

Geldanamycin treatment inhibits hemorrhage-induced increases in KLF6 and iNOS expression in unresuscitated mouse organs: role of inducible HSP70

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¹Division of Military Casualty Research, Walter Reed Army Institute of Research, Silver Spring 20910-7500; Departments of ²Medicine and ³Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799; and ⁴United States Army Institute of Surgical Research, San Antonio, Texas 78234-6315

Submitted 25 February 2004; accepted in final form 10 April 2004

Kiang, Juliann G., Phillip D. Bowman, Brian W. Wu, Nyasa Hampton, Andrew G. Kiang, Baiteng Zhao, Yuang-Taung Juang, James L. Atkins, and George C. Tsokos. Geldanamycin treatment inhibits hemorrhage-induced increases in KLF6 and iNOS expression in unresuscitated mouse organs: role of inducible HSP70. *J Appl Physiol* 97: 564–569, 2004. First published April 16, 2004; 10.1152/jappphysiol.00194.2004.—The aim of this study was to determine whether hemorrhage affects the levels of a variety of stress-related proteins and whether changes can be inhibited by drugs reported to provide protection from ischemia and reperfusion injury. Male Swiss Webster mice were subjected to a 40% hemorrhage without resuscitation. Western blot analysis indicated that c-Jun (an AP-1 protein), Kruppel-like factor 6 (KLF6), and inducible nitric oxide synthase (iNOS) were upregulated sequentially in that order. Pretreatment of mice with geldanamycin (GA) 16 h before hemorrhage effectively inhibited the expression of the proteins KLF6 and iNOS, whereas caffeic acid phenethyl ester did not. GA pretreatment increased inducible heat shock protein (HSP) 70 but not HSP90 in both sham and hemorrhagic tissues. The overexpressed inducible HSP70 formed complexes with KLF6 and iNOS. These results suggest that GA may be therapeutically useful for reducing hemorrhage-induced injury when used as a presurgical treatment or when added to resuscitation fluids.

apoptosis; caffeic acid phenethyl ester; c-Jun; c-Fos; nuclear factor- κ B; hypoxia-inducible factor-1; Kruppel-like factor 6 transcription factor

HEMORRHAGE LEADS TO systemic inflammatory response syndrome, multiple organ dysfunction syndrome, and organ failure (1). A variety of biomolecules are known to be involved in this response. In rodents, increases in inducible heat shock protein 70 (HSP70i) stimulated by heat stress limit injury to tissues caused by ischemia and reperfusion (34). Likewise, inhibition of nitric oxide production results in significant reduction of local tissue damage, polymorphonuclear neutrophil infiltration, and leukotriene B₄ generation caused by ischemia-reperfusion (3). Mice deficient in inducible nitric oxide synthase (iNOS) also demonstrate limited hemorrhage/resuscitation-induced injury (10, 25). Therefore, remedies that induce HSP70i and/or inhibit iNOS might prove very useful for reducing hemorrhage- or hemorrhage and resuscitation-induced injury in humans.

These observations are consistent with the idea that the low oxygen supply resulting from conditions such as ischemia or

hemorrhage affects the expression of iNOS, which then influences the expression of proteins that alter cell viability. We showed that hypoxia results in alteration of iNOS, Bcl-2, and p53 mRNA expression in cultured human intestinal epithelial T84 cells and Jurkat T cells, alterations that can be modulated by treatment with a NOS inhibitor (17). We 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 (17).

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, HSP70i, and hypoxia-inducible factor (HIF)-1 α has not been previously reported in an in vivo model. The relationship between iNOS and KLF6 in the in vivo model has also not been studied. Geldanamycin (GA) has been shown to suppress caspase-3 activity and DNA cleavage in human megakaryocytic leukemia CMK-7 cells treated with actinomycin D and colcemid (31) and to reduce fluid accumulation and iNOS overexpression in rat lung after hemorrhage and resuscitation (27). In this study, we report that hemorrhage in mice stimulates increased expression of c-Jun, KLF6, iNOS, HSP70i, and HIF-1 α proteins. Treatment of mice with GA before hemorrhage inhibits expression of iNOS and KLF6 proteins. GA treatment increases levels of HSP70i, which in turn form complexes with KLF6 and iNOS. The results suggest that the inhibitory effect of GA is probably mediated by its capacity to induce HSP70i, a well-characterized cytoprotectant (14, 16).

