Accelerated recovery after endotoxic challenge in heat shock-pretreated mice

CHARLES N. PAIDAS,1 MARIA LOURDES MOONEY,1 NICHOLAS G. THEODORAKIS,1 AND ANTONIO DE MAIO1,2
1Division of Pediatric Surgery and the 2Department of Physiology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287

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Paidas, Charles N., Maria Lourdes Mooney, Nicholas G. Theodorakis, and Antonio De Maio. Accelerated recovery after endotoxic challenge in heat shock-pretreated mice. Am J Physiol Regulatory Integrative Comp Physiol 282: R1374–R1381, 2002; 10.1152/ajpregu.00280.2001.—The inflammatory response induced by bacterial lipopolysaccharide (LPS) has profound metabolic and physiological effects. Thus hepatic glucose production is depressed after LPS administration, which is, at least in part, due to the downregulation of phosphoenolpyruvate carboxykinase (PEPCK) expression. PEPCK is a key regulatory enzyme of the gluconeogenic pathway. Expression of heat shock proteins (hsps) is a well-conserved response to stress correlated with protection from subsequent insults including inflammation. In this study, the expression of PEPCK was observed to be preserved after injection of LPS in heat shock-pretreated mice. Protection of PEPCK expression was limited to the time after heat shock treatment that displayed hsp70. Comparison of the transcription rate and mRNA levels of PEPCK after LPS injection between mice that were heat shock pretrained or not indicated that the preservation of PEPCK expression was not due to initial protection from the LPS challenge. On the contrary, it was mediated by a rapid recovery after the LPS insult at the level of transcription. These observations suggest that the mechanism of heat shock-mediated protection (stress tolerance) after LPS challenge is due to an increase in the capacity of the organism to recover rather than deterrence from the insult.

Protection; inflammation; endotoxin; phosphoenolpyruvate carboxykinase

The genesis and progression of the systemic inflammatory response syndrome (SIRS) leading to multiple organ dysfunction syndrome (MODS) and death is one of the most vexing problems that challenge clinicians in the intensive care unit (2, 23). The development of SIRS is unequivocally a major health problem particularly in critically ill and severely injured patients. SIRS and its sequel MODS constitute the principal causes of mortality and morbidity of these patients (3). Some of the symptoms observed in SIRS patients, such as hyperpyrexia (>38°C), tachycardia (>90 beats/min), and leukocytosis (>12,000 cells/mm3), can be reproduced by the administration of bacterial lipopolysaccharide (LPS) (1, 6). LPS is a component of the external walls of gram-negative bacteria, which are shed after infection or is released after bacterial lysis. LPS induces an inflammatory response characterized by the orderly appearance of different cytokines in circulation and the expression of gene families such as the acute phase within the liver (41). Simultaneously with the increase in expression of genes involved in the inflammatory response, the expression of other genes, such as albumin, is downregulated to maintain hemostasis at the level of gene expression. This phenomenon was coined the adaptive response to stress (40). Previous studies showed that the gene encoding for phosphoenolpyruvate carboxykinase (PEPCK), a key regulatory enzyme of the hepatic gluconeogenic pathway (15, 17), is also downregulated after administration of LPS (16, 40). This decrease in PEPCK expression compromised the ability of the liver to synthesize glucose. In the short term, a decrease in gluconeogenesis may not be detrimental. However, the long-term incapacity of the liver to produce glucose will compromise patient recovery.

Expression of heat shock or stress proteins (hsps) is a well-conserved response to injury. Hsps are composed of several members with different sizes and localized in different subcellular compartments. Some of them are present in unstressed cells and are involved in the folding and translocation of polypeptides across membranes. These hsps are named molecular chaperones (8, 26). After the initial expression and accumulation of hsps, cells become resistant to subsequent stresses. This phenomenon has been termed stress tolerance. The mechanism of stress tolerance is not completely understood (8). Because hsps act as molecular chaperones, one possibility is that they participate in the refolding of proteins that are denatured as a consequence of the stress. In particular, hsp104 has been observed to resolublize protein aggregates that are formed after a stress (28). Experiments in vitro have also shown resolubilization of denatured protein by the addition of hsp13 (13). The other possi-
bility is that hsps are involved in the stabilization of cellular processes such as transcription and translation (8). In addition, cellular structures, such as microfilaments and centrosomes, that are affected by the stress have also been observed to be stabilized by the presence of hsps (21, 37).

