

Inducible heat shock protein 70 kD and inducible nitric oxide synthase in hemorrhage/resuscitation-induced injury

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ABSTRACT

Inducible heat shock protein 70 kD (HSP-70i) has been shown to protect cells, tissues, and organs from harmful assaults in *in vivo* and *in vitro* experimental models. Hemorrhagic shock followed by resuscitation is the principal cause of death among trauma patients and soldiers in the battlefield. Although the underlying mechanisms are still not fully understood, it has been shown that nitric oxide (NO) overproduction and inducible nitric oxide synthase (iNOS) overexpression play important roles in producing injury caused by hemorrhagic shock including increases in polymorphonuclear neutrophils (PMN) infiltration to injured tissues and leukotriene B₄ (LTB₄) generation. Moreover, transcription factors responsible for iNOS expression are also altered by hemorrhage and resuscitation. It has been evident that either up-regulation of HSP-70i or down-regulation of iNOS can limit tissue injury caused by ischemia/reperfusion or hemorrhage/resuscitation. In our laboratory, geldanamycin, a member of ansamycin family, has been shown to induce HSP-70i overexpression and then subsequently to inhibit iNOS expression, to reduce cellular caspase-3 activity, and to preserve cellular ATP levels. HSP-70i is found to couple to iNOS and its transcription factor. Therefore, the complex formation between HSP-70i and iNOS may be a novel mechanism for protection from hemorrhage/resuscitation-induced injury.

Keywords: inducible HSP-70, iNOS, eNOS, hemorrhage, caspase-3, ATP, KLF6, resuscitation

INTRODUCTION

Hemorrhagic shock is the leading cause of death and complications in combat casualties and civilian trauma. It has been shown to cause systemic inflammatory response syndrome, multiple organ dysfunction, and multiple organ failure [1]. It is an old problem and to find useful remedies that are capable of reducing its casualty is a most important task in combat medicine and trauma injury [2]. In this paper, the role of inducible heat shock protein 70 kD (HSP-70i) and inducible nitric oxide synthase (iNOS) in hemorrhage/resuscitation-induced injury is discussed in details.

EFFECTS OF HEMORRHAGIC SHOCK

Hemorrhage/resuscitation is a common scenario in the clinical practice of medicine and occurs in virtually all organ systems. Its hallmarks are relatively consistent

across patient populations and organ systems. They lead to systemic inflammatory response syndrome, multiple organ dysfunction, and multiple organ failure [1]. A variety of biomolecules is known to be involved in this response. In rodents, increases in HSP-70i by heat stress limit tissue injury such as tissue integrity, polymorphonuclear neutrophil (PMN) infiltration and leukotriene B₄ (LTB₄) generation caused by ischemia reperfusion [3, 4], hemorrhage [5], or hemorrhage plus resuscitation [6]. In transgenic mice expressing HSP-70i, protection against myocardial dysfunction after a brief ischemia was reported [7]. Likewise, inhibition of nitric oxide (NO) production results in significant reduction of local tissue damage, PMN infiltration, and LTB₄ generation caused by ischemia/reperfusion [8]. Mice deficient in inducible NO synthase (iNOS) also demonstrate limited hemorrhage/resuscitation-induced injury [9, 10]. Therefore, remedies that induce HSP-70i and/or inhibit iNOS might prove very useful for reducing hemorrhage/resuscitation-induced injury in man.

The above observations are consistent with the idea that the low oxygen supply resulting from conditions such as

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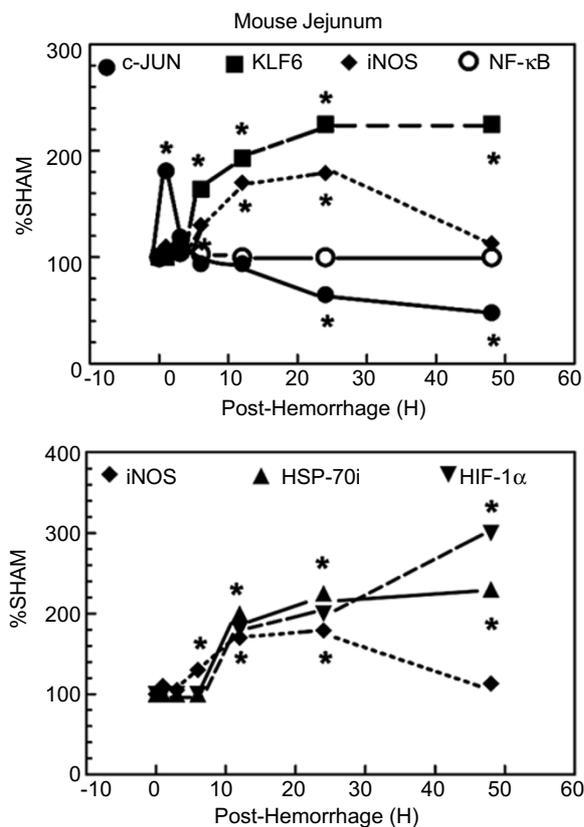


Fig. 1 Time course study of stress-related proteins in hemorrhaged mouse jejunum. The albino mouse was subjected to a cardiac puncture and its 40% of total blood was removed. The mouse was then allowed for various periods of time to respond to the hemorrhage before a small portion of jejunum was dissected out for immunoblotting analysis [13]. The sham-operated mice were exposed to the same procedure except blood withdrawal. * $P < 0.05$ vs the data observed at time = 0 h of post-hemorrhage.

ischemia or hemorrhage affects the expression of iNOS, which then influences the expression of other proteins that alter cell viability. It has been shown that hypoxia results in alteration of iNOS, Bcl-2, and P53 mRNA expression in cultured human intestinal epithelial T84 cells and Jurkat T cells [11, 12], alterations that can be modulated by treatment with a NOS inhibitor [11]. It is also found that hypoxia increases the activity of caspase-3, an aspartate-specific cysteinyl protease involved in apoptosis, an activity that is blocked by NOS inhibitors [11].

A full time-course study of the effect of hemorrhage on a series of stress-related proteins such as c-JUN, Kruppel-like factor 6 (KLF6), iNOS, HSP-70i, and hypoxia inducible factor 1 α (HIF-1 α) has been reported in a hemorrhage mouse model [13]. Based on Western blot data obtained from mouse lung, jejunum, heart, kidney, liver, and brain, c-JUN, KLF6, iNOS, HSP-70i, and HIF-1 α are

up-regulated (Fig. 1). In jejunum, c-JUN protein was over-expressed within 1 h, but levels returned to baseline values 3 h later. KLF6 began to increase significantly 6 h later, reached the maximum at 24 h, and remained at that level at 48 h after hemorrhage. iNOS increased at 6 h, reached a maximum at 12 - 24 h, and returned to baseline values at 48 h. HSP-70i increased at 12 h and remained at elevated levels at 48 h. HIF-1 α also increased at 12 h and continued to increase at 48 h. The sequence of protein appearance was c-JUN, KLF6, iNOS, HSP-70i, and HIF-1 α . KLF4 (a repressor to iNOS, [12] and c-FOS (an AP-1 protein) were not detected, and NF- κ B 65 kD was detected but not affected by hemorrhage.

