Protection against endotoxemia by HSP70 in rodent cardiomyocytes

SANDY S. LAU, TINA M. GRIFFIN, AND RUBEN MESTRIL
Division of Endocrinology and Metabolism, Department of Medicine,
University of California, San Diego, La Jolla, California 92039-0618

Lau, Sandy S., Tina M. Griffin, and Ruben Mestril. 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 \( \hat{S}(t) = 0.538 \), transgenic \( \hat{S}(t) = 0.787 \), \( P < 0.05 \)]. 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

DESPITE RECENT PROGRESS in the treatment of septic shock in intensive care units, septic shock remains one of the main causes of mortality and morbidity after major surgery. The causes of death among patients afflicted with septic shock are severe hypotension associated with a decrease in cardiac output, severe hypotension due to a decrease in systemic vascular resistance, and multiple organ system failure syndrome (27). Therefore, cardiovascular disorders during septic shock or endotoxemia seem to play an important role in the outcome of this disease. In addition, myocardial performance is known to be seriously compromised in both septic shock and experimental endotoxemic shock. Myocardial dysfunction during endotoxemia is characterized by diminished contractility due to both reduced left and right ventricular ejection fractions and increased end-diastolic ventricular volumes (25, 26). The depressed myocardial performance persists in isolated cardiomyocytes and in isolated perfused hearts of endotoxin lipopolysaccharides (LPS)-induced experimental animals. Isolated perfused hearts from LPS-treated rats present a significant decrease in left ventricular developed pressure (35) and depressed left ventricular function (10). Cardiomyocytes isolated from LPS-treated animals exhibit an increased synthesis of inducible nitric oxide synthase (iNOS; see Ref. 33). This increase in iNOS results in the accelerated metabolic conversion of arginine to citrulline and nitric oxide (NO; see Ref. 5). NO then increases the level of cellular cGMP, which results in an attenuation of the cardiomyocyte’s contractility (3, 4). Although the exact mechanism of how LPS activates iNOS is not completely known, a study has shown that iNOS induction requires the activation of the transcription factor nuclear factor-\( \kappa B \) (NF-\( \kappa B \); see Ref. 40).

Other 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 increases the level of the inducible heat shock protein 70 (HSP70) but also produces several other alterations, including increases in catalase enzyme activity, ATP alterations, and increased expression of other heat shock proteins and related stress proteins. Therefore, it is currently unclear if solely the increased HSP70 levels can lead to protection against endotoxic shock. One study has utilized sodium arsenite to induce the heat shock response in rats and found that these animals became tolerant to septic shock (30). Another report has found that sodium arsenite induces the expression of heat shock proteins in isolated rat hearts (12). Therefore, it is currently unclear if solely the increased HSP70 levels can lead to protection against endotoxemia. One study has utilized sodium arsenite to induce the heat shock response in rats and found that these animals became tolerant to septic shock (30). Another report has found that sodium arsenite induction of the heat shock proteins inhibits NO expression and results in the attenuation of hypotension in LPS-challenged rats (12). However, sodium arsenite not only induces heat shock proteins but also produces many other changes in the cell. It then becomes obvious that the observed protective effect against endotoxemic or septic shock by the induction of the heat shock response may not necessarily be due solely to the increase in HSP70. We have previously used a stably transfected myogenic cell line that overexpresses significant amounts of exogenous HSP70 (21) and have shown that this cell line exhibits a significant increase in resistance to cellular injury after exposure to LPS (6). In addition, another 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-\( \kappa B \) and thus re-
duces 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/l 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 (AdHSP70) and the control adenoviral construct (Ad5R) 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 against iNOS (Santa Cruz Biotechnology). Blots were then reacted with an anti-rabbit IgG biotin-streptavidin, horseradish peroxidase-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 enzyme activity present in cardiomyocytes. CK enzyme activity was measured using a standard CK assay kit (Sigma). Cardiomyocytes were either preheat treated (42°C for 60 min) or were pretreated with herbimycin A (0.5 mg/ml, 4 h) before LPS exposure, or left untreated (control). CK activity was then normalized by the amount of protein in each plate (U/mg). Values are means ± SE from 6 independent experiments and represent percentage of creatine kinase released compared with control myocytes without (-) lipopolysaccharide (LPS), which were taken as 100%.

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) before LPS exposure, or left untreated (control). CK activity released to the media and that remaining in the myocytes for each individual plate were measured. 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 assay.
Table 2. Survival of rat neonatal cardiomyocytes treated with herbimycin A or heat shock and exposed to LPS

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<th>-LPS</th>
<th>+LPS</th>
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<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>
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<tr>
<td>Heat shock-treated myocytes</td>
<td>95.5±6.1</td>
<td>61.3±6.9*</td>
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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.

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.
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 L-citrulline due to enhanced metabolic conversion of L-arginine to L-citrulline after LPS treatment. Although in nontransgenic littermate mice we found an increase in citrulline in the heart of nontransgenic littermates than in transgenic mice exposed to LPS, 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 improve-
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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