The expression of hsps has also been correlated with protection from lethal doses of LPS (18, 20, 33), induced lung injury (38), and sepsis (32, 39). These observations are of clinical relevance because sepsis, endotoxemia, and acute respiratory dysfunction syndrome are associated with SIRS and MODS and frequently result in death or significant morbidity (1, 2). Thus it is important to understand the possible mechanisms of heat shock (HS)-mediated protection from these conditions because they may be pertinent to patient care. In the present study, the mechanism of HS pretreatment in the protection of LPS-induced inflammation was evaluated. Because PECK expression is reduced after administration of LPS (40), we tested whether or not the expression of this gene was preserved after LPS injection in HS-pretreated mice.

We found that HS pretreatment did not prevent the initial reduction in PECK expression after LPS challenge. However, HS pretreatment resulted in a rapid recovery after the LPS insult.

EXPERIMENTAL PROCEDURES

Whole body hyperthermia. BALB/c mice (Jackson Laboratories) were individually weighed and anesthetized with ketamine-HCl (80–100 mg/kg ip). A corn oil–LPS probe was inserted via the rectum into the colon to monitor temperature. After the initial colonic temperature was recorded, mice were warmed by placing them onto a heating blanket until core body temperature reached 42°C. This temperature was maintained constant for 10 min. Animals were placed back in their cages and allowed to recover for 24 h with water ad libitum. Most animals awakened within 1 h after hyperthermia and behaved normally on recovery from anesthesia. This treatment did not result in mortality within 24 h. Control animals were anesthetized but not subjected to thermal stress. These animal studies were conducted according to protocols reviewed and approved by the Institutional Animal Care and Use Committee and adhered to guidelines promulgated by the National Institutes of Health.

LPS-induced inflammation. Mice were fasted for 16 h with water provided ad libitum. Mice were then weighed and injected with LPS (1, 5, 10, 15, 20, or 25 mg/kg ip) using a 26-gauge needle. Three doses were chosen to evaluate the effects of different levels of magnitude of stress as evidenced by the mortality data. Escherichia coli LPS (B 026:B6) was obtained from Sigma-Aldrich. The nucleic acid precipitate was resuspended in 10 mM Tris, pH 8, 0.3 M sucrose, 3 mM CaCl2, 2 mM Mg(AC2), 0.1 mM EDTA, 0.1 mM PMSF using a Potter-Elvehjem homogenizer and a Teflon pestle at low speed. The homogenate was rapidly filtered through plastic mesh (0.1 mm), layered gently onto 5 ml of solution A (10 mM Tris·HCl, pH 8, 0.3 M sucrose, 3 mM CaCl2, 2 mM Mg(AC2), 0.1 mM EDTA, 0.1 mM PMSF) using a Potter-Elvehjem homogenizer and a Teflon pestle at low speed. The homogenate was rapidly filtered through plastic mesh (0.1 mm), layered gently onto 5 ml of solution B (50 mM Tris·HCl, pH 8, 2 M sucrose, 5 mM Mg(AC2), 0.1 mM EDTA, 0.1 mM PMSF) in a polylamellar tube, and centrifuged (SW41 rotor) at 100,000 g for 30 min at 4°C. After the centrifugation, the top layer was aspirated, the bottom sucrose cushion was poured off by inversion of the tube, and the bottom of the tube containing the nuclear pellet was cut off, keeping the tube inverted. The nuclear pellet was resuspended (250 μl) with glycerol storage solution (25% glycerol, 5 mM Mg(AC2), 0.1 mM EDTA, 5 mM DTT) on ice and quickly frozen at −70°C until use (40). Nuclei were counted after staining with propidium iodine using a fluorescence microscope. Alternatively the amount of nuclei was quantitated by directly measuring the DNA content. Nuclei were solubilized in 0.2 M NaOH, 2% SDS, and the absorbance was read at 260 nm. This parameter was used to normalize the nuclear run-off reaction by the concentration of nuclei that are used. Nuclei in storage buffer (50 μl) were mixed with an equal volume of 50 mM HEPES, pH 7.4, 5 mM MgCl2, 5 mM DTT, 150 mM KCl, 1% glycerol, 0.7 mM ATP, GTP, and CTP, 0.8 μM UTP, and 0.1 mM [α-32P]UTP and incubated at 25°C for 30 min. The reaction was stopped by adding with DNAse I (10 U) for 10 min at 37°C, followed by the addition of 4 vol of 10 mM Tris, pH 8, 0.35 M LiCl, 1 mM EDTA, 7 M urea, and 2% SDS. The reaction was further incubated with Proteinase K (500 μg/ml) at 45°C for 60 min. At the end of the incubation period, tRNA (50 μg) was added and the total mixture was precipitated with TCA (10%). The nucleic acid precipitate was resuspended in 10 mM Tris, pH 8, 1 mM EDTA, 0.5% SDS and hybridized to DNA targets. Hybridization was carried out in 50% formamide, 6× SSC, 10× Denhardt’s, and 0.2% SDS at 42°C for 72 h. After hybridization, filters were washed in 50 mM Tris, pH 8.6, 1 M NaCl, 2 mM EDTA, and 1% SDS at 42°C for 30 min and 65°C for 15 min. Washes were continued with 2× SSC, 0.1% SDS
at 65°C for 15 min twice, and 0.1× SSC, 0.1% SDS at 65°C for 5 min. Filters were exposed to X-ray films (Kodak X-OMAT AR) at −70°C in the presence of intensifying screens (36).