METHODS

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals* (NRC Publication, 1996 edition).

Experimental protocol. We conducted experiments using the mouse model described by Song et al. (32). Male Swiss Webster mice weighing 25–35 g were briefly anesthetized with isoflurane, and 40% of the calculated blood volume was removed over a 1-min period by cardiac puncture with a 26-gauge needle. Mean arterial blood pressure fell from 80 to 40 mmHg in the 2 h after treatment. Treated mice were allowed to respond for 1, 3, 6, 12, 24, and 48 h. Sham-treated animals underwent cardiac puncture, but no blood was removed. In different experiments, mice were pretreated with caffeic acid phenethyl ester (CAPE; 1 μ g/g body wt) or GA (1 μ g/g body wt) in 10% DMSO

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1. REPORT DATE 01 AUG 2004		2. REPORT TYPE N/A		3. DATES COVERED -	
4. TITLE AND SUBTITLE Geldanamycin treatment inhibits hemorrhage-induced increases in KLF6 and iNOS expression in unresuscitated mouse organs: role of inducible HSP70				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Kiang J. G., Bowman P. D., Wu B. W., Hampton N., Kiang A. G., Zhao B., Juang Y. T., Atkins J. L., Tsokos G. C.,				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) United States Army Institute of Surgical Research, JBSA Fort Sam Houston, TX 78234				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT SAR	18. NUMBER OF PAGES 6	19a. NAME OF RESPONSIBLE PERSON
a REPORT unclassified	b ABSTRACT unclassified	c THIS PAGE unclassified			

saline by intraperitoneal injection 16 h before hemorrhage (27). These mice were then subjected to 40% hemorrhage and allowed to respond for 6 h before they were killed. Small portions of lung, jejunum, kidney, heart, brain, and liver were removed from the treated mice and frozen immediately at -70°C until used.

Tissues were sonicated for 15 s and then centrifuged at 10,000 g for 10 min. The supernatant was saved for determining the total amount of protein in each lysate sample and performing immunoblot analysis. Proteins and moieties assessed by immunoblotting included c-Fas, 65-kDa nuclear factor- κB (NF- κB), c-Jun, KLF4, KLF6, iNOS, HSP70i, HSP90, HIF-1 α , phosphotyrosine, and phosphothreonine.

Immunoprecipitation. Tissue lysates containing 300 μg of protein were incubated with the specified antibody (5 μl), chilled on ice for 1 h, mixed with protein A/G agarose beads (50 μl ; Santa Cruz Biotechnology, Santa Cruz, CA), and incubated overnight on a nutator at 4°C . The immunoprecipitate was collected by centrifugation at 12,500 g for 10 min, washed twice with 500 μl of stop buffer, and once with 500 μl of Tris wash buffer. The pellet was resuspended in the 50 μl of electrophoresis sample buffer without 2-mercaptoethanol, boiled for 5 min, and then centrifuged for 30 s to remove the agarose beads. The supernatant was incubated with 5% 2-mercaptoethanol at 37°C for 1 h. Twenty-five microliters of sample were loaded onto precast 10% Tris-glycine polyacrylamide gels for Western blots.