A similar time-course observation on these stress-related proteins was also obtained in mouse lung, kidney, liver, and heart. Sham-treated mouse organs displayed no changes in the basal levels of c-JUN, KLF6, and iNOS. In addition to changes in this series of stress-related proteins listed above, hemorrhage also increases cellular caspase-3 activity [14], reduces cellular ATP levels [14-18], and elevated mRNA of p38-MAPK, p53, and Bcl-2 [18]. Fig. 2 shows that 5-lipoxygenase, lipid peroxidation, cyclooxygenase, cNOS, LTB₄, PGE₂, interleukins, tumor necrosis factor- α , caspases, and complement activation occur relatively at different time points after hemorrhage [3-6, 9, 10, 19]. The sequence of their occurrence provides the useful information for studying the mechanism(s) underlying the hemorrhage-induced injury as well as therapeutic targets to prevent or ameliorate the injury.

HEAT SHOCK PROTEINS

Heat shock proteins (HSPs) are present in most cells. They represent multi-gene families that range in molecular size from 10 to 174 kD. Based on their molecular weights, they are divided into families of HSP-10, HSP-20, HSP-40, HSP-60, HSP-70, HSP-90, and HSP-110. Tab. 1 lists the members of each family, their locations, and functions in the cell [20-23].

Structure of HSP-70i and its functions

The family of HSP-70 contains HSP-72, HSP-73, HSP-75, and HSP-78. HSP-72 is an inducible form and also abbreviated as HSP-70i. The others are constitutive. The molecular structure of HSP-70i is very similar to that of HSP-73. They both contain a globular unit linked to a β -sheet and an α -helical tube. The globular unit is the ATPase domain (1-386 aa, 44 kD); the β -sheet and the α -helix tube (384-543 aa, 18 kD) are the peptide binding domain; and the tail (542-646 aa, 11 kD) of HSP-73 has the signal peptide of EEVD, but not the tail (542-640 aa, 10 kD) of HSP-70i. Proteins in the cytoplasm recognize the EEVD of HSP-73 as a chaperone protein, whereas HSP-70i has no such EEVD as a chaperone but instead acts as a cytoprotectant. It should be noted that there is a distinct

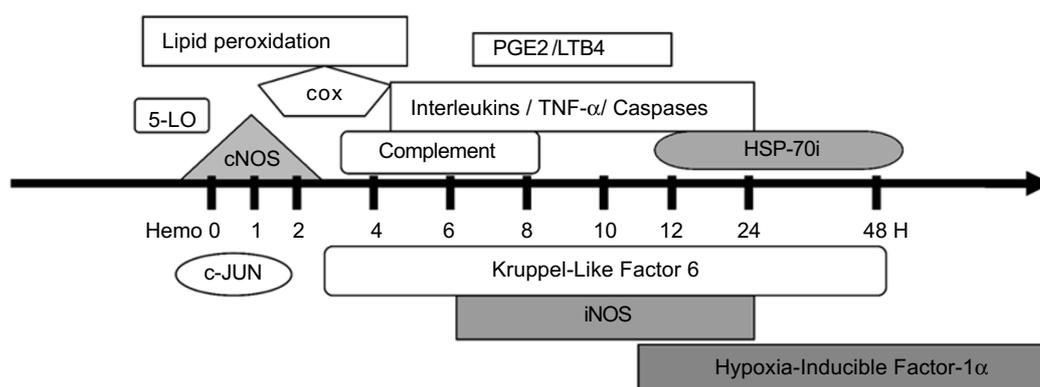


Fig. 2 Hemorrhage increases stress-related protein expression and activity at different time points after hemorrhage. Hemo: hemorrhage; 5-LO: 5-lipoxygenase; cNOS: constitutive nitric oxide synthase; COX: cyclooxygenase; PGE2: prostaglandin E2, LTB4: leukotriene B4; iNOS: inducible nitric oxide; TNF- α : tumor necrosis factor- α .

Tab. 1 Members of HSPs and Their Locations and Functions [20-23]

HSP	Members	Locations	Functions
HSP-110	HSP-110/104	Cytosol/nucleus	Cytoprotection
HSP-90	GRP-94	Endoplasmic reticulum	Chaperone
	HSP-90 α	Cytosol/nucleus	Endogenous steroid receptor antagonist
	HSP-90 β	Cytosol/nucleus	Cytoprotection
HSP-70	GRP-78 (Bip)	Endoplasmic reticulum	Chaperone
	HSP-75 (GRP-75)	Mitochondria	Chaperone
	HSP-73	Cytosol/nucleus	Chaperone
	HSP-72	Cytosol/nucleus	Cytoprotection
HSP-60	HSP-60	Mitochondria	Cohort to HSP-75
	HSP-56	Cytosol	Binds to steroid receptors and FK506
HSP-40	HSP-47	Endoplasmic reticulum	Collagen chaperone
HSP-20	HSP-27	Cytosol/nucleus	Chaperone; cytoprotection; Lysine- and glutamine-donor
	HSP-24	Cytosol/nucleus	Chaperone
	HSP-20	Cytosol/nucleus	Chaperone; Lysine- and glutamine-donor
	HSPB8	Cytosol	Lysine- and glutamine-donor
	HSPB2	Cytosol	Glutamine-donor
	HSPB3	Cytosol	---
	α B-crysatlline	Cytosol	Lysine-donor
HSP-10	HSP-10	Mitochondria	Cohort to HSP-60
Ubiquitin		Cytosol/nucleus	Involves nonlysosomal ATP dependent protein degradation pathway

difference between being a chaperone and a cytoprotectant. A chaperone protein is involved in bringing a protein to the targeted location for its specified purpose, whereas cytoprotection usually involves the modulation of particular protein activity or overall cell functionality thereby leading to cell protection against injury or cell death. The functions and regulations of HSP-73, -75, and -78 have been reviewed in detail [20].

HSP-70 gene regulation involves phosphorylation, heat shock transcriptional factor, heat shock elements, and ions. Each of these components is described briefly below.

