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HEAT SHOCK PROTEINS AND SEPSIS: A HOT STORY

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Sepsis represents one of the most challenging problems in the field of intensive care medicine. Even with the use of powerful anti-microbial agents, sepsis continues to represent the most common cause of multiple system organ dysfunction and death in most patients admitted to intensive care units. Cumulative experimental and clinical evidence indicates a major role for cytokine production and systemic effects of sepsis-induced inflammatory responses. Thus, blocking cytokine activation and/or pharmacological effects with specific cytokine-receptor antagonists represents a logical strategy for the treatment or attenuation of sepsis-related inflammation [1-3]. While strategies which make it possible to selectively down-regulate the effects of specific cytokines would be particularly attractive from the therapeutic perspective, achieving this objective is far from straightforward since the effects of specific cytokine inhibition *in vivo* has been demonstrated to be extremely unpredictable. For example, despite data from animal studies showing the dramatic efficacy of antibodies against endotoxin and tumor necrosis factor (TNF)-α, and interleukin (IL)-1 receptor antagonist (IL-lra) in the treatment of sepsis, corresponding beneficial effects have not been observed in recent human trials [4,5]. Accordingly, it appears that information concerning cytokine biology will need to be considerably enhanced before the development of a 'magic bullet' that attenuates inflammatory responses during organ injury and prevents organ dysfunction.

In previous chapters of this book the cellular events involved in organ inflammation have been extensively discussed; damage and repair are ultimately controlled at the molecular level and cannot be fully understood

without consideration of the functions of the relevant genes and their products. It is now widely recognized that various cellular stimuli mediate physiologic effects by the induction of complex intracellular signalling cascades which culminate in the activation or induction of a particular gene or subset of genes. As a result, activation leads to the synthesis of particular sets of proteins and a consequent change in cellular behavior. Cytokines are a critical set of proteins involved in directing the inflammatory response with a direct or indirect influence on tissue damage or repair. Depending on the nature of the pathological perturbation, these steps represent potential targets for intervention and potentially novel therapeutic strategies.

THE UNIVERSAL HEAT SHOCK OR STRESS RESPONSE

During their life span, all living organisms are exposed to environmental stresses to which they must adapt with varying degrees of structural and functional changes in order to survive. All forms of life on Earth, from bacteria to man, have evolved mechanisms of response to maintain homeostasis in the face of diverse and complex environmental stresses which can jeopardise their survival. The oldest response is the heat shock or stress response. The heat shock response is a complex, transient reprogramming of cellular activities which: i) protects essential cell components from irreversible stress injury; ii) helps to ensure survival during the stress period; and iii) allows a rapid and complete resumption of normal cellular activities in the recovery period [6]. The general theme of the stress response is the rapid and almost exclusive transcription and translation of a set of highly conserved proteins known as the heat shock or stress proteins (HSP); concomitantly, there is a corresponding decrease in the production of most other cellular polypeptides [6,7].

The heat shock response was originally described in 1962 by Ritossa, an Italian scientist, as a phenomenon of inducible gene expression after brief heat treatment of *Drosophila* larvae, as judged by the changes in puffing patterns observed in the salivary gland polytene chromosomes [8]. These observations, although initially considered to be experimental tricks, opened the way to the description of a general stress response system in eukaryotic cells with the discovery of the HSP in 1974 by Tissières et al. [9]. Almost two dozen proteins are induced in response to a range of different stresses. The best studied of these stresses is heat shock. Mammalian cells have been shown to synthesize HSPs after a brief period of hyperthermia at temperatures 3-5°C above normal body temperature. HSP induction is observed both *in vivo* and *in vitro*. Besides heat shock, the induction of the

heat stress response can be achieved by hypoxia, glucose starvation followed by refeeding, ethanol, sodium arsenite, cadmium, dexamethasone, heavy exercise, viral infection and agents which affect cell cycle [6]. HSPs may be induced directly by such agents or indirectly, by virtue of increased expression of other proteins which in turn provoke HSP gene expression. Thus for example, increases in HSP expression in injured cardiac muscle cells have been linked to increases in TNF-α and IL-1 production [10]. Synthesis of almost all HSPs can be detected in normal, unstressed cells, thereby implicating their probable participation in cellular processes distinct from physiological stress. The formation of induced HSP is usually dependent on a concomitant formation of new cytoplasmic mRNA. This finding is supported by the observation that HSP synthesis is inhibited by actinomycin D [6].

