).

Using TNF α (TNF) as the inflammatory stimulus, we found that the addition of heat shock (HS) markedly decreased I κ B, drove activation of NF κ B and increased expression of iNOS, along with the expected increase in HSPs. HS or TNF alone or HS followed by TNF did not decrease I κ B. Using DNA binding decoys for HSF, we dissected the role of HSF activation in these changes. The results of these experiments are reported here. To our knowledge, this is the first report of the heat shock paradox in cardiac myocytes.

Materials and Methods

Isolated Adult Rat Cardiac Myocytes

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. Cardiac myocytes were isolated from adult male Sprague Dawley rats as previously described.(6)

Experimental Protocol

Experimental groups consisted of: 1. Control, untreated cells; 2. HS at 42°C for 1 h followed by 18 h of recovery at 37°C; 3. TNF (10 ng/ml, R&D Systems) for 19 h.; 4. TNF with 1 h of HS at 42°C at 6 h and then 12 h of recovery at 37°C; 5. HS at 42°C for 1 h followed by TNF (10 ng/ml) for 18h at 37°C. Heat shock was delivered in a special CO₂ incubator set to 42°C (Brunswick). All protein samples were collected at the end of the experiment, 19 h, for analysis. NF κ B activation was measured 1 h. after the last treatment (HS or TNF).

Western Blotting

was performed as previously described.(7) Antibodies were used to HSP72 (StressGen, 1:1000) HSP60 (SPA806, 1:1000, StressGen), heme oxygenase 1 (HO-1, also known as HSP32, 1:1000, StressGen), I κ B (1:1000, Cell Signaling), and iNOS (1:1000, BD Transduction). Antibody to GAPDH (1:40,000, Fitzgerald Industries) was used as a loading control. Nitrocellulose membranes after blocking and antibody incubations were developed with a chemiluminescent system (Pierce). Densitometry was done on scanned images using U-Scan It software.

NF κ B Activation Assay

This assay measures binding of nuclear extract to a microtiter plate coated with the DNA binding element for NF κ B (Pierce). The plate is washed and developed with antibody for p65 following the instructions of the manufacturer. Previously, we have found this assay to correlate well with the results of EMSA, but to provide more precise quantification and to allow for the comparison of multiple samples.(8)

LDH Release

was measured as an index of necrosis as previously described.(7)

Apoptosis

was assessed by measuring caspase 3 activity using an assay that detects cleavage of AC-DEVD-AMC (Calbiochem, San Diego, CA). DNA fragmentation was used as a second index of apoptosis and was measured by the Cell Death ELISA (Roche) following instructions of the manufacturer. This assay measures oligosome formation, an index of DNA fragmentation. The results were normalized to control, untreated cells to correct for any variation amongst cardiac myocyte isolations.

Cell Viability

was determined with the Live/Dead Assay (Invitrogen) as previously described.(7)

HSF Decoy

DNA binding decoys act as a cytosolic sponge, binding activated transcription factor.(9;10) A scrambled nonspecific sequence was used as a control as previously described.(11) The phosphorothioate DNA was synthesized and purified by Trilink (San Diego). The small DNA dimers consist of oligonucleotides encoding a consensus DNA binding domain for HSF as previously detailed.(11) Cells were pretreated with 2 μ M HSF1 decoy or a scrambled control sequence for 6h, as reported previously, prior to the start of the experiment.(8;11)

Statistics

Data was analyzed by an ANOVA followed by a Student Neuman Keuls test. Normalized data was analyzed by an ANOVA on RANKS followed by an appropriate secondary test. A $p < 0.05$ was considered significant. Results are expressed as mean $\pm$ the standard error of the mean (SEM).

Results

The effect of heat shock (HS), TNF, HS/TNF and TNF/HS on cardiac myocyte injury were compared. Figure 1 summarizes the time line for the treatment protocols. As shown in figure 2A, caspase 3 activation occurred with all treatments, but the greatest activation was seen with TNF treatment, which was used as an inflammatory injury, followed by HS (TNF/HS). Similarly, DNA fragmentation, as measured by the CDD assay, was increased with all treatments, but greatest with TNF /HS (figure 2B). LDH release was mild to moderately increased by the treatments, but in contrast to the apoptosis indices, TNF and TNF plus heat shock resulted in similar amounts of LDH release (figure 2C). To assess the percent of cells undergoing apoptosis/cell death, the Live/Dead Assay was used, as shown in figure 2D. TNF/HS was associated with the highest cell death with almost 30% of the cells dead, compared to 12.3% with HS/TNF. Thus, HS prior to TNF treatment diminished the injury caused by TNF alone for both apoptosis and necrosis parameters (figure 2A–D).