Heat shock elements

Genomic footprinting of the human HSP-70 promoter has revealed that heat stress induces a quick binding of heat shock transcriptional factors (HSFs) to a region encompassing five nGAAn sequences named heat shock elements (HSEs), three of them are GAA (also called perfect HSEs) and other two are GAC and GGG (also called imperfect HSEs). The sequences of HSEs at site 3 and site 4 are dyad symmetrical, and HSFs preferably bind to HSEs at sites 3 and 4 [20]. In mammalian cells, HSEs are usually bound by HSF4 to keep other HSFs away.

Heat shock transcriptional factors

Four different HSFs have been identified in vertebrate: HSF1, HSF2, HSF3, and HSF4 [24, 25]. HSF2 has two isoforms due to splicing: HSF2A and HSF2B [26]. HSFs have a binding domain, a helical trimerization surface, and a short conserved element. The binding domain is to bind protein and HSEs; the trimerization surface has leucine zipper coiled-coil motifs for trimer formation. There are two genes in mice and humans encoding HSFs. The sizes for human HSF1, HSF2, HSF4, and chicken HSF3 are 529 aa, 510 aa, 463 aa, and 467 aa, respectively. Human HSF1 can be activated by various stimuli, human HSF2 only by hemin, chicken HSF3 only by heat, while human HSF4 is not activated.

It has been reported that mouse HSF1, chicken HSF3, and mouse HSF4 bind to all 5 HSEs, whereas mouse HSF2 interacts with only 4 HSEs. Activation of HSF1, 2, or 3 up-regulates HSP-70i [20]. In contrast, HSF4 is responsible for down-regulation of HSP-70i [25]. During the process of HSF trimerization, homotrimers or heterotrimers have been found and influence the overall expression of HSP-70i [26].

HSP-70i Autoregulation

Normally, HSFs that reside in the cytosol of mammalian cells are bound to HSPs under unstressed conditions. Under stress conditions such as hemorrhage, HSFs are separated from HSPs. Then, these HSFs are available for phosphorylation by protein kinase C or other serine/threo-

nine kinases. They form homotrimers [27] or heterotrimers [26]. The trimers enter the nucleus, bind to HSEs located on the promoter region of HSP genes, and become further phosphorylated by HSF kinases. Transcription is then initiated and translation is resulted. Therefore, new HSP-70i is synthesized in the cytosol of cells. Soon, the elevated HSP-70i is bound by cytosolic HSFs. The complex of HSP-70i and HSFs turns off the further increase in HSP-70i. The steps of HSFs phosphorylation, trimerization, and its translocation from the cytosol to the nucleus are Ca^{2+} -dependent [28].

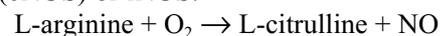
Protein Regulation of HSP-70i

HSP-70 family is present in every cell type and tissue under both unstressed and stressed conditions. HSP-70i is induced by physiological stressors, pathological stressors, and environmental stressors [20]. The degree of its induction depends on the level and duration of exposure to stressors. The increase usually is transient, but how long it persists is different in various cell types, ranging from hours, days, or weeks [20]. It can be regulated by hormones [29], intracellular pH [30], cellular cAMP levels [31], intracellular concentrations of Ca^{2+} [32, 33], and activation of protein kinase C [33-37], protein kinase A [33-38] and protein tyrosine kinase [4, 13, 39-44]. Aging is also known to result in over-expression of HSP-70i [45] that in turn desensitizes Ca^{2+} machinery and turns off new synthesis of HSP-70i [46-48]. Bioflavonoid such as quercetin prevents HSF1 binding to HSEs, thereby leading to attenuation of HSP-70i gene expression [49].

Anti-convulsant and mood-stabilizing drugs such as valproic acid has been shown to up-regulate HSP-70i in the cortex and striatum of both ipsilateral and contralateral sides of middle cerebral artery occlusion rats and of normal rats. The increased HSP-70i reduced infarct size and caspase-3 activation [50]. Lithium, a drug primarily used to treat bipolar mood disorder also has been reported to up-regulated HSP-70i by an increasing DNA binding activity of HSF1 [51]. Like sublethal heat stress [20], sublethal hemorrhage induced significantly the HSP-70i over-expression [52, 53].

NO AND NOS

Like ischemia and reperfusion or hypoxia [11], hemorrhage increases NO production and NOS overexpression [13]. The substrate of NO is L-arginine with O_2 and the enzymes involved in this process are either constitutive NOS (cNOS) or iNOS.



The chemical biology of NO includes its direct effects and indirect effects [19, 54]. Tab. 2 shows that the direct effects, also named the primary mode of NO action, are NO reacts with heme-containing proteins. These reactions

Tab. 2 NO Chemical Biology [19, 54]

Primary mode of NO action (i.e. Direct effect)	Secondary mode of NO action (i.e. Indirect effect)
React with heme-containing proteins	React with NO ₂ or O ₂ ⁻
At a low [NO]	At a high [NO]
Rapid act	Slow act
Physiological effects, e.g. vasodilation	Pathophysiological effects, e.g. organ dysfunction and failure
Involves cNOS	Involves iNOS

Tab. 3 Types of NOS and Their Characteristics [19, 55, 56]

NOS	Sizes(kD)	Chromosome	Features	Tissues
nNOS (NOS1)	160	12	Ca ²⁺ -dependent NADPH, FAD, FMN, Heme Calmodulin PKC phosphorylation Gene has no TATA box GC-rich promoter Regulation by Sp1	Neurons
eNOS (NOS3)	135	7	Ca ²⁺ -dependent NADPH, FAD, FMN, Heme Calmodulin PKC phosphorylation Myristonylation and palmitonylation Gene has no TATA box GC-rich promoter Regulation by Sp1	Endothelium
iNOS (NOS2)	130	17	Ca ²⁺ -independent NADPH, FAD, FMN, Heme Calmodulin PKC phosphorylation Gene has a TATA box	Macrophages

are generally rapid, require low concentrations of NO, and are the genesis of most of the physiological effects of NO such as vasodilation. In contrast, the indirect effects, namely the secondary mode of NO action, include formation of N₂O₃, ONOO⁻, NO₂, and HNO that react with cellular targets and may result in a major configuration change in critical molecules. It has been shown that indirect effects require much higher concentrations of NO than direct effects. It appears that NO produced at low concentrations for short periods primarily mediates direct effects, whereas high local NO concentrations sustain over prolonged periods mediate indirect reactions and cause

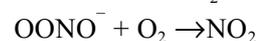
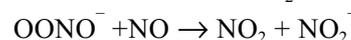
pathophysiological effects such as organ dysfunction and failure.