Western blots. For samples not requiring immunoprecipitation, lysate was mixed with an equal volume of Tris buffer (pH 6.8) containing 1% SDS and 1% 2-mercaptoethanol. Aliquots containing 20 μg of protein were resolved on SDS-polyacrylamide slab gels (precaster 10% gel, Invitrogen, Carlsbad, CA). Protein was blotted onto a nitrocellulose membrane (type NC, 0.45 μm , Schleicher and Schuell) using a Novex blotting apparatus and the manufacturer's protocol. The nitrocellulose membrane was blocked by incubation for 90 min at room temperature in PBS containing 5% nonfat dried milk. The blot was then incubated for 60 min at room temperature with the selected monoclonal antibody against p53, Bcl-2, c-Fas, c-Jun, KLF4, KLF6, 65-kDa NF- κB , HSP90, actin (Santa Cruz), HSP70i (Amersham, Arlington Heights, IL), iNOS, HIF-1 α (BD Transduction Laboratories, San Diego, CA), phosphotyrosine, or phosphothreonine (Zymed Laboratories, San Francisco, CA) at 1 $\mu\text{g}/\text{ml}$ in PBS-5% BSA. The blot was washed three times (10 min each) in TBS-0.1% Tween 20 before incubating for 60 min at room temperature with a 1,000 $\times$ dilution of species-specific IgG peroxidase conjugate (Santa Cruz) in PBS-1% gelatin. The blot was washed six times (5 min each) in TBS-0.1% Tween 20 before detection of the peroxidase activity using the enhanced chemiluminescence kit (Amersham Life Science Products).

Expression of the protein actin was not altered by hemorrhage; therefore, actin was used as a sample loading control, and data for immunoblotting analysis were normalized by actin (13).

Statistical analysis. All data are expressed as means $\pm$ SE. One-way ANOVA, two-way ANOVA, Studentized-range test, Bonferroni's inequality, and Student's *t*-test were used for comparison of groups with 5% as a significant level.

Chemicals. Chemicals used in this study were GA and CAPE (Sigma Chemical, St. Louis, MO).

RESULTS

Hemorrhage increases c-Jun, KLF6, iNOS, HSP70i, and HIF-1 α . iNOS expression is known to increase in rodent lung after hemorrhage (10, 25), but the time course of the increase has not been studied. Figure 1 demonstrates how hemorrhage affects expression of several stress-related proteins in our mouse hemorrhage model over time. On the basis of Western blot data from jejunum lysates, c-Jun protein was overexpressed within 1 h, but levels returned to baseline values 3 h later. KLF6 began to increase significantly after 6 h, reached a