Both HS and TNF activate HSF-1 and this would be expected to increase HSP levels.(6) Expression of HSP72, HSP60 and HO-1 (HSP32) were examined by western blotting at 19h. HO-1 levels did not change significantly, but the combination of TNF/HS increased HSP60 levels by greater than 2.5 fold (figure 3A and B). HSP72, which is more readily induced by stresses such as heat, was significantly increased by all four treatments with TNF/HS having the greatest effect (figure 3C, $p < 0.05$), and HS exceeding the effect of TNF or HS/TNF.

Activation of NF κ B is thought to have a role in the heat shock paradox. We examined NF κ B activation after treatment with TNF, HS/TNF and TNF/HS. For each treatment, NF κ B activation was examined 30 min. after treatment. As the TNF/HS combination has the second treatment (HS) at 6 h, NF κ B 7.5 h after TNF treatment alone was examined for

comparison (figure 4A). TNF treatment increased NF κ B activation, but the difference was not significant. There was no change in NF κ B activation when TNF was given after HS. In contrast, NF κ B activation was markedly increased when TNF was followed by HS ($p < 0.05$). This was 7.5 h after initial TNF treatment. TNF treatment alone by 7.5h had no evidence of NF κ B activation. To investigate the underlying mechanism, levels of I κ B were examined. Only TNF followed by HS resulted in a decrease in I κ B expression (figure 4B, $p < 0.05$). In addition, as shown in figure 4C/D, all four treatments increased expression of iNOS.

HS and TNF both activate HSF1.(6) HSF DNA binding decoys, as previously described, were used to inhibit the effects of HSF1 activation. The decoys are small phosphorothioate oligonucleotide dimers encoding the binding element (heat shock element, HSE) for HSF. The decoys act as a sponge for activated HSF1. For the decoy experiments, we focused on apoptosis. Cells were pretreated with HSF1 decoy or a scrambled control sequence for 6h, as reported previously.(8;11) The HSF1 decoy inhibited caspase 3 activation after TNF, HS/ TNF or TNF/HS (figure 5A). Similarly, DNA fragmentation was greatly reduced by the HSF decoy (figure 5B). The scrambled control decoy had no effect.

Western blotting was done to analyze the effect of the HSF decoy on the protein changes seen with the treatments. HO-1 expression was not significantly changed, similar to the baseline experiments. HSP60 expression was decreased in the HS/TNF and TNF/HS groups (figure 6A and B). This is in contrast to the increase in HSP60 expression seen with TNF/HS in the absence of the decoy. HSP72 expression was decreased in all groups (figure 6C). The HSF decoy blocked the decrease in I κ B seen with TNF/HS (figure 7A). iNOS levels were not affected by the HSF decoy (figure 7B).

Discussion

This study investigates the role of HSF in the heat shock paradox, where induction of the heat shock response after an inflammatory insult is unexpectedly more injurious than the inflammatory insult alone.(5) While most experimental studies focus on a single injury, in the intensive care setting it is common to have multiple sequential injuries, such as the trauma victim, who develops a fever, infection and other complications, or in the elderly, who at baseline have elevation of inflammatory cytokines such as IL-6.(12) Thus, the heat shock paradox may be quite important in the hospital course of severely ill patients. In the current study, we report for the first time to our knowledge that adult cardiac myocytes exhibit the heat shock paradox. TNF/HS, an inflammatory insult followed by heat shock, caused more apoptotic injury than any of the other treatments. Overall, HS prior to TNF was protective, as would be expected. Interestingly, only TNF/HS significantly increased HSP60 expression. In contrast, all groups had increased HSP72, but the greatest increase was seen with TNF/HS. TNF/HS caused a marked increase in NF κ B activity. Only in this group was I κ B expression significantly decreased. iNOS was used as another index of an inflammatory response. All treatment groups had an increase in iNOS.

Inhibition of HSF and the Heat Shock Paradox

A DNA binding decoy was used to block the effect of any activated HSF. Others have investigated the role of NF κ B activation in the deleterious effects of the heat shock paradox by inhibiting NF κ B; however, many inhibitors of NF κ B are actually inducers of the heat shock proteins, which are known to inhibit activation of NF κ B.(13–15) Thus, it is difficult to separate the effects of NF κ B inhibition from the induction of the heat shock response by the inhibitors. In the current study, we focused on the role of HSF activation. The DNA binding decoys are more selective than chemical inhibitors. HSF1 is the main mediator of the stress response, but HSF2 can be activated as well. HSFs are inactive in the cytosol of the normal, unstressed cell. Phosphorylation, trimerization and translocation to the nucleus