When NO reacts with NO₂ resulting in nitrosative stress, while it does with O₂⁻ causing oxidative stress.

Nitrosative Stress —



Oxidative Stress —



NOS

NO production is mediated by NOS (Tab. 3). NOS can be divided into two major categories: constitutive form (cNOS) and inducible form (iNOS) [55, 56]. cNOS includes nNOS and eNOS and is Ca²⁺-dependent, whereas iNOS is not. Both cNOS and iNOS contain a reductase domain and an oxidase domain. However, eNOS has myristonylation and palmitonylation sites, whereas nNOS and iNOS do not. The iNOS promoter has a TATA site and differs in that from the cNOS promoters which are TATA-less promoters. The cNOS promoters are GC rich and are regulated primarily by Sp1 and other members of the Sp1-like family. In contrast, NF- κ B plays a crucial role in the regulation of iNOS. In the murine iNOS promoter, the downstream NF- κ B binding site (-76 to -85 bp) seems to be the most important one [57], however, the upstream NF- κ B site (-974 to -960 bp) also seems to have some functionality and cooperativeness with the downstream site [58]. For the human iNOS promoter, the NF- κ B motif at -5.8 kb is most critical for cytokine-induced promoter activity, whereas the sites at -5.2, -5.5, -6.1, and -8.2 kb have a cooperative effect [59, 60].

cNOS is thought to generate low levels of NO at sub-micromolar range for short durations and perform physiological effect. iNOS generates NO for prolonged periods and at local concentrations as high as 1-5 μ M and causes pathophysiological effects. Since the chemistry and biological outcome depend on the concentration of NO, the proximity of a biological target to the NO source becomes critical. For example, cells or tissues close to macrophages that produce high levels of NO will be subjected to direct and indirect effects due to the primary and the secondary modes of NO actions. In contrast, if they are far from the NO source, they will experience only direct effects as the primary mode of NO action.

Hemorrhage induces sequential increases in the levels of stress-related proteins c-JUN, cNOS, KLF6, iNOS and then HSP-70i and HIF-1 α (Figs. 1 and 2). Both cNOS gene and iNOS gene get activated by hemorrhage and they are early response genes, though cNOS responds to hemorrhage earlier than iNOS. HSP-70i and HIF-1 are considered to be the late response genes and their overexpression probably play a role to control the post-hemorrhage tissue damage.

iNOS has previously been shown to be overexpressed after hemorrhagic shock in rodent lung [6, 18, 61] human liver [62] and murine tissues [13, 62, 63]. Warke *et al* [12] reported that the binding of KLF6 to the iNOS promoter increased significantly in cultured cells after chemical hypoxia, heat stress, serum starvation, and phorbol 12-myristate 13-acetate and A23187 ionophore stimulation. Using GeneBank, we identified four CACCC sites on the mouse iNOS promoter (-253 to -257, -818 to

-822, -856 to -860, and -1556 to -1600) that potentially bind KLF6. Furthermore, both the KLF6 promoter and iNOS promoter have AP-1 binding sites (KLF6: -364 to -371; iNOS: -644 to -650 and -1225 to -1231) that probably also bind c-JUN and/or c-FOS. In our study, no c-FOS was detected using immunoblotting analysis. Because iNOS promoters include two AP-1 sites and are involved in processes such as cell injury, wound repair, embryogenesis, tissue differentiation, and suppression of tumorigenesis, the observation that c-JUN overexpression occurs earlier than KLF6 and iNOS overexpression suggests two possibilities. 1) Hemorrhage activates c-JUN, which up-regulates KLF6 expression, leading to iNOS expression; or 2) hemorrhage activates c-JUN, which then directly binds to the iNOS promoter to induce iNOS expression.

Down-regulation of iNOS

A series of NOS inhibitors have been designed and extensively studied [64]. Among them, L-NNA (N-nitro-L-arginine), L-NMA (N-methyl-L-arginine), L-NIL [N-(1-iminoethyl)-L-lysine], and L-NIO [N-(1-iminoethyl)-L-ornithine] are shown to effectively inhibit iNOS gene expression, NO production, and caspase-3 activity [10, 11].

In addition to the use of NOS inhibitors, iNOS deficiency can be caused by deletion of the iNOS gene. This has been shown to reduce or prevent hemorrhage-induced injury and death [9]. This result further confirms that iNOS is responsible for the hemorrhage-induced injury. Therefore, down-regulation of iNOS by any means will be beneficial to patients suffering from hemorrhage.

In addition to treatment with iNOS inhibitors or iNOS gene deletion, compounds such as ansamycin can inhibit iNOS activity and gene expression. Both geldanamycin and 17-allylamino-17-demethoxygeldanamycin, members of the ansamycin family, have been demonstrated to reduce NO production, iNOS mRNA, and cytokine-induced activation of the iNOS gene promoter in cultured cells [49], in rats [6], and in mice [13].

INTERACTION BETWEEN HSP-70I AND INOS

Using immunoprecipitation and immunoblotting analysis, it has been found that HSP-70i forms a complex with iNOS and its transcription factor KLF6 after hemorrhage [13], probably by iNOS and KLF6 binding to the peptide binding domain of HSP-70i. No complex formation is detected between HSP-70i and p53 or Bcl-2 proteins, suggesting that HSP-70i specifically couples to iNOS and KLF6. Treatment with geldanamycin increases HSP-70i expression and decreases the hemorrhage-induced increase in iNOS expression. The complex formation between HSP-70i and iNOS is still observed. It is possible that the complex formed

between HSP-70i and iNOS might decrease the enzymatic activity of iNOS and subsequently decrease NO production. As a result of a low level of NO, the morbid sequelae caused by hemorrhagic shock is markedly diminished.

Our preliminary data show that up-regulation of HSP-70i by HSP-70 gene transfection to human intestinal cells inhibited the hypoxia-induced increase in caspase-3 activity and reduced apoptosis. Likewise, treatment of human intestinal epithelial cells with iNOS inhibitors decreased caspase-3 activity and apoptosis [11]. Taking these data together, one can conclude that the inhibition of HSP-70i on caspase-3 and apoptosis is via its inhibition on iNOS that leads to less NO production.