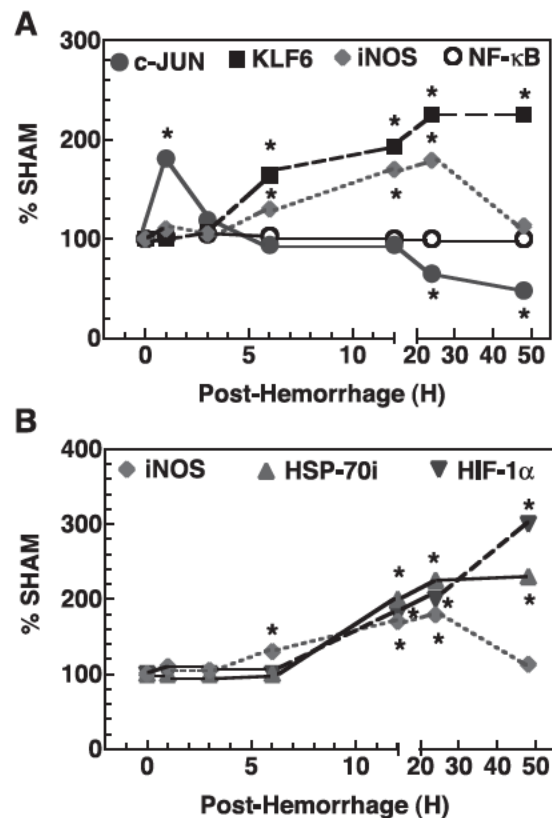
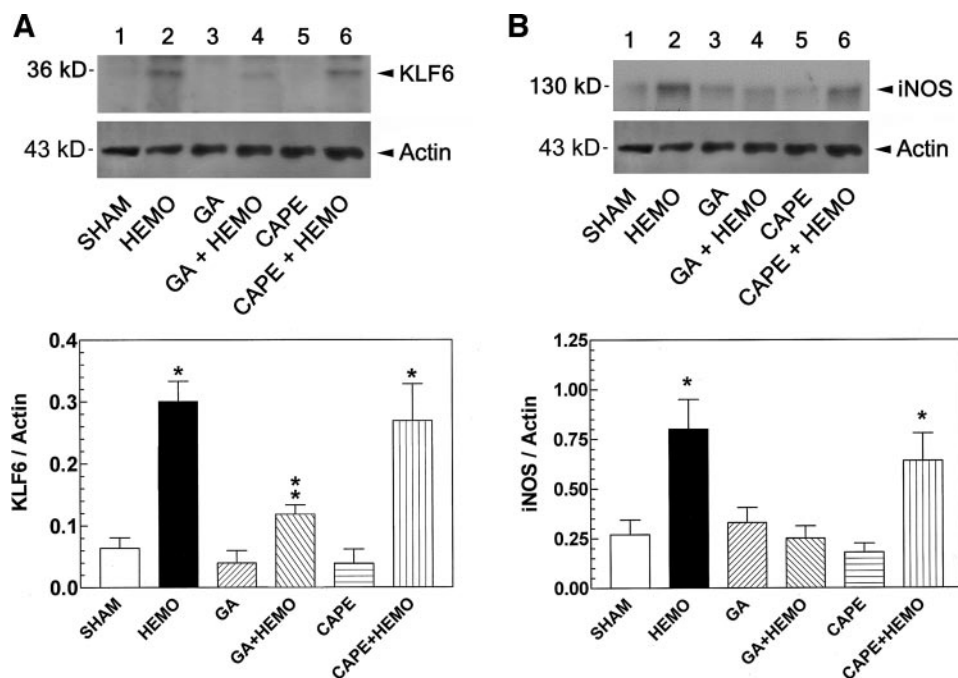


Fig. 1. Time course of stress proteins after hemorrhage in mouse jejunum. Mice were exposed to hemorrhage and allowed to respond for 1, 3, 6, 12, 24, or 48 h ($n = 6$). Data collected from sham-operated mouse are represented at 0 h. Immunoblot analyses using antibodies against c-Jun, Kruppel-like factor 6 (KLF6), inducible nitric oxide synthase (iNOS), and 65-kDa nuclear factor- κB (NF- κB) (A), and iNOS, inducible heat shock protein (HSP) 70 (HSP70i), and hypoxia-inducible factor (HIF)-1 α (B) were conducted. Protein bands were quantitated densitometrically and normalized with actin band. * $P < 0.05$ vs. time = 0 h (Student's *t*-test).

maximum at 24 h, and remained at that level 48 h after hemorrhage. iNOS increased at 6 h, reached a maximum between 12 and 24 h, and returned to baseline values by 48 h. HSP70i increased at 12 h and remained elevated at 48 h. HIF-1 α also increased at 12 h and continued to increase at 48 h. The sequence of protein appearance was, therefore, c-Jun, KLF6, iNOS, HSP70i, and HIF-1 α . KLF4 and c-Fos (an AP-1 protein) were not detected in jejunum (data not shown), and 65-kDa NF- κB was detected, but it was not affected by hemorrhage (Fig. 1). Similar time courses for these stress-related proteins were observed in lung, heart, kidney, liver, and heart (data not shown). Sham-treated mouse organs displayed no changes in the basal levels of c-Jun, KLF6, and iNOS (data not shown).