are important steps in activation. Both activated HSF1 and HSF2 will be blocked by the DNA binding decoy, which acts as a cytosolic sponge for activated HSF.(9) Interestingly, the HSF DNA binding decoy significantly decreased apoptosis in all groups, but did not completely prevent apoptosis. Although brief heat shock is thought to induce protection, even the HS/TNF group had a marked decrease in apoptosis with the HSF DNA binding decoy. These results suggest that HSF activation can contribute to injury, as well as activating protective responses. The HSF binding decoy also blocked the increases in HSP60 and HSP72, and tended to decrease the expression of the three HSPs in all groups, likely reflecting inhibition of normal synthesis. The binding decoy had no effect on iNOS expression, indicating that the upregulation of iNOS was independent of HSF.

NFκB and the HSPs: A Complex Dance

There is an intertwining of the actions of the HSPs, HSF1, TNF and NFκB.(14;16) These interactions may help maintain the balance between health and inflammation. HSF1 inhibits both NFκB and the expression of TNF.(17) There is a chronic increase in TNF levels in HSF1 knockout mice, and this is associated with increased sensitivity to endotoxin.(18) The TNF promoter has an HSF1 binding site, and HSF1 functions as a repressor of TNF expression.(19;20) In contrast, TNF can inhibit the activation of HSF-1 via activation of phosphatases mediated by TNF-R1.(21) Low dose TNF increases HSP72 levels in cardiac myocytes, and we have found that TNF can activate HSF1 in cardiac myocytes (Chang and Knowlton, unpublished).(22) Others have reported that TNF increases resistance to heat injury in cancer through induction of MnSOD, increased HSF1 binding and greater HSP72 expression.(20;23) Activation of HSF1 is regulated by a complex mixture of phosphorylation and desphosphorylation, with phosphorylations at distinct sites promoting and inhibiting activity.(24–26) The heat shock paradox reflects a disassembly of the balance between HSPs and NFκB, viability and apoptosis, health and inflammation. The involved signaling pathways are complex. HSF1 is primarily known as the transcription factor for the HSPs. Slowly additional genes regulated by HSF1 are being identified including most recently, FasL, which has heat shock elements (HSE) in its promoter and is upregulated by HSF1. (20) HSF1 has also been found to block the activation and recruitment of SP1 and AP1.(20;27) In vascular smooth muscle cells knockdown of HSF1 with siRNA increased angiotensin II induced inflammation. Our understanding of the interaction of these fundamental pro- and anti-inflammatory proteins and pathways is incomplete, and much remains to be understood.

HSF, NFκB Activation and HSP60

In the current study, HS prior to TNF attenuated injury, but HS post-TNF increased injury. All the treatments increased expression of HSP72, although this increase was greatest with TNF/HS. HSP60 was only increased with the combination of TNF/HS, and this likely reflects the strong sequential activation NFκB occurring with first TNF and then HS combined with HSF1 activation. HS prior to TNF was associated with lower activation of NFκB. We have recently reported that the HSP60 gene contains two NFκB binding sites, and that these drive expression of HSP60 in cardiac myocytes.(28) Thus, TNF can increase the expression of HSP60. The HSF DNA binding decoy inhibited all of the changes in HSPs seen with the different treatments, and significantly decreased basal expression of both HSP60 and HSP72, likely through inhibition of underlying HSF1 mediated expression of these proteins.

These findings have important implications for our understanding of evolving illness in the critical care setting. In the absence of chest pain or pulmonary edema, most emphasis is placed on the function of organs other than the heart, assuming that without these signs and symptoms the heart is normal. However cardiac dysfunction is amongst the criteria for

MODS, and rising TNF levels have been found in patients who go on to develop MODS. (29;30) MODS and other critical illness states, for which the criteria remain in flux, are associated with autonomic dysfunction with loss of the normal variability of heart rate and other parameters.(31;32) Increased troponin I leak, suggesting cardiac myocyte damage, in the absence of acute coronary syndrome or heart failure is associated with poorer outcomes. (33) However, cardiac myocyte apoptosis, as seen in the current study with TNF/HS, will not result in the release of troponin I, but has the potential to negatively impact cardiac function both acutely and in the long term. Our results support a more aggressive monitoring of cardiac function with cardiac echo, as well as invasive monitoring, such as cardiac output and pressure measurement, which is more routinely done in the setting of MODS.