OTHER PROTECTIVE MECHANISMS

Other protective mechanisms of HSP-70i have also been proposed. For example, TNF- α overexpression caused by sepsis, endotoxemia, hemorrhagic shock, and ischemia/reperfusion [52, 53, 65] has been found. *In vitro* induction of HSP-70i in monocytes or macrophages inhibits TNF- α production following bacteria lipopolysaccharide stimulation, and *in vivo* induction of HSP-70i down-regulates tissue TNF- α production following an injurious insult [5, 52, 53, 66]. The underlying mechanism of the inhibitory action of HSP-70i on TNF- α over-production induced by sepsis, endotoxemia, hemorrhagic shock, or ischemia/reperfusion is not clear. It is highly likely that over-expression of iNOS gene may be responsible for the TNF- α over-production via p38-MAPK overexpression [18]. Since it is known that HSP-70i couples to iNOS and inhibits iNOS protein levels [13], a possibility of a down-regulation of iNOS resulting in decreases in TNF- α levels can not be ruled out.

Fig. 3 is a schematic representation of our model for the interaction between hemorrhage-induced changes, indicating points where HSP-70i might block these changes. Based on our data, KLF6 and HSP-70i are first normally associated with each other in some way. After hemorrhage, increases in c-JUN protein up-regulate expression of KLF6 and iNOS proteins and, somehow, caspase-3 activity and TNF- α expression. Overexpression of HSP-70i protein results in increased complex formation with KLF6 and decreased KLF6 expression. As a result, less KLF6 is available to bind to the iNOS promoter and less iNOS is expressed. Both caspase-3 activity and TNF- α are inhibited directly (through less availability of iNOS) or indirectly (through other intermediate proteins such as p38-MAPK [18, 53]). Further studies are needed to understand the underlying mechanism in detail.

RESUSCITATION FLUIDS

Currently, the major cause of death in potentially salvageable battlefield casualties is hemorrhage [67]. About

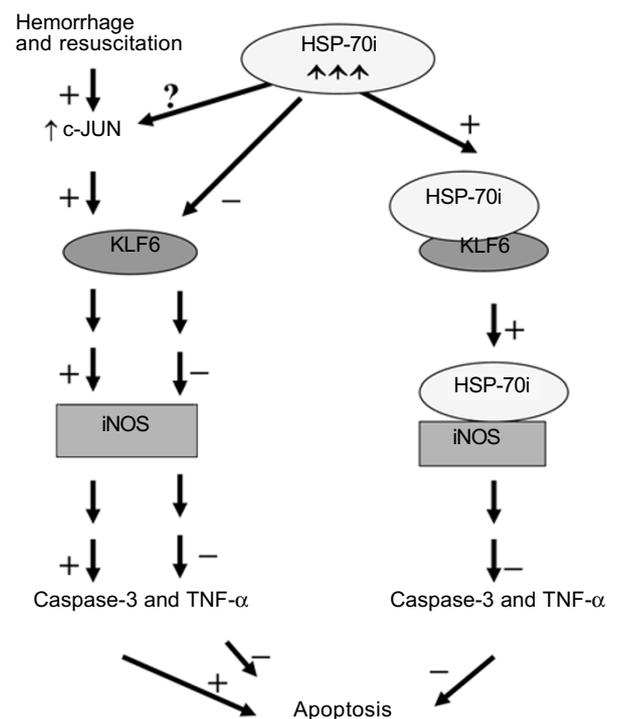


Fig. 3 The Schematic representation of model for interaction between hemorrhage-induced changes and points where overexpressed HSP-70i might block changes. KLF6 and HSP-70i are normally associated with each other. Hemorrhage increases c-JUN that upregulates expression of KLF6 and iNOS proteins and subsequently probably increases caspase-3 activity and TNF- α . Overexpressed HSP-70i leads to increased complex formation between HSP-70i and both KLF6 and iNOS. Therefore, less free KLF6 available to bind iNOS promoter and less iNOS is expressed, which leads to inhibition of caspase-3 activity [11] and probably the TNF- α level. +: stimulation; -: inhibition; \uparrow : increase.

20% of these deaths are preventable if the bleeding can be quickly controlled or minimized [68, 69] and sufficient resuscitation fluid is administered in time to maintain critical tissue perfusion. However, it has been recognized that resuscitation fluids are not innocuous and that they may actually potentiate the cellular injury caused by hemorrhagic shock [70]. It has been proposed that some resuscitation fluids may contribute to delayed multiple organ dysfunction [1].

To search for an optimal resuscitation fluid is still an ongoing research program. Questions have been raised particularly about the use of lactated Ringer fluid (LR). LR has been shown to increase neutrophil activation. Hemorrhage elevates mRNA levels of iNOS, KLF6, p-38 MAPK, p53, Bcl-2, and caspase-3 genes in rat lung and ileum. The LR resuscitation does not effectively block their increases. Instead it further increases some of them [18, 71]. Alam *et al* [72] also reported that 51 genes were altered

in rats treated with hemorrhage plus LR resuscitation. Most importantly, resuscitation with LR but not whole blood is shown to restore the ATP loss and preserve the increased levels of p53 and Bcl-2 in rat lung caused by hemorrhagic shock [18], suggesting that LR may be a relatively good resuscitation fluid.

As mentioned above, resuscitation may actually potentiate the cellular injury caused by hemorrhagic shock [70] since delayed multiple organ dysfunction and failure occur and mortality results after resuscitation [1]. Because iNOS is known to be responsible for the hemorrhage-induced injury and HSP-70i is shown to offer cytoprotection, it is likely that agents or remedies that can reduce iNOS expression and/or increase HSP-72 will be beneficial in conjunction with administration of resuscitation. iNOS inhibitors such as L-NIL, and L-NIO can be potentially useful for this purpose. Likewise, HSP-70i inducers such as herbimycin A [4, 39-44, 73], heat stress [20], ethanol [66-69], and geldanamycin [4, 6, 13, 39, 42] are also in the list for consideration.

CONCLUSIONS

Hemorrhage/reperfusion-associated pathophysiology is complicated and poorly understood. Many adverse effects of hemorrhage/resuscitation are common to ischemia/reperfusion and hypoxia/reoxygenation. Resuscitation eventually does not completely reverse the hemorrhage-induced changes. The complexity of the cellular response to hemorrhage complicates efforts to design approaches to treat or prevent injury resulting from resuscitation. Nevertheless, an additive in resuscitation fluids, which can induce HSP-70i, block iNOS, and restores ATP depletion, may be potentially therapeutic to salvageable patients.

Hemorrhage induces overexpression of iNOS and HSP-70i proteins, increases in cellular caspase-3 activity [14], and reduction in ATP [15-18]. iNOS overexpression appears early and it leads to the NO production and its direct and indirect effects. HSP-70i overexpression appears 12 h after occurrence of hemorrhage [13]. Because of its late appearance, it is possible that this HSP-70i is not serving its usual protective role in hemorrhage and resuscitation, but that it is rather facilitating tissue repair for salvaging the damage caused by hemorrhage and resuscitation. However, a pre-treatment to induce HSP-70i overexpression and inhibit iNOS induction prior to hemorrhage would be an ideal one to prevent the resulting injury. On the other hand, its post-treatment is still useful in order to minimize the morbid sequelae such as multiple organ dysfunction and failure.