HSPs are classified into five protein families based on molecular mass in SDS (sodium dodecyl sulfate) polyacrylamide gels. These include the large molecular weight HSPs (100 kD), the HSP90 family, the highly conserved HSP70 family which represents the most prominent eukaryotic group of HSPs, the HSP 60 family which is found in bacteria, chloroplasts, and mitochondria, and the small HSP family, expressed predominantly in plants. Comparison of the sequences of the respective heat shock genes from bacteria, plants, flies, and man, have indicated these genes to be among the most highly conserved proteins in nature. All major HSP genes have been cloned and investigated by restriction mapping and sequence analysis. Pairwise comparisons of HSP sequences from almost any two organisms reveals that about half of the amino acids residues are identical and that many of the remaining residues are similar. The ancestors of humans and mycobacteria diverged about 1500 million years ago but their HSP65 have identical amino acids in about 50% of the protein sequence and the similarity approaches 65% when conservative substitutes are included. The HSP70 of drosophila and yeast have a 72% amino acid identity and their HSP 84 have a 63% identity. The derived protein sequence of a human HSP70 gene is found to be 73% homologous with *drosophila* HSP70 and that from maize is 68% homologous[11].

With few exceptions, HSP70 is a constitutive protein of eukaryotic cells, part of it being closely associated with the microtubular and intermediate filament cytoskeletal systems and with the plasma membrane. Four members of the human HSP70 protein family have been identified: HSP70, HSP72, HSP73, and grp78 [12]. At least ten HSP70 related genes have been found in human cells, some of which map between the complement and TNF-α and β genes on chromosome 6. HSP70 is both the major heat-inducible protein and a cell cycle regulated protein. HSP72 is a protein expressed only after heat shock. HSP73 is constitutively expressed at high levels in growing cells.

Grp78 is a glucose-regulated protein located in the endoplasmic reticulum. HSP have been identified as components of the plasma membrane, the Golgi apparatus, cytoplasmic mRNP, the nucleus and the nucleolus. Moreover, HSP70 is an integral component of the cytoskeleton which may be a primary target of heat stress or shock treatments.

HEAT SHOCK PROTEINS ARE RESPONSIBLE FOR ACQUISITION OF STRESS TOLERANCE AND SURVIVAL

The HSPs appear to manifest many diverse functions. HSP genes have been implicated in a variety of cellular processes which include DNA replication, the transport of proteins across membranes, the assembly or disassembly of protein complexes, the binding of proteins in the endoplasmic reticulum, and the uncoating of coated vesicles. The finding that HSP70-related proteins reside in the endoplasmic reticulum and in mitochondria and that HSP70 is translocated into the nucleus upon heat shock suggests that 70 kDa proteins perform important functions in all cellular compartments. The induction of the HSP genes, particularly the HSP70 gene, appears to be necessary for cell division even in the absence of stress. It has been suggested for example that members of the HSP70 family act in the protection of cellular damage by binding to denatured or abnormal proteins following heat shock and thereby preventing protein aggregation.