The heat shock paradox, or two-hit hypothesis, has been studied primarily in cell culture. It has also been demonstrated that mice have the heat shock paradox when heat follows cecal ligation and puncture (CLP).(5) Repetitive injury is not only likely important in the clinic, it may very well be the norm. In the current study we demonstrate that adult cardiac myocytes manifest the heat shock paradox, and that this is accompanied by a significant increase in HSP60. HSP60 itself can cause inflammation and apoptosis, and further work will need to address the importance of the increase in this protein.(34) (14;35)

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Figure 1.

Treatment protocols are summarized in diagram. For CDD assay, live dead assay and westerns, samples were studied at 19 hr., which is the completion of all protocols, on the far right. Caspase 3 activity, which increases before DNA fragmentation based on pilot studies, was measured at 16 hr. NF κ B activity was postulated to peak early after treatments. Therefore, NF κ B activity was measured at 7.5 h, which is 30 min. after completion of the last HS treatment. Dark grey bar represents TNF treatment. In the TNF groups, TNF was added at the beginning of experiment and not removed at any point, nor was new TNF added after HS. For the HS/TNF group, as HS was the first treatment, TNF added after completion of the 1h HS. Light grey bar represents 1 hr heat shock. HS - heat shock at 42°C for 1 h followed by 18 h at 37EC in a standard cell culture incubator; TNF - TNF α 10 ng/ml; HS/TNF - heat shock at 42°C for 1 h followed by the addition of TNF 10 ng/ml for 18 h; TNF/HS - TNF α 10 ng/ml for 6 h followed by heat shock at 42°C for 1 h, and then 12 h at 37EC in a standard cell culture incubator.

Figure 2.

A) Caspase 3 activity at 16h. B) DNA fragmentation by CDD or cell death assay (Roche) at 19h. C) LDH release at 19 h. D) Live Dead Assay. Percent of cells dead based on ethidium bromide uptake vs. metabolism of calcein in live cells at 19 h. C - control; HS - heat shock; HSTNF - 1 h hs followed by TNF treatment; TNFHS - 6 h TNF followed by 1 h hs. * p < 0.05 vs. all others, + p < 0.05 vs. C, HS, HSTNF, # p < 0.05 vs. C. Graphs summarize 3–6 experiments with 9–18/group.

Figure 3.

Heat shock protein expression at 19 h. Expression was analyzed by western with GAPDH as a loading control. Graphs summarize results of 4–6 separate experiments. A) HO-1. B) HSP60. C) HSP72. C - control; HS - heat shock; HSTNF - 1 h hs followed by TNF treatment; TNFHS - 6 h TNF followed by 1 h hs. * $p < 0.05$ vs. all others. + $p < 0.05$ vs. control. N = 4–6/group.

Figure 4.

A) NFκB activation was measured by assay 30 min. after completion of all treatments. TNF at 30 min. (black bar) and at 7.5 h (cross-hatched bar) are shown for comparison. * p < 0.05 vs. C (control). B) IκBα expression was examined at 19h. Levels were decreased in TNF/HS group only. Graph summarizes results. Representative western with GAPDH on the same blot shown on right. * p < 0.05 vs. all. C) iNOS levels were increased in all groups. * p < 0.05 vs. C. D) Representative western for iNOS. GAPDH for same blot shown below. All protein samples collected at 19 h. Graphs summarize results of 3–5 separate experiments. C - control; HS - heat shock; HS/TNF - 1 h hs followed by TNF treatment; TNF/HS - 6 h TNF followed by 1 h hs.

Figure 5.

Effect of HSF binding decoy on apoptosis. A) Caspase 3 activity at 16h. B) DNA fragmentation at 19h. Graphs summarize 3 separate experiments with 9–15/group. C - control; HS - heat shock; HS/TNF - 1 h hs followed by TNF treatment; TNF/HS - 6 h TNF followed by 1 h hs. SCR - scrambled sequence decoy (i.e. nonfunctional). * $p < 0.05$ vs. all. # $P < 0.05$ vs. C. + $p < 0.05$ vs. respective decoy.

Figure 6. HSF DNA binding decoy and HSP expression at 19 h. A) HO-1 protein levels were unchanged. B) HSP60 protein expression was decreased in all groups compared to control. C) HSP72 expression was decreased in all groups compared to control. Graphs summarize results of multiple westerns. Data normalized to control group. Representative western for each protein and GAPDH on same blot shown. N = 3–4/group. * p < 0.05 vs. C.

Figure 7.

Effect of HSF binding decoy on IκB and iNOS expression at 19 h. A) HSF binding decoy blocked the decrease in IκB seen with TNF/HS. B) HSF binding decoy had no effect on iNOS expression. Graphs summarize results of multiple westerns. Data normalized to control group. Representative western for each protein and GAPDH on same blot shown. N = 4/group. * p < 0.05 vs. C.