PERSPECTIVE

Hemorrhage/resuscitation is a common scenario in the clinical practice of medicine and occurs in virtually all

organ systems. Its hallmarks are relatively consistent across patient populations and organ systems. In our murine model [13, 14, 18], treatment with geldanamycin is known to increase HSP-70i and reduce iNOS, cellular caspase-3 activity and ATP loss. Though the underlying mechanism is not completely elucidated, geldanamycin treatment inhibits iNOS by inhibiting KLF6 and induces HSP-70i by activating HSF1. HSP-70i is shown to complex with iNOS and its transcription factor KLF6 and understanding the interaction between HSP-70i and iNOS may provide an insight for a therapeutic design to prevent the hemorrhage/resuscitation-induced injury. Taking together the data obtained from geldanamycin or agents such as ethanol [74-77], glutamine or crocetin [78], adjunct therapy with the optimal resuscitation fluid such as ATP-MgCl₂ [79] or ethyl pyruvate [80] may be a novel approach to address the problems raised from assaults under circumstances such as hemorrhage/resuscitation.

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REFERENCES

- 1 Baue AE, Durham R, Faist E. Systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), multiple organ failure (MOF): are we winning the battle? *Shock* 1998; **10**:79-89.
- 2 Rhee P, Alam HB, Ling GSF. Hemorrhagic shock and resuscitation. In: Tsokos GC, Atkins JL. Eds. *Combat Medicine – Basic and clinical research in military, trauma, and emergency medicine*, Humana Press, New Jersey, 2003: 177-218.
- 3 Stojadinovic A, Kiang JG, Smallridge RC, Galloway RL, Shea-Donohue T. Induction of heat-shock protein 72 protects against ischemia/reperfusion in rat small intestine. *Gastroenterology* 1995; **109**:505-15.
- 4 Conde AG, Lau SS, Dillmann WH, Mestrlil R. Induction of heat shock proteins by tyrosine kinase inhibitors in rat cardiomyocytes and myogenic cells confers protection against simulated ischemia. *J Mol Cell Cardiol* 1997; **29**:1927-38.
- 5 Mizushima Y, Wang P, Jarrar D, et al. Preinduction of heat shock proteins protects cardiac and hepatic functions following trauma and hemorrhage. *Am J Physiol Regul Integr Comp Physiol* 2000; **278**:R352-9.
- 6 Pittet JF, Lu LN, Geiser T, et al. Stress preconditioning attenuates oxidative injury to the alveolar epithelium of the lung following