RESULTS

LPS induced an inflammatory response in BALB/c mice. We first characterized the effect of LPS in BALB/c mice. No mortalities were observed 24 h after injection of LPS (1 or 5 mg/kg). However, higher doses of LPS (10, 15, and 20 mg/kg) resulted in 50, 60, and 80% mortality, respectively. The two lowest doses of LPS (1 or 5 mg/kg) were used in further studies to eliminate the possibility that the observed changes in gene expression were the result of premorbid events, such as hemodynamic changes associated with a reduction in cardiac output and alterations in blood flow. A marker of the LPS-induced inflammatory response is the increase in the expression of the acute phase gene β-fibrinogen (β-fib) (40). An elevation of β-fib mRNA levels was observed after injection of LPS (1 or 5 mg/kg), peaking at 3 h and remaining elevated until 24 h of the challenge (Fig. 1A). These observations indicated that an inflammatory response was induced after the administration of LPS. Simultaneously with the increase of β-fib mRNA levels, a decrease in PEPCK mRNA was observed after injection of LPS (1 or 5 mg/kg). This reduction was evident within 3 h of LPS injection, reaching minimal levels within 6 h of the insult. PEPCK mRNA levels remained depressed for 24 h after injection of LPS (Fig. 1B). This decrease of PEPCK mRNA is consistent with previous studies in rats (40).

Expression of hsp70 was increased in BALB/c mice after whole body hyperthermia. Mice were warmed until colonic temperature reached 42°C and were maintained at this temperature for 10 min. No mortalities were observed within 24 h of this thermal stress. After whole body hyperthermia, animals were killed immediately or 1, 2, 4, 6 h, or 1, 2, 3, 5, 6, and 7 days after the stress. Liver samples were homogenized and analyzed by Western blotting for the presence of hsp70 (Fig. 2A). The signal intensity of hsp70 observed in the autoradiogram of the Western blot in linear range of exposure was quantitated using a laser scanner densitometer (Fig. 2B). Hsp70 was detected within 4 h of thermal stress and maximal levels were observed within 24 h of the insult. Hsp70 levels were reduced by 3 days and undetectable 5 days after the insult. Subsequent experiments were performed within 24 h of the thermal stress because hsp70 expression was maximal at this time point.

PEPCK mRNA levels were not reduced after exposure to LPS in HS-pretreated mice. Mice were thermally stressed as described above and challenged with LPS (1 mg/kg) 24 h after the HS pretreatment. PEPCK mRNA levels were evaluated by Northern blots of RNA isolated from liver samples obtained 6 h after the LPS injection. No reduction in PEPCK mRNA levels was observed in these HS-pretreated mice. In contrast, mice that did not undergo HS pretreatment showed the typical decrease of PEPCK mRNA levels after administration of LPS. Also, no reduction in PEPCK mRNA levels was observed in mice injected with saline with or without HS pretreatment (Fig. 3). The inflammatory response in these mice was evaluated by the increase in β-fib mRNA levels. No difference in β-fib mRNA levels was observed in mice that were HS pretreated or not after LPS injection. This observation suggests that the preservation of PEPCK mRNA levels in HS-pretreated mice was not due to an impairment of the inflammatory response induced by LPS injection (Fig. 3). Mice that were HS pretreated but not injected with LPS also showed higher levels of β-fib mRNA, probably due to a secondary inflammatory effect after whole body hyperthermia.