Heat shock and other stress proteins are almost certainly involved in protecting cells from the deleterious effects of heat and other stresses. Perhaps the most compelling argument that HSPs have protective functions is the phenomenon of thermotolerance [13]. Thermotolerance represents a property of all living cells and refers to the capacity of cells to survive or recover from normally lethal exposures to abrupt, severe heat shock or stress conditions if prior to the lethal stress, the cells are exposed to a more mild or shorter period of heat/stress. It is well known that to function optimally under cellular conditions of increased temperature, proteins must retain a degree of structural flexibility that allows rapid and reversible changes in conformation and assembly [14]. By being only marginally stable at physiological temperatures, proteins clearly face the risk of significant thermal damage. Body temperatures in vertebrates span in a range from -2°C (in the case of a Antarctic notothenioid fish) to 47°C (for the desert iguana). HSP are induced at temperatures near the upper range of an organism´s normal body temperature as part of an adaptative response. The basic observation is that a group of cells or organisms die rapidly when shifted directly from their normal growing temperature to a much higher temperature. However, a

matched group that is given a mild preheat treatment to induce HSP dies much more slowly. For example, this approach to cellular stress has been shown to markedly reduce the extent of heat-induced central nervous system (CNS) injury [15].

Moreover, treatment with heat as the stressor can induce tolerance to other forms of stress. Accordingly, recent studies [16,17] have addressed the question as to whether the induction of the stress response might protect animals against subsequent injury. In this context, following their demonstration that a brief period of warming induced HSP72 gene and protein expression in a time-related fashion in the rat lungs (Figure. 1), Villar et al. [16] examined the effects of the induction of HSP in attenuating lung damage and outcome in an animal model of acute lung injury (ALI) induced by intratracheal instillation of phospholipase A_2, a phospholipid-degrading enzyme and a potent mediator of inflammation. These authors reported that a brief exposure of experimental animals to transient hyperthermia, resulting in HSP72 protein accumulation in the rat lung, attenuated lung damage and significantly decreased mortality. Under control conditions, 27% of the unheated animals died by 48 hours after intratracheal instillation of phospholipase A_2, compared to zero mortality in the heat-treated group.

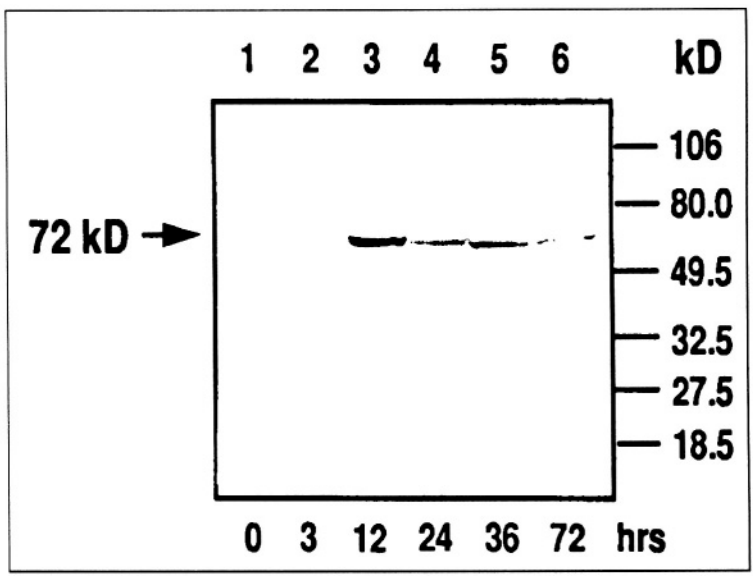

Figure 1. *Time course of HSP72 protein accumulation in rat lungs by Western analysis. HSP72 peaks between 12 and 24 hours after heating and remains at high levels up to 72 h (from [16] with permission).*

In an experimental model of hyperoxia-induced ALI, Winston et al [17] were also able to reduce mortality rate after exposing pre-heated animals to 100% oxygen for 60 hours.

