Protection against endotoxemia by HSP70 in rodent cardiomyocytes

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Protection against endotoxemia by HSP70 in rodent cardiomyocytes. Am J Physiol Heart Circ Physiol 278: H1439–H1445, 2000.—Clinical and experimental studies have shown that myocardial dysfunction is an early event during endotoxemia or septic shock. Several reports have shown that rodents submitted to a mild heat shock become resistant to lipopolysaccharides (LPS) or sepsis. The most abundant of the heat shock proteins (HSP), the HSP70, has been postulated to be the principal mediator of the observed protection against endotoxemia. We have tested the hypothesis that a protective effect against endotoxemia is achievable by the increased presence of the HSP70 in rodent cardiomyocytes. We have found that a transgenic mouse line overexpressing the rat HSP70 gene in the heart exhibits an increased tolerance to LPS treatment [control estimated survival function \( S(t) = 0.538, \text{transgenic } S(t) = 0.787, P < 0.05 \)]. Interestingly, the increased presence of the HSP70 in the hearts of these mice results in a decrease in the activation of the inducible nitric oxide synthase (iNOS) after LPS treatment. We conclude that HSP70 protection against LPS is most probably mediated through the modulation of iNOS activation and the subsequent decreased synthesis of nitric oxide in cardiomyocytes.

heat shock proteins; lipopolysaccharides; septic shock; cross-protection...
Induces iNOS induction (9). In the present study, we demonstrate a direct relationship between the level of expression of HSP70, resistance against LPS, and the levels of iNOS in the hearts of transgenic and control mice exposed to LPS. These results indicate that a major part of the protection against LPS exposure by the increased presence of the HSP70 is attributable to the modulation of iNOS activation by HSP70.

METHODS

Cell culture of rat neonatal cardiomyocytes. Neonatal rat ventricular myocytes were prepared as previously described by us (15). Tissue culture plates of neonatal cardiomyocytes were preheat treated at 42°C for 60 min or were pretreated with herbimycin A (0.5 mg/ml for 4 h) after which they were left to recuperate at 37°C for 8 h. Control plates were left untreated. Subsequently, one-half of the pretreated plates and control plates was exposed to LPS (L-2262; Sigma Chemical, St. Louis, MO) at a final concentration of 200 µg/ml for 16 h in complete Hanks’ buffered salt solution, after which LPS-induced cell injury was evaluated.

Adenoviral vector constructs and infection of myocytes. Rat neonatal cardiomyocytes are known to be permissive to adenovirus infection (34). The adenoviral construct containing the rat HSP70 gene (AdHSP70i) and the control adenoviral construct (AdS5R) have been previously described by us (23). Adenoviral particles at high titers were used to infect neonatal cardiomyocytes in DMEM with 2% heat-treated FBS. A time span of 2 days is required to obtain adequate transgene expression. Therefore, cells were left for 2 days, subsequently placed in serum-free medium, and exposed to LPS.

Protein analysis. Protein samples (40 µg each) were fractionated for Western blot analysis on an 8% SDS-polyacrylamide gel and were electrotransferred to nitrocellulose using a semidry electrotransfer apparatus (Bio-Rad). The nitrocellulose blots were reacted with a polyclonal antibody that binds to the nitric oxide synthase, horseradish peroxydase-conjugated antibody and developed with diaminobenzidine tetrahydrochloride (DAB kit; Vector Laboratories).

Quantitation of creatine kinase release. After exposure to LPS, medium and cardiomyocytes were sampled, and creatine kinase (CK) enzymatic activity was determined. CK release in the medium is expressed as a fraction of the total enzymatic activity present in cardiomyocytes. CK enzyme activity was then normalized by the amount of protein in each plate (U/mg). As can be seen, preheat treatment or pretreatment with herbimycin A resulted in lower CK release as compared with control and nontransgenic (n = 39) littermate mice were injected intraperitoneally with 20 mg/kg body wt of LPS (Sigma). Animals were then placed under close supervision for the duration of the experiment (5 days). Death of any animal during the study was logged, and an immediate postmortem autopsy was performed to obtain tissues for further analysis. To assure ourselves that the transgenic mice used in our study were overexpressing the rat HSP70 transgene, we routinely saved a piece of the myocardium, brain, and muscle at the end of each experiment. The pieces were used for Northern and Western blot analysis, as previously described by us (19).