Apparent protective effect of HS disappeared after 5 days of the pretreatment. Mice were thermally stressed as described above and allowed to recover for 1 day (maximal hsp70 expression) or 5 days (no detectable hsp70 levels) and injected with LPS (1 mg/kg) and
killed 6 h later. Steady-state PEPCK mRNA levels were preserved in mice that were injected with LPS 1 day after HS pretreatment. However, no preservation of PEPCK mRNA levels was observed in mice that were injected with LPS 5 days after HS pretreatment. No significant differences in the inflammatory response, evaluated by the increase of β-fib expression, were observed between mice injected with LPS after 1 or 5 days of HS pretreatment (Fig. 4). These observations correlate the preservation of PEPCK mRNA levels after LPS challenge with the presence of hsp70 (Fig. 2B).

Fig. 2. Kinetics of heat shock protein 70 (hsp70) expression after whole body hyperthermia in mice. Mice were warmed to 42°C, maintained at this temperature for 10 min (whole body hyperthermia), and allowed to recover at ambient temperature with access of water and food ad libitum. Mice were killed at 0, 1, 2, 4, 6 h or 1, 2, 3, 5, 6, and 7 days after whole body hyperthermia and liver samples were collected. Liver samples were homogenized and analyzed by Western blotting using a monoclonal antibody (C92) specific for hsp70 and 125I-labeled goat anti-mouse monoclonal antibody as secondary antibody (A). The signal intensity for hsp70 in the autoradiogram, in linear range of exposure, was quantitated using a laser scanner (B).

Fig. 3. Effect of heat shock (HS) pretreatment on PEPCK mRNA levels after injection of LPS. Mice (n = 5 each group) were subjected to whole body hyperthermia as previously described and allowed to recover for 24 h. Mice were injected (intraperitoneally) with LPS (1 mg/kg) or saline. Another group of mice that was not HS pretreated was injected with LPS or saline. Livers were harvested 6 h later. Total RNA, isolated from liver samples, was analyzed by Northern blotting/hybridization using a cDNA probe for PEPCK (A) or β-fib (B) followed by autoradiography. The autoradiogram, in the linear range of normal, was quantitated using a laser scanner densitometer. The signal intensity for PEPCK and β-fib mRNAs was normalized to the 18S rRNA determined after staining the blot with methylene blue.
HS pretreatment did not prevent the initial reduction in PEPCK mRNA levels after injection of LPS. To better characterize the interrelationship between HS pretreatment and preservation of PEPCK gene expression, mRNA levels of this enzyme were analyzed at different time points (0, 1.5, 3, 6, and 24 h) after LPS injection in HS-pretreated mice. Mice were thermally stressed, recovered 24 h, injected with LPS (1 mg/kg), and liver samples were harvested at different time points (0, 1.5, 3, 6, and 24 h) after LPS injection. Total RNA isolated from these samples was analyzed for PEPCK mRNA levels by Northern blotting. A similar reduction in PEPCK mRNA levels was observed within 1.5 h of LPS injection in the presence or absence of HS pretreatment (37 vs. 42%, respectively). PEPCK mRNA levels were also reduced 3 h after LPS injection in both groups. A clear increase in PEPCK mRNA levels was observed within 6 h of LPS injection in HS-pretreated mice, whereas PEPCK mRNA levels were further reduced in non-HS-pretreated mice under identical conditions of LPS challenge. This observation is consistent with data presented in Figs. 3 and 4.