An interesting clinical observation of the implications for the HSP response resulted from the potential benefits and the observed problems of localized hyperthermia as a means of cancer therapy [18]. Human and other mammalian tumor cells die after a modest degree of hypothermia of 4-8°C above normal. To be successful, this procedure will require a basic understanding of the biology of fractionated heating procedures as well as the development of the thermotolerance that will ensue. Unfortunately, the development of thermotolerance is a major impediment to the effective application of clinical hyperthermia. Chemical agents are being developed for clinical use which will increase the cell-killing powers of hyperthermia and eliminate the development of thermotolerance. Besides hyperthermia, the expression of HSP70 can be induced by oncogene products of the DNA tumor virus adenovirus 5 as well as by the rearranged c-*myc* mouse oncogene [19]. Such oncogenes are implicated in the immortalization of primary cells in culture and it may be that HSP70 has a critical role to play in animal cell growth. Irrespective of the mechanisms by which the stress response provides cytoprotection, the capacity of HSP to subserve this function is of considerable interest from the perspective of elucidating the pathophysiology of organ damage and dysfunction in different diseases states.

HEAT STRESS RESPONSE REPLIES TO THE IMMUNE SYSTEM AND KEEPS QUIET THE SYSTEMIC INFLAMMATORY RESPONSE

The observations on HSP regulation suggest that there is a connection between in vivo stress proteins and disease states. Although the mechanism for HSP-mediated cytoprotection is not completely understood, one possible explanation is that this protective effect relates to the capacity of HSPs to block the synthesis and/or release of cytokines that play key roles in the febrile and inflammatory responses to stress [20]. Immunodominant antigens from a wide variety of pathogens have been found to be HSP. The major stress protein antigen recognized by antibodies in bacterial infections is HSP60 [21]. The sequence of the DNA clones has revealed that mycobacterial HSP70 and the HSP60 are the major targets of the murine antibody response to both *Mycobacterium tuberculosis* and *M. leprae*. The high degree of sequence conservation between host and pathogen stress proteins may provide a link between infection and autoimmunity. HSP65 is a

molecule that is part self and part foreign to any organism with an immnune system. It is immunologically dominant and it elicits strong immune responses in individuals infected or exposed to various bacteria. Moreover, T-cell responses to HSP65 seem to be associated with autoimmune arthritis in both rats and humans. There is some evidence to suggest that a subset of human T cells specific for the 65 kDa antigen of *M. leprae* is able to recognize the corresponding HSP in human cells [22]. Murine macrophages stressed by viral infection or interferon (IFN)-γ become targets for anti-65 kDa cytotoxic T cells. This suggests that upregulation of HSP may indeed play a role in the pathology associated with autoimmune disease.

HSP have also been identified as immune targets in most major human parasitic infections. Antibodies to HSP70 have been identified in the sera of patients suffering from malaria, trypanosomiasis, leishmaniasis, schistosomiasis, and filariasis [23]. HSP90 is a target for antibodies in trypanosomiasis and a member of the small HSP family has been recognized in some patients with schistosomiasis. During viral infections there is increased synthesis of HSP that could, of course, be simply part of a non-specific induction of host genes. However, specific host-coded HSP may play a crucial role in viral replication. All types of mutual interactions are observed: viral induction of HSP synthesis, enhanced synthesis of viral proteins, and interference of heat shock and viral stress. Whether mammalian HSP70 is required for mammalian virus replication is not yet known. The sensitivity of virus multiplication to heat stress is well documented. Heat shock inhibits virus multiplication. Brief heat shock of host cells before viral infection inhibits the subsequent replication of herpes and pseudorabies virus [6]. The synthesis of the influenza B virus M protein in mammalian cells is specifically inhibited at 39°C. The inhibitory effects of fever on viral infections result i) from inhibition of the replicase, and ii) from liberation of lysosomal enzymes including RNAses which destroy viral RNA. Hence a reduced production of viral particles is observed. On the other hand, cytotoxic T lymphocytes that recognize HSP induced by the virus could limit the spread of the virus by killing infected cells, possibly before substantial amounts of mature virus are assembled, and by secreting IFN-γ [24].

In view of these observations, could immune responses to stress proteins have a role in protection against infection? One view is that they do not, since infection by any one pathogen does not generally protect an individual against infection by another. An alternative view is that immune responses to conserved stress protein determinants that developed early in life, probably during the establishment of natural microbial flora on the skin and in the gut, could provide a general level of protection against infection. This may help to explain the observation that, for many pathogens, only a fraction of infected individuals progress to clinical disease.