Determination of the mRNA levels of iNOS. Total RNA was prepared from transgenic and control mice treated with LPS. Total RNA was analyzed by Northern blots that were hybridized with specific cDNA probes to the rat iNOS gene, the rat HSP70, and the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The level of expression of each mRNA was quantitated by scanning the Northern blots.

Statistics. Results are expressed as means with SE determined by conventional methods. Statistical comparisons were performed between the different experimental groups by using the Student’s two-tailed, unpaired t-test. To analyze the survival differences between the two experimental mouse groups, estimated survival functions [S(t)] for each group at each time point were computed and used to generate a Kaplan-Meir product-limit estimate of the survival curve for each group (11). A $\chi^2$ test was then applied to 2 × 2 contingency tables to obtain a probability value. A probability value of < 0.05 was considered to be statistically significant.

RESULTS

Initially, we established if neonatal rat cardiomyocytes could be rendered tolerant to LPS exposure. Our results show that a preheat treatment or pretreatment with herbimycin A, a known inducer of the heat shock response (7, 24), is able to protect neonatal rat cardiomyocytes against LPS exposure, as shown in Table 1. Cardiomyocytes were either preheat treated (42°C for 60 min), treated with herbimycin A (0.5 µg/ml, 4 h) 8 h before LPS exposure, or left untreated (control). CK activity was then normalized by the amount of protein in each plate (U/mg). As can be seen, preheat treatment and herbimycin A treatment result in a significant decrease in CK release after LPS exposure. To confirm these results, we also used a cytotoxicity

Table 1. Creatine kinase released from LPS-treated rat neonatal cardiomyocytes

<table>
<thead>
<tr>
<th>Condition</th>
<th>U/mg</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>8.3</td>
</tr>
<tr>
<td>Herbimycin A-treated</td>
<td>89.1</td>
<td>8.5</td>
</tr>
<tr>
<td>Heat shock-treated</td>
<td>88.4</td>
<td>5.6</td>
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</tbody>
</table>

Values are means ± SE from 6 independent experiments and represent percentage of creatine kinase released compared with control myocytes without (−) or with (+) LPS, which were taken as 100%. *P < 0.05 vs. control myocytes +LPS.
assay kit (XTT; Sigma). As presented in Table 2, pretreatment of cardiomyocytes with herbimycin A or a heat shock preserves cell viability after LPS exposure, as measured by the XTT cytotoxicity assay kit. Presumably, this protective effect is linked to the induction of the heat shock proteins by herbimycin A and the pre-heat shock treatment.

Our previous results have shown that a preheat treatment and a herbimycin A treatment are able to induce the heat shock proteins, particularly the HSP70 in isolated neonatal cardiomyocytes (7, 15). To test if the HSP70 by itself is capable of protecting the cardiomyocyte against LPS in a similar fashion as we previously showed in the myogenic H9c2 cell line (6), we introduced an exogenous copy of the HSP70 gene in adenoviral vectors. We have recently been successful in infecting cardiomyocytes with an adenoviral construct containing the rat inducible HSP70 gene, which is highly expressed in cardiomyocytes (23), as shown in Fig. 1. Interestingly, rat neonatal cardiomyocytes infected with the adenoviral HSP70 construct (AdHSP70i) exhibited increased tolerance to LPS exposure compared with myocytes infected with a control adenoviral construct (AdSR), as shown in Fig. 2. To investigate the possible mechanism of how the overexpression of HSP70 protects against LPS exposure, we performed Western blots on protein extracts from cardiomyocytes infected with our control and our HSP70-containing adenoviral constructs before and after LPS treatment. Blots were reacted with antibodies against iNOS, a protein known to be increased by LPS and the inducible HSP70. Results of these experiments are presented in Fig. 3. As can be observed, LPS treatment induces the expression of the iNOS in cardiomyocytes, but, interestingly, in the presence of the adenoviral-mediated increase in HSP70, the level of iNOS expression is considerably reduced. It should also be noted that direct LPS treatment on the isolated cardiomyocytes also weakly induces the endogenous HSP70, as can be observed in Fig. 3.