Transcription of PEPCK gene was initially reduced, after LPS challenge, and followed by a rapid recovery in HS-pretreated mice. The reduction in PEPCK mRNA levels after administration of LPS could be due to alterations in the transcription rate of the gene or to accelerated degradation of PEPCK mRNA. Accordingly, the beneficial effects of HS pretreatment could be the result of PEPCK transcription stabilization or blocking of PEPCK mRNA degradation. The kinetics of transcription were evaluated after administration of LPS by the nuclear run-off technique. Mice, HS pretreated or not, were injected with LPS (1 mg/kg). Nuclei were isolated from liver samples obtained 0, 1, 2, 3, 4, and 6 h after LPS injection and used for the nuclear run-off reaction. The kinetics of PEPCK transcription after LPS injection were similar in HS-pretreated or nonpretreated mice. However, PEPCK gene transcription appears to recover more rapidly in HS-pretreated mice compared with nonpretreated mice (Fig. 6).
mally stressed (F percentage of the maximal PEPCK transcriptional activity that quantitated using a densitometric scanner. Data are presented as ground. The autoradiograms in the linear range of exposure were containing PEPCK cDNA, 18S rRNA, and an empty vector for back-

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C for 48 h to immobilized plasmids kines, which modi-

 cascade of events initiated by the secretion of cyto-

LPS challenge in a mouse model. LPS stimulates a present study, we investigated a possible mechanism response, such as after sepsis and endotoxemia. In the

DISCUSSION

The expression of hsps is a well-conserved response to different stresses including clinically relevant situ-

ations, such as circulatory shock, ischemia-reper-

fusion, and inflammation (8, 25). The expression of hsps has been associated with protection from sub-

sequent stresses, a phenomenon coined stress tolerance (8). The mechanism underlying this phenomenon is not well understood. This is particularly true for the protection observed in cases of exaggerated inflammatory response, such as after sepsis and endotoxemia. In the present study, we investigated a possible mechanism for the effect of HS pretreatment in the protection from LPS challenge in a mouse model. LPS stimulates a cascade of events initiated by the secretion of cyto-

kines, which modifies gene expression in different organ systems, such as the acute phase genes within the liver. Additionally, LPS induces fever, increases cardiac output, decreases arterial pressure, and lowers systemic vascular resistance (12). LPS alters hepatic metabolism in a variety of ways, including alterations in regional acinar blood flow, which then alters the oxygen delivery to periportal and pericentral hepatocytes (10). Hepatocytes surrounding the periportal areas are exposed to oxygen-rich blood that induces the preferential expression of enzymes involved in aerobic metabolism. An example of metabolic alteration is the hepatic level of gluconeogenesis, which involves the formation of glucose from less than six carbon precur-

sors such as pyruvate, lactate, glycerol, and amino acids. Gluconeogenesis is more active in pericentral hepatocytes than in the periportal area. This difference in hepatic gluconeogenesis has been correlated with zonal expression of PEPCK, a key regulatory enzyme of the gluconeogenic pathway (15, 17). PEPCK expression is higher in pericentral hepatocytes with respect to periportal hepatocytes (31). The expression of this enzyme is suppressed after LPS administration (16, 40). This reduction in PEPCK expression has been found to compromise the capacity of the liver to produce glucose during inflammatory conditions (5, 9, 22, 27).

Levels of PEPCK mRNA, which were decreased after LPS injection in untreated mice, were preserved in HS-pretreated mice after the injection. This protection was correlated with the presence of hsp70, because it disappeared within 5 days after HS pretreatment when hsp70 levels were undetectable. The preservation of PEPCK expression in HS-pretreated mice was not due to neutralization of the response to LPS. On the contrary, HS-pretreated mice showed similar inflammatory response and an initial downregulation of PEPCK expression after injection of LPS compared with non-HS-pretreated mice. HS-pretreated mice re-

covered more rapidly from the insult than animals that were not pretreated. These results suggest that stress tolerance, at least tolerance to LPS, is mediated by an accelerated readjustment to normal situations after an insult. Prior studies also suggest a recovery rather than prevention as a potential mechanism of stress tolerance. Thus hsp104 has been demonstrated to par-

ticipate in the resolubilization of protein aggregates that are formed after HS (28). Hsp70, hsp40, and hsp90 have been shown to be resolubilized denatured proteins that are unfolded as a consequence of a stress (13, 24). Another cellular pathway that is protected in HS-

primed cells is translation. Stabilization of translation also seems to be part of a mechanism of rapid recovery (4). Thus cells that are subjected to an HS showed a rapid decrease in protein synthesis followed by a re-

covery of translation that parallels the synthesis of hsps. This effect can be blocked by administration of inhibitors of transcription during HS (unpublished observations). However, our data only present a corre-

lation between the presence of hsps and protection mediated by HS pretreatment. Further studies ad-

dressing the direct role of hsps in this process are needed to elucidate the possible molecular mechanisms of the protective phenomenon.