Irrespective of the mechanisms by which hyperthermia provides protection against subsequent noxious exposure, these observations are of interest from the perspective of the pathophysiology and clinical importance of fever. In most hospitalized patients, body temperature is recorded at least once daily. Fever has been recognized as a manifestation of disease since the dawn of civilization in the Fertile Crescent. Hippocrates said that fevers were the worst diseases. In the Antiquity, people died of fever, not microorganisms, which were then unknown. However, in the Modern Age fever was often regarded as favorable to the patient´s survival. Fever is defined as an elevation of core body temperature above the normal level (>37.5°C in humans). Irreversible cell protein denaturation occurs at 42.5°C. Fever in the absence of detectable infection is a common finding in clinical practice. Trauma, drugs, inflammatory processes, malignant diseases, burns, dehydration, cell disruption, surgery, myocardial infarction or placental detachment are among the most common clinical conditions associated with a febrile response. The normal febrile response tends to be limited both in magnitude and in duration. Core temperatures in mammals can reach 40-41°C during fever, but it has been unclear whether such temperatures benefit or harm the host.

There is no evidence that fever is detrimental or that antipyretic therapy offers any significant benefit. Currently, fever is seen in the ICU environment as portending sinister outcomes. In many cases, it is treated as the origin of, rather than the response to, an illness. Many ICU physicians and nurses believe mistakenly that lowering the fever will improve the course of the illness. The observed correlation between hyperthermia and cytoprotection is consistent with data suggesting that the failure to mount an appropriate fever response is associated with increased mortality [25]. One of the first studies to examine the significance of hyperthermia in response to a bacterial infection was done by Kluger et al [26]. They used lizards inoculated with a bacterial suspension of live *Aeromonas hydrophila* to determine whether elevation in body temperature increases the resistance of the host to this infection. Infected animals were placed in a constant temperature chamber at different ambient temperatures, ranging from 34 to 42°C. An elevation in temperature following the experimental bacterial infection resulted in a significant increase in host survival throughout a 7-day period. Within 24 hours, 50% of the lizards maintained at 38°C were dead. However, lizards maintained at 40° and 42° had only 14 and 0 percent mortality, respectively (Figure 2).

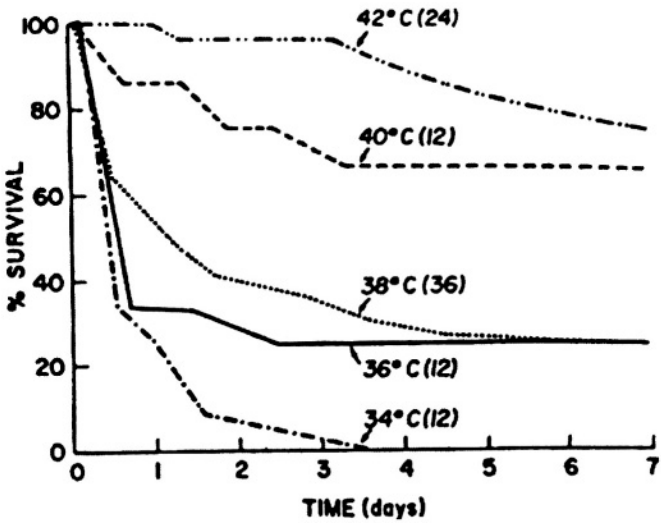

Figure 2. *Percentage survival of lizards injected with A. Hydrophila and maintained at temperatures of 34° to 42°C. The number of animals in each group is given in parentheses (From [26] with permission).*