In an attempt to determine if our previous in vitro results in isolated cardiomyocytes would be relevant in an in vivo model, we used a transgenic mouse line overexpressing the rat HSP70 gene (14, 19). This transgenic mouse line has been characterized and shown to express high levels of the exogenous rat HSP70 in cardiac and skeletal muscle and in brain. We therefore used these transgenic mice to test the hypothesis that the sole increased presence of the HSP70 is able to protect against induced endotoxemia in vivo. Our present results show an increased tolerance in these transgenic mice overexpressing the rat HSP70 compared with nontransgenic control mice. The results obtained are presented in Fig. 4. A Kaplan-Meir product-limit estimate of the survival curve (11) was computed.

Table 2. Survival of rat neonatal cardiomyocytes treated with herbimycin A or heat shock and exposed to LPS

<table>
<thead>
<tr>
<th></th>
<th>−LPS</th>
<th>+LPS</th>
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<tbody>
<tr>
<td>Control myocytes</td>
<td>100±13.9</td>
<td>42.2±2.4</td>
</tr>
<tr>
<td>Herbimycin A-treated myocytes</td>
<td>98.1±12.2</td>
<td>94.8±7.4*</td>
</tr>
<tr>
<td>Heat shock-treated myocytes</td>
<td>95.5±6.1</td>
<td>61.3±6.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE from 6 independent experiments and represent percentage of tetrazolium salt measurements (see Methods) compared with control myocytes −LPS, which were taken as 100%. *P < 0.01 vs. control myocytes +LPS.

Fig. 1. Western blot analysis of protein extracts from infected neonatal rat myocytes. Western blot reacted with a polyclonal antibody specific for both the inducible and constitutive 70-kDa heat shock protein (HSP70). Protein extracts are from cardiomyocytes heat shocked for 60 min at 42°C and then left at 37°C for 8 h (HS), untreated cardiomyocytes (Co), cardiomyocytes infected with adenoviral HSP70 [multiplicity of infection (MOI) of 1:1; Ad70i], and cardiomyocytes infected with the control adenoviral-SR construct (MOI 1:1; AdSRi). Protein (40 µg) was loaded in each lane. Western blot was developed as described in Methods. HSP70c, control HSP70; HSP70i, inducible HSP70.

Fig. 2. Creatine kinase (CK) released from lipopolysaccharide (LPS)-treated rat neonatal cardiomyocytes infected with the adenoviral constructs. Shown is the percentage of total CK released from adenoviral-infected cardiomyocytes after a 16-h exposure to LPS (200 µg/ml). Myocytes were infected with the adenoviral construct containing the rat inducible HSP70 (AdHSP70i) or the control adenoviral construct (AdSR). Myocytes were infected 48 h before LPS exposure. Results are from 6 independent experiments (*P < 0.05).

Fig. 3. Western blot of cardiomyocytes treated with LPS. Uninfected cardiomyocytes and cardiomyocytes infected with control (AdSR) and AdHSP70i adenoviral constructs were either treated with LPS (200 µg/ml) or left untreated. Protein extracts from these cardiomyocytes were fractionated by 8% SDS-PAGE, transferred to nylon membrane, and reacted with antibodies against inducible nitric oxide synthase (iNOS) or the inducible HSP70.
for the 5-day period after intraperitoneal injection with LPS (20 mg/kg body wt) for both transgenic and control mice. By convention, the survival function is drawn as a series of step changes, with the steps occurring at the times of known deaths (11).