- haemorrhage in rats. *J Physiol* 2001; **538**:583-97.
- 7 Trost SU, Omens JH, Karlson WJ, et al. Protection against myocardial dysfunction after a brief ischemic period in transgenic mice expressing inducible heat shock protein. *J Clin Invest* 1998; **101**:855-62.
 - 8 Chabrier PE, Demerle-Pallardy C, Auguet M. Nitric oxide synthases: targets for therapeutic strategies in neurological diseases. *Cell Mol Life Sci* 1999; **55**:1029-35.
 - 9 Hierholzer C, Harbrecht B, Menezes JM, et al. Essential role of induced nitric oxide in the initiation of the inflammatory responses after hemorrhagic shock. *J Exp Med* 1998; **187**:917-28.
 - 10 Moore WM, Webber RK, Jerome GM, et al. L-N⁶-(1-iminoethyl) lysine: a selective inhibitor of inducible nitric oxide synthase. *J Med Chem* 1994; **37**:3886-8.
 - 11 Kiang JG, Warke VG, Tsokos GC. NaCN-induced chemical hypoxia is associated with altered gene expression. *Mol Cell Biochem* 2003; **245**:211-6.
 - 12 Warke VG, Nambiar MP, Krishnan S, et al. Transcriptional activation of the human inducible nitric-oxide synthase promoter by Kruppel-like factor 6. *J Biol Chem* 2003; **278**:14812-9.
 - 13 Kiang JG, Bowman PD, Wu BW, et al. Geldanamycin treatment inhibits hemorrhage-induced increases in KLF6 and iNOS expression in un-resuscitated mouse organs: Role of inducible HSP-70. *J Appl Physiol* 2004; **97**:564-9.
 - 14 Kiang JG, Bowman PD, Wu BW, et al. Geldanamycin inhibits hemorrhage-induced increases in caspase-3 activity, KLF6 and iNOS expression in unresuscitated organs of mice: Role of inducible HSP-70. *FASEB J* 2004; **18**:A220-1.
 - 15 Chaudry IH, Planer GJ, Sayeed MM, Baue AE. ATP depletion and replenishment in hemorrhagic shock. *Surg Forum* 1973; **24**:77-9.
 - 16 Chang CG, Van Way CW 3rd, Dhar A, Helling T Jr, Hahn Y. The use of insulin and glucose during resuscitation from hemorrhagic shock increases hepatic ATP. *J Surgery* 2000; **92**:171-6.
 - 17 Paxian M, Bauer I, Rensing H, et al. Recovery of hepatocellular ATP and "pericentral apoptosis" after hemorrhage and resuscitation. *FASEB J* 2003; **17**:993-1002.
 - 18 Kiang JG, Lu X, Tabaku LS, et al. Resuscitation with lactated Ringer's solution limits the expression of molecular events associated with lung injury after hemorrhage. *J Appl Physiol* 2005; **98** (Epub ahead of print).
 - 19 Shea-Donohue T, Anderson J, Swiecki C. Ischemia/reperfusion injury. In: Tsokos GC, Atkins JL. Eds. *Combat Medicine – Basic and clinical research in military, trauma, and emergency medicine*, Humana Press, New Jersey, 2003:219-48.
 - 20 Kiang JG, Tsokos GC. Heat shock protein 70 kD: Molecular Biology, Biochemistry, and Physiology. *Pharmacol Ther* 1998; **80**:183-201.
 - 21 Boros S, Kamps B, Wunderink L, et al. Transglutaminase catalyzes differential crosslinking of small heat shock proteins and amyloid- β . *FEBS Lett* 2004; **576**:57-62.
 - 22 Mayr M, Chung YL, Mayr U, et al. Loss of PKC- δ alters cardiac metabolism. *Am J Physiol Heart Circ Physiol* 2004; **287**:H937-45.
 - 23 Papaconstantinou AD, Brown KM, Noren BT, et al. Mercury, Cadmium, and arsenite enhance heat shock protein synthesis in chick embryos prior to embryotoxicity. *Birth Defects Res Part B Dev Report Toxicol* 2003; **68**:456-64.
 - 24 Morimoto RI, Kroeger PE, Cotto JJ. The transcriptional regulation of heat shock genes: a plethora of heat shock factors and regulatory conditions. *Experientia* 1996; **77** (Suppl.):139-63.
 - 25 Nakai A, Tanabe M, Kawazoe Y, et al. HSF4, a new member of the human heat shock factor family which lacks peptides of a transcriptional activator. *Mol Cell Biol* 1997; **17**:469-81.
 - 26 He H, Soncin F, Grammatikakis N, et al. Elevated expression of heat shock factor (HSF) 2A stimulates HSF1-induced transcription during stress. *J Biol Chem* 2003; **278**:35465-75.
 - 27 Kroeger PE, Sarge KD, Morimoto RI. Mouse heat shock transcription factors 1 and 2 prefer a trimeric binding site but interact differentially with the HSP70 heat shock elements. *Mol Cell Biol* 1993; **13**:3370-83.
 - 28 Kiang JG, Carr FE, Burns MR, McClain DE. HSP-72 synthesis is promoted by increase in [Ca²⁺]_i, or activation of G proteins but not pHi or cAMP. *Am J Physiol* 1994; **267**:C104-14.
 - 29 Kiang JG, Gist ID, Tsokos GC. 17 β -Estradiol-induced increases in glucose-regulated proteins protect human breast cancer T47-D cells from thermal injury. *Chin J Physiol* 1997; **40**:213-9.
 - 30 Kiang JG, McKinney LC, Gallin EK. Heat induces an intracellular acidification in human A-431 cells: role of Na⁺/H⁺ exchanger and metabolism. *Am J Physiol* 1990; **259**:C727-37.
 - 31 Kiang JG, Wu YY, Lin M. Hyperthermia elevates cAMP levels in human epidermoid A-431 cells. *Biochem J* 1991; **276**:683-9.
 - 32 Kiang JG, Koenig ML, Smallridge RC. Heat shock increases cytosolic free Ca²⁺ concentration via the Na⁺/Ca²⁺ exchange in human epidermoid A-431 cells. *Am J Physiol* 1992; **263**:C30-8.
 - 33 Ding XZ, Smallridge RC, Galloway RJ, Kiang JG. Increases in HSF1 translocation and synthesis in human epidermoid A-431 cells: role of protein kinase C and [Ca²⁺]_i. *J Investig Med* 1996; **44**:144-53.
 - 34 Kiang JG, Gist ID, Tsokos GC. Cytoprotection and regulation of heat shock proteins induced by heat shock in human breast cancer T47-D cells: role of [Ca²⁺]_i and protein kinases. *FASEB J* 1998; **12**:1571-9.
 - 35 Kiang JG, Gist ID, Tsokos GC. Biochemical requirement of heat shock protein 72 kD expression in human breast cancer MCF-7 cells. *Mol Cell Biochem* 1999; **199**:179-88.
 - 36 Kiang JG, Gist ID, Tsokos GC. Heat shock induces expression of heat shock protein 72 kD and 90 kD in human breast cancer MDA-231 cells. *Mol Cell Biochem* 2000; **204**:169-78.
 - 37 Kiang JG, Kiang SC, Juang YT, Tsokos GC. N ω -nitro-L-arginine inhibits the inducible heat shock protein 70 kD via Ca²⁺, PKC, and PKA. *Am J Physiol* 2002; **282**:G415-23.
 - 38 Kiang JG, McClain DE. Heat Stress. In: Tsokos GC, Atkins JL. Eds. *Combat Medicine – Basic and clinical research in military, trauma, and emergency medicine*, Humana Press, New Jersey, 2003:83-101.
 - 39 Jones DT, Addison E, North JM, et al. Geldanamycin and herbimycin A induce apoptotic killing of B chronic lymphocytic leukemia cells and augment the cells' sensitivity to cytotoxic drugs. *Blood* 2004; **103**:1855-61.
 - 40 Sachidhanandam SB, Lu J, Low KS, Mochhala SM. Herbimycin A attenuates apoptosis during heat stress in rats. *Eur J Pharmacol* 2003; **474**:121-28.
 - 41 Hamel L, Kenney M, Jayyosi Z, et al. Induction of heat shock protein 70 by herbimycin A and cyclopentenone prostaglandins in smooth muscle cells. *Cell Stress Chaperones* 2000; **5**:121-31.
 - 42 Kim HR, Kang HS, Kim HD. Geldanamycin induces heat shock protein expression through activation of HSF1 in K562 erythroleukemic cells. *IUBMB Life* 1999; **48**:429-33.
 - 43 Briant D, Ohan N, Heikkila JJ. Effect of herbimycin A on hsp30 and hsp70 heat shock protein gene expression in *Xenopus* cultured cells. *Biochem Cell Biol* 1997; **75**:777-82.
 - 44 Morris SD, Cumming DV, Latchman DS, Yellon DM. Specific