In order to investigate whether the therapeutic benefits of fever might be engendered by HSP synthesis, Villar et al. [27] showed that thermal treatment of animals with intra-abdominal sepsis, produced by cecal ligation and perforation, induces the synthesis of HSP in the lung, heart and liver and is associated with the attenuation of organ damage and reduced mortality. This experimental model mimics many features of the human septic syndrome with the presence of enteric microorganisms and endotoxin in the blood. The authors studied two groups of animals (heated and unheated), and evaluated survival rate and pathological changes in the lung, heart and liver before and after cecal perforation, after cecum removal, and at 7 days. At 18 hours after perforation, 25% of the unheated animals died whereas none of the heated animals died. Seven days after cecal perforation, the protection was still evident, with 20% mortality compared to 70% in the non-stressed group. In addition, heated animals showed less histological evidence of lung and liver damage (Figure 3).

Since whole-body warming could be associated with a number of non-specific mechanisms unrelated to the induction of the heat shock response, Ribeiro et al [28] used sodium arsenite as a non-thermal means to induce the heat shock response and examined whether this could also provide protection in the same model of intra-abdominal sepsis. Following a single intravenous

injection of sodium arsenite, HSP72 was detected in the lungs with a peak between 18 and 24 hours. Administration of 6 mg/kg of sodium arsenite 18 hours prior to performing cecal ligation and perforation was associated with a marked decrease in mortality at 18 and 24 hours after sepsis. The protection in the sodium arsenite treated animals appeared to follow the time course of HSP72 protein levels. Therefore, these studies support the hypothesis that HSPs are cytoprotective *in vivo*.

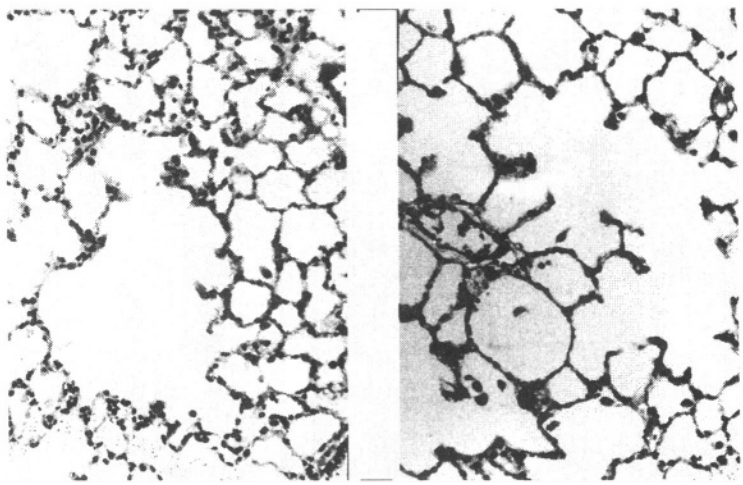

Figure 3. *Histopathologic features of sepsis-induced acute lung injury. Left panel: unheated animal; representative example of the pathology of the lungs 18 hrs. after cecal ligation and perforation showing evidence of atelectasis, early hyaline membrane formation and acute inflammatory infiltrates. Right panel: heated animal; relatively normal architecture with occasional neutrophils present in the septa (HS stain x400) (from [27] with permission).*

The mechanisms by which the heat shock response might provide cytoprotection are not known. However, Ribeiro et al [29] have demonstrated in endotoxin-stimulated alveolar macrophages that TNF-α levels were lower in the supernatant of LPS-treated cells, and that HSP72 coprecipitated with TNF-α from cells which had received stress treatment (heat stress and sodium arsenite) prior to endotoxin exposure. This finding suggests that HSP may participate in post-translational control of TNF-α release, binding with nascent TNF-α, and preventing its release from macrophages. Therefore, HSP determine whether TNF-α is released from the cell or is sent to the lysosomal machinery for degradation.