As shown, overexpression of the HSP70 confers protection against LPS, but the important question is how does the HSP70 render the cardiomyocyte tolerant to LPS. Our prior results in isolated cardiomyocytes indicated that iNOS induction by LPS is reduced by the increased presence of HSP70. Therefore, we measured the level of NO, a main mediator of LPS-induced cell injury in the presence of LPS. NOS activity was determined as an increase in \(\text{L-citrulline} \rightarrow \text{L-arginine} \rightarrow \text{L-citrulline} \) after LPS treatment. Although in nontransgenic littermate mice we found an increase in citrulline in the heart after 16 h of LPS exposure of about 27 fmol citrulline/mg protein, in the heart of transgenic mice exposed to LPS we found no significant increase in citrulline. In addition, Northern blots of total RNA from the hearts of both transgenic and nontransgenic littermate mice exposed to LPS for varying periods of time were probed with a cDNA probe to iNOS, to HSP70, and GAPDH. Interestingly, these Northern blots showed that there is a higher level of iNOS in nontransgenic littermates than in transgenic mice exposed to LPS. A representative Northern blot is shown in Fig. 5A. In addition, Northern blots for heat-shocked and control mice are presented in Fig. 5A to show the levels of iNOS and the position of the heat-induced endogenous mouse HSP70s in relation to the exogenous rat HSP70 in the transgenic mice. Figure 5B presents the quantitative results of three independent experiments.

**DISCUSSION**

Recent studies have shown that a prior hyperthermic treatment of rodents results in a significant improvement in the survival rate after experimentally induced endotoxic or septic shock (13, 31, 38). A pre-heat shock treatment significantly increases the level of the inducible HSP70 but also causes several other alterations, including an increase in catalase enzyme activity, ATP alterations (8), and increased expression of other heat shock proteins and related stress proteins (22). It is then currently unclear if the sole increase in HSP70 levels can lead to protection against endotoxic shock. Another study has utilized sodium arsenite to induce the heat shock response in rats and found that these animals became tolerant to septic shock (30). Unfortunately, sodium arsenite not only induces the heat shock proteins but also a related stress protein, heme oxygen-

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**Fig. 4.** Survival of transgenic and control mice after LPS treatment. Estimated survival function \(\hat{S}(t)\) was computed, and a Kaplan-Meir product-limit estimate of the survival curve (11) was plotted for both transgenic mice overexpressing the rat HSP70 transgene and nontransgenic littermate mice over 5 days after ip injection with 20 mg/kg body wt of LPS. There were 33 mice in the transgenic group and 39 mice in the nontransgenic group. Statistical difference between groups was calculated by the \(\chi^2\) test on \(2 \times 2\) contingency tables (* \(P < 0.05\).)

**Fig. 5.** Expression of the HSP70 transgene and iNOS in both transgenic and nontransgenic mice treated with LPS. Transgenic (+) and nontransgenic (−) mice were treated with LPS (20 mg/kg) for 1, 3, and 24 h, at which point animals were killed, and total RNA was prepared from their hearts. A: total RNA was analyzed by Northern blot that was hybridized sequentially with cDNA probes to the rat HSP70, the rat iNOS, and the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. Position of exogenous HSP70, iNOS, and GAPDH is shown on right. Northern blot of control and heat-shocked (42°C, 15 min) mice is shown on left. Arrowheads indicate position of endogenous mouse HSP70s. B: Northern blots were scanned, and expression of HSP70 and iNOS was normalized by the expression of GAPDH. Level of normalized HSP70 and iNOS is shown for transgenic (+) and control (−) mice. Hours refer to length of LPS treatment for each mouse. Results are means ± SE from 3 mice.
ase (36). Heme oxygenase has recently been established to be a mediator of antioxidant defense mechanisms in mammalian cells (37). It is then obvious that the observed protective effect against endotoxic or septic shock by the induction of the heat shock response may not necessarily be due solely to the increase in HSP70. Nonetheless, thermotolerance is mediated through the increase in expression of heat shock proteins and especially that of the HSP70. Direct evidence that increased expression of HSP70 protects against a lethal heat shock in rat fibroblasts (17) and in simian CV-1 cells (1) has been previously shown. These studies showed that the sole presence of HSP70 protein before a lethal heat shock is the main cause for the protection seen during thermotolerance. We have shown that a myogenic cell line (H9c2) stably transfected with a human HSP70 gene is also rendered tolerant to LPS exposure (6). This indicates that the HSP70 plays an important role in protecting the cell against LPS-induced damage.