A rapid recovery in the transcription rate may be the major component in the mechanism of HS tolerance to LPS treatment. The rate of PEPCK degradation within 1.5 h of LPS injection is identical between mouse HS pretreated or not. Because PEPCK transcription is similarly depressed during this early period of LPS treatment, the decay of PEPCK mRNA reflects its true degradation rate. Even more, the recovery of PEPCK mRNA in HS-pretreated mice is coincidental with the increase of PEPCK transcription in the pretreated mice. Protection of the overall rate of transcription has

Fig. 6. Steady-state PEPCK nuclear run-off analysis of isolated nuclei from HS pretreatment followed by LPS-induced inflammation. Mice were subjected to whole body hyperthermia as previously de-

scribed (C). Another set of mice was anesthetized but was not ther-

mally stressed (C). Both groups of mice were allowed to recover for 24 h. Mice (n = 2 each group) were injected with LPS (1 mg/kg) and at 0, 1, 2, 3, 4, and 5 h after injection, nuclei were isolated from liver samples. Nuclei were used for the nuclear run-off reaction and the labeled transcripts were hybridized to PEPCK and β-actin cDNA tar-

gets. The transcription reaction was performed with the isolated nuclei in the presence of a [α-32P]UTP for 30 min at 30°C. Radiolabeled transcripts were purified and equal amounts of radioactivity were used to hybridize at 42°C for 48 h to immobilized plasmids containing PEPCK cDNA, 18S rRNA, and an empty vector for back-

ground. The autoradiograms in the linear range of exposure were quantitated using a densitometric scanner. Data are presented as percentage of the maximal PEPCK transcriptional activity that corresponds to mice not injected with LPS.
been observed in thermotolerant cells (36). Obviously, the present study cannot completely rule out a contribution of PEPCK mRNA stability in the rapid recovery after LPS treatment in HS-pretreated mice. Similar to transcription, stability of mRNA plays an equally important role in gene expression. Thus a decrease in the expression of a particular gene is accomplished by a reduction in transcription and an increase in the degradation of the encoding mRNA. Previous studies have shown that PEPCK mRNA can be regulated at the level of mRNA stability, particularly via cAMP (17, 34). Changes in mRNA stability have been shown to play an important role in the response to LPS and other insults that produce an inflammatory response such as connexin 32 (14, 35).

In summary, this study illustrates that protection by HS pretreatment is potentially mediated by an accelerated recovery process. If hsps are indeed involved, this idea of recovery is consistent with the chaperone capacity of these proteins. Thus cells already containing hsps may be more capable to repair the stress-induced damage than cells that required the synthesis of these proteins for injury control.

**Perspectives**

The emerging hypothesis that the HS-mediated protection is, at least in part, due to a rapid recovery from the stress may have an important impact in any attempt to use HS expression as a therapeutic intervention. Because the presence of hsps is a natural evolutionary defense mechanism, its induction could potentially be used in the prevention of several clinically relevant situations such as organ transplantation, severe injury, burns, and critical illness (8). Early work in this regard has centered in prevention. In other words, an HS pretreatment can prevent death from sepsis (39). In addition, LPS (18, 33) has been shown to improve the success of a variety of organ transplants (29, 30). Any clinical benefit of the HS response depends on the expression of these genes in a nontraumatic manner, which seems to be a very impractical idea limited to few clinical scenarios, such as elective surgery. Most notably, it would be impossible to induce the expression of hsps before a stochastic event, such as injury and trauma. On the other hand, if hsps are important in the recovery process, the expression of hsps after injury may be beneficial. It is possible that survival from severe hemorrhage or other traumatic injuries may be dependent on the capacity of the patient to mount the HS response. Thus the HS response may facilitate a rapid recovery from the injury. The induction of the HS response (in a nontraumatic form) in critically ill patients may improve their clinical outcome. An important aspect that needs to be addressed in the precise mechanism, if any, that involves hsps. Consequently, knowledge of the detailed molecular mechanism that is involved in the HS expression and protection is necessary to design strategies that may be used to improve survival of critically ill patients.

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