- induction of the 70 kD heat stress proteins by the tyrosine kinase inhibitor herbimycin-A protects rat neonatal cardiomyocytes. A new pharmacological route to stress protein expression? *J Clin Invest* 1996; **97**:706-12.
- 45 Fonager J, Beedholm R, Clark BF, Rattan SI. Mild stress-induced stimulation of heat-shock protein synthesis and improved functional ability of human fibroblasts undergoing aging in vitro. *Exp Gerontol* 2000; **37**:1223-8.
- 46 Kiang JG, Ding XZ, McClain DE. Thermotolerance attenuates heat-induced increases in $[Ca^{2+}]_i$ and HSP-72 synthesis but not heat-induced intracellular acidification in human A-431 cells. *J Investig Med* 1996; **44**:189-92.
- 47 Kiang JG, Koenig ML. Characterization of intracellular calcium pools in thermotolerant and their desensitization in thermotolerant cells. *J Investig Med* 1996; **44**:352-61.
- 48 Kiang JG, Ding XZ, McClain DE. Overexpression of HSP-70 attenuates increases in $[Ca^{2+}]_i$ and protects human epidermoid A-431 cells after chemical hypoxia. *Toxicol Appl Pharmacol* 1998; **149**:185-94.
- 49 Murphy P, Sharp A, Shin J, et al. Suppressive effects of ansamycins on inducible nitric oxide synthase expression and the development of experimental autoimmune encephalomyelitis. *J Neurosci Res* 2002; **67**:461-70.
- 50 Ren M, Leng Y, Jeong M, Leeds PR, Chuang DM. Valproic acid reduces brain damage induced by transient focal cerebral ischemia in rats: potential roles of histone deacetylase inhibition and heat shock protein induction. *J Neurochem* 2004; **89**:1358-67.
- 51 Ren M, Senatorov VV, Chen RW, Chuang DM. Postinsult treatment with lithium reduces brain damage and facilitates neurological recovery in a rat ischemia/reperfusion model. *Proc Natl Acad Sci U S A* 2003; **100**:6210-5.
- 52 Carter Y, Liu G, Stephens WB, et al. Heat shock protein (HSP72) and p38 MAPK involvement in sublethal hemorrhage (SLH)-induced tolerance. *J Surg Res* 2003; **111**:70-7.
- 53 Carter Y, Liu G, Yang J, Fier A, Mendez C. Sublethal hemorrhage induces tolerance in animals exposed to cecal ligation and puncture by altering p38, p44/42, and SAPK/JNK MAP kinase activation. *Surg Infect (Larchmt)* 2003; **4**:17-27.
- 54 Thomas DD, Miranda KM, Citrin D, Espey MG, Wink DA. Nitric oxide. In: Tsokos GC, Atkins JL. Eds. *Combat Medicine – Basic and clinical research in military, trauma, and emergency medicine*, Humana Press, New Jersey, 2003:23-60.
- 55 Griffith OW, Stuehr DJ. Nitric oxide synthases: properties and catalytic mechanism. *Annu Rev Physiol* 1995; **57**:707-36.
- 56 Nathan C, Xie Q. Regulation of biosynthesis of nitric oxide. *J Biol Chem* 1994; **269**:13725-8.
- 57 Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF- κ B/Rel in induction of nitric oxide synthase. *J Biol Chem* 1994; **269**:4705-8.
- 58 Spink J, Cohen J, Evans TJ. The cytokine responsive vascular smooth muscle cell enhancer of inducible nitric oxide synthase. Activation by nuclear factor- κ B. *J Biol Chem* 1995; **270**:29541-7.
- 59 Taylor BS, de Vera ME, Ganster RW, et al. Multiple NF- κ B enhancer elements regulate cytokine induction of human inducible nitric oxide synthase gene. *J Biol Chem* 1998; **273**:15148-56.
- 60 Marks-Konczalik J, Chu SC, Moss J. Cytokine-mediated transcriptional induction of the human inducible nitric oxide synthase gene requires both activator protein 1 and nuclear factor kappa B-binding sites. *J Biol Chem* 1998; **273**:22201-8.
- 61 Hierholzer C, Billiar TR. Molecular mechanisms in the early phase of hemorrhagic shock. *Langenbecks Arch Surg* 2001; **386**:302-8.
- 62 Collins JL, Vodovotz Y, Hierholzer C, et al. Characterization of the expression of inducible nitric oxide synthase in rat and human liver during hemorrhagic shock. *Shock* 2003; **9**:117-22.
- 63 Rajnik M, Salkowski CA, Thomas KE, et al. Induction of early inflammatory gene expression in a murine model of non resuscitated, fixed-volume hemorrhage. *Shock* 2002; **17**:322-8.
- 64 Salerno L, Sorrenti V, Di Giacomo C, Romeo G, Siracusa MA. Progress in the development of selective nitric oxide synthase (NOS) inhibitors. *Curr Pharm Des* 2002; **8**:177-200.
- 65 Meng X, Harken AH. The interaction between HSP70 and TNF- α expression: a novel mechanism for protection of the myocardium against post injury depression. *Shock* 2002; **17**:345-53.
- 66 Meng X, Banerjee A, Ao L, et al. Inhibition of myocardial TNF- α production by heat shock: a potential mechanism of stress-induced cardioprotection against post-ischemic dysfunction. *Ann NY Acad Sci* 1999; **874**:69-82.
- 67 Bellamay RF. The causes of death in conventional land warfare: implications for combat casualty care research. *Mil Med* 1984; **149**:55-62.
- 68 Bellamay RF. How shall we train for combat casualty care? *Mil Med* 1987; **152**:617-21.
- 69 Bellamay RF. Combat trauma. In: Zajtchuck R and Grande CM. Eds. *Textbook of Military Medicine; vol 4: Anesthesia and Perioperative Care of Combat Casualty*. Washington DC, TMM Publications, 1995:1-42.
- 70 Committee on Fluid Resuscitation for Combat Casualty. Fluid resuscitation: State of science for treating combat casualties and civilian injuries. Report of the institute of medicine. Washington DC, National Academy Press, 1999.
- 71 Kiang JG, Lu X, Tabaku LS, et al. Hemorrhage and resuscitation increase caspase-2 activity and alter stress-related gene expression in rat. *Shock* 2004; **21**(suppl 2):44.
- 72 Alam HB, Stegalkina S, Rhee P, Koustova E. cDNA array analysis of gene expression following hemorrhagic shock and resuscitation in rats. *Resuscitation* 2002; **54**:195-206.
- 73 Kiang JG. Genistein inhibits herbimycin A-induced inducible heat shock protein 70 kD. *Mol Cell Biochem* 2003; **245**:191-9.
- 74 Saika M, Ueyama T, Senba E. Expression of immediate early genes, HSP70, and COX-2 mRNAs in rat stomach following ethanol ingestion. *Dig Dis Sci* 2000; **45**:2455-62.
- 75 Calabrese V, Testa G, Ravagna A, Bates TE, Stella AM. HSP70 induction in the brain following ethanol administration in the rat: regulation by glutathione redox state. *Biochem Biophys Res Commun* 2000; **269**:397-400.
- 76 Tunci P, Schiaffonati L, Rabellotti E, et al. *In vivo* modulation of 73 kD heat shock cognate and 78 kD glucose-regulating protein gene expression in rat liver and brain by ethanol. *Alcohol Clin Exp Res* 1999; **23**:1861-7.
- 77 Sato M, Maulik N, Das DK. Cardioprotection with alcohol: role of both alcohol and polyphenolic antioxidants. *Ann N Y Acad Sci* 2002; **957**:122-35.
- 78 Van Way CW 3rd, Dhar A, Morrison DC, Longorio MA, Maxfield DM. Cellular energetics in hemorrhagic shock: restoring adenosine triphosphate to the cells. *J Trauma* 2003; **54** (Suppl):S169-76.
- 79 Nalos M, Asfar P, Ichai C, Radermacher P, Leverve XM. Adenosine triphosphate-magnesium chloride: relevance for intensive care. *Int Care Med* 2003; **29**:10-8.
- 80 Fink MP. Ethyl pyruvate: a novel treatment for sepsis and shock. *Minerva Anesthesiol* 2004; **70**:365-71.