Our aim in the present study was to determine if the increased expression of inducible HSP70 in rat cardiomyocytes could exert a protective effect against cellular injury induced by exposure to LPS. Initially, we determined if the protective effect induced by a heat shock response could be translated into a protective effect against exposure to LPS in neonatal rat cardiomyocytes. The preconditioning of the cardiomyocytes either by a prior heat shock or herbimycin A treatment markedly increased the expression of the heat shock proteins and especially the inducible HSP70 and rendered the cells resistant to the presence of LPS (Tables 1 and 2). It should be noted that, although herbimycin A is a good inducer of the heat shock response when used at 0.5 µg/ml for 4 h, longer time exposures to herbimycin A have been found to have cytotoxic effects on the isolated cardiomyocytes. In addition, herbimycin A is known to be toxic in mice (LD50 of 19 mg/kg in mice from MSDS Life Technologies).

To investigate the role of the inducible HSP70 in cross protecting against cellular injury caused by LPS, we chose an adenoviral vector system that permits us to overexpress significant amounts of the exogenous HSP70. We have previously shown that an adenoviral construct containing the HSP70 renders cardiomyocytes more tolerant to ischemia-induced injury compared with a control adenoviral construct (23). Figure 1 shows the level of HSP70 expression achieved with our adenoviral constructs, compared with heat shock and control, in neonatal rat cardiomyocytes. Our present results show that cardiomyocytes infected with the adenoviral/HSP70 construct exhibit a significant increase in resistance to cellular injury after exposure to LPS, as measured by CK release (Fig. 2). These results indicate that a major part of the protection conferred by a heat or herbimycin A pretreatment against a subsequent LPS exposure is most likely attributable to the increased presence of the inducible HSP70. In addition, we found that the adenoviral-mediated increased HSP70 expression considerably reduced the induction of iNOS by LPS treatment in isolated cardiomyocytes (Fig. 3).

The overexpression of a HSP70 gene in transgenic mouse models has previously been achieved and has been shown to confer protection against cardiac ischemia-reperfusion injury (14, 19, 28, 29). We therefore used the same transgenic mouse line to determine if the sole increased presence of the HSP70 is capable of protecting against endotoxemia in vivo. Our results show that these transgenic mice are more tolerant to treatment with LPS compared with nontransgenic littermates (Fig. 4). Our results also show that the observed protection due to the increased presence of the exogenous HSP70 correlates with a decrease in the expression of the iNOS gene in the hearts of mice treated with LPS (Fig. 5). This reduced expression of the iNOS results in a reduction in the synthesis of NO in cardiac tissue. As mentioned previously, an increase in NO has been implicated in the attenuation of cardiomyocyte contractility (3, 4). Interestingly, a recent report has shown that NO production by the induction of iNOS due to LPS exposure is modulated by a heat shock and that HSP70 directly interferes with the activation of NF-κB and thus reduces iNOS induction (9). This would indicate that part of the protection conferred by a pre-heat shock treatment against a subsequent LPS exposure may be due to the increased presence of the inducible HSP70 and its effect on the activation of NF-κB. Although it has been established that increased production of NO during sepsis and endotoxemia is responsible for changes in vasomotor tone, decreased vasopressor responsiveness, and decreased myocardial function, approaches to inhibit NOS have not always proven to be effective. For example, studies on the effects of LPS treatment in iNOS knockout mice have been inconclusive. Although some studies have found that the lack of iNOS in homozygotic knockout mice confers protection against LPS exposure (18, 39), another report has shown the contrary (16). Nonetheless, the use of iNOS inhibitors in clinical studies has shown some promise of being effective against the cardiovascular derangement during sepsis in humans (2). Therefore, it is possible that the reduction in iNOS expression during LPS treatment in the presence of HSP70 overexpression may be one of the mechanisms by which the HSP70 protects against endotoxemia, but not necessarily the only one.

In summary, the results in rodents have shown that a whole body heat treatment confers a protective effect against lethal injury due to endotoxic or septic shock in vivo (13, 30, 31, 38). Our present studies in both isolated cultured cardiomyocytes and transgenic mice directly implicate the inducible HSP70 as responsible for this protective effect. Given the number of deaths associated with septic shock in the intensive care units in the United States, it is of interest to study any possible prophylactic agent or strategy that may protect patients who have developed sepsis. Therefore, research to discover pharmacological agents that are able to increase the level of HSP70 may become an important means of treating patients at high risk of developing sepsis.
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