Several studies [30-32] have shown that the induction of the stress response by transient whole-body hyperthermia prior to the experimental sepsis can render animals resistant to the lethal effects of bacterial endotoxin. Ryan et al. [30] reported that administration of endotoxin from *E. coli* to nonheated rats resulted in 71% lethality; in contrast, all rats subjected to a single nonlethal heat stress 24 hr before endotoxin inoculation survived. This acquired resistance to endotoxin was not associated with the presence of endotoxemia immediately after the heat stress treatment. However, although they did not measure the expression of HSP, it is possible that in this model, the reduced lethality might be related to the reduced responsiveness of cells to endotoxin, resulting in reduced production of cytokines. Chu et al. [32] examined the effects of heat stress after the administration of *E. coli* endotoxin in rats. They found that survival rates in the heated animals were roughly double that of septic, unheated animals. This increase in survival could be related to the attenuation of plasma IL-1β concentrations, which were significantly lower at 2 hr after endotoxin administration in the heat stressed rats. In light of the broad cytoprotective role of heat shock response in animal models, Wong et al. [33] showed that the induction of HSP70 gene expression protected sheep pulmonary artery endothelial cells from cell death. In addition, they found inhibition of endotoxin-mediated superoxide anion generation, suggesting that an early step in endotoxin-induced apoptosis may be sensitive to HSP expression. On the other hand, the lack of HSP72 gene expression in the lungs of animals with fecal peritonitis might account for the high mortality rate of the septic insult [34]. Although the regulation of HSP expression in sepsis can be a multi-step process, Durand et al. [35] have recently reported that patients with the acute respiratory distress syndrome (ARDS) have an inability to mount a stress response, measured as HSP70 levels in blood monocytes, that correlates with disease severity and recovers over time while the patients are mechanically ventilated.

In patients with sepsis and septic shock, lactic acidosis develops as a result of organ hypoperfusion. Ischemia-reperfusion and hypoxia-reoxygenation can cause cellular damage and stress responses in several organs. Aoe et al. [36] analyzed HSP70 gene expression in the isolated rat liver exposed to various periods of ischemia/hypoxia-reperfusion. They found that HSP70 mRNA increased as the reperfusion period increased, suggesting that the accumulation of this messenger could be considered a marker of injury since it reflects the severity of reperfusion injuries. Deshpande et al. [37] have recently reported that the induction of heat shock response, prior to sepsis, markedly decreased lactate concentration in the plasma of septic rats. Several published clinical studies during the 70s and 80s showed a correlation between high levels of lactate and poor outcome in patients with sepsis and septic shock. By contrast, low levels of lactate and/or

an ability to increase lactate clearance has been associated with good prognosis. Although the mechanisms by which the induction of the heat shock response might improved organ perfusion and attenuate organ damage are not fully understood, it has been demonstrated that the heat shock response inhibits cytokine-mediated expression of inducible nitric oxide synthetase (iNOS) [38]. As Deshpande et al. [37] have pointed out, organ protection may depend of the degree and duration of the heat stress. While a mild stress induces a protective response, a more potent stress stimulus induces apoptosis and an even stronger one leads to necrosis.

HEAT SHOCK PROTEINS: A NOVEL THERAPEUTIC AGENT FOR SEPSIS?

One cannot help wondering whether the genes and proteins involved in the complexities of the heat shock response might be important in human disease states and whether medically significant mutations that affect heat shock loci exist [39]. It is quite possible that this system of proteins is simply so important that such variations are incompatible with life. It has been shown that fibroblasts injected with monoclonal antibodies to HSP70 were unable to survive after a brief period of heat stress whereas cells injected with control antibodies survived a similar heat shock [40]. On the other hand, in transgenic animals engineered to express constitutively high levels of human inducible HSP70, the recovery of myocardial contractility after 30 min of ischemia is significantly better than in nontransgenic hearts. With the explosion of knowledge and technology in the fields of cellular and molecular biology, we will be able to study particular aspects of disease mechanisms in finer and finer detail. A further understanding of the role of the heat shock response may allow for the development of rational pharmacologic agents and to make them potential targets for therapeutic interventions. Future research could focus on novel strategies to turn on the HSP genes, as a potential therapy for sepsis, ALI and other critical care conditions.

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