GA inhibits hemorrhage-induced increases in KLF6 and iNOS expression. GA is a known inhibitor of the ATPase activity of HSP90. It has been shown to inhibit caspase-3 activity in human leukemia CMK-7 cells treated with actinomycin D and colcemid (31) and to reduce fluid accumulation in rat lung after hemorrhage and resuscitation (27). CAPE is an active anti-inflammatory component of propolis and rutin (plant-derived natural products). It is reported to be a potent inhibitor of NF- κB (26) as well as a natural antioxidant (6). We treated mice with GA or CAPE 16 h before hemorrhage to

Fig. 2. Geldanamycin (GA) inhibits hemorrhage-induced increase in KLF6 (A) and iNOS (B) protein expression in mouse jejunum. Mice were pretreated with GA (1 μ g/g body wt) or caffeic acid phenethyl ester (CAPE; 30 μ g/g body wt) in 10% DMSO saline by intraperitoneal injection 16 h before hemorrhage ($n = 3-5$). KLF6 and iNOS in lysates of mouse jejunum were detected using immunoblot analysis, quantitated densitometrically, and normalized with actin band. A: representative Western blot of jejunum KLF6. $*P < 0.05$ vs. sham, GA, GA + hemorrhage (HEMO), and CAPE. $**P < 0.05$ vs. sham, HEMO, GA, CAPE, and CAPE + HEMO determined by two-way ANOVA and Studentized range test. B: representative Western blot of jejunum iNOS. $*P < 0.05$ vs. sham, GA, GA + HEMO, and CAPE determined by two-way ANOVA and Studentized range test.



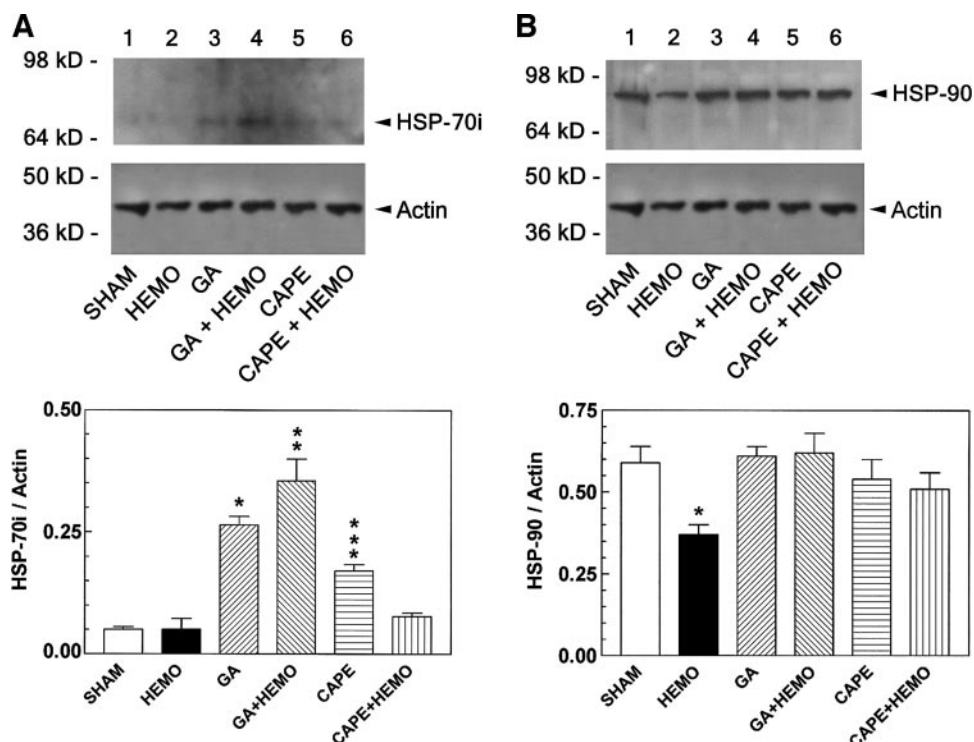
determine how the respective activities inhibited by GA and CAPE affect our mouse hemorrhage model.

Figure 2 shows that hemorrhage is associated with overexpression of KLF6 and iNOS. Pretreatment with GA significantly reduced the hemorrhage-induced increase in KLF6 protein (Fig. 2A) and completely inhibited the hemorrhage-induced increase in iNOS protein (Fig. 2B). CAPE pretreatment did not affect the basal expression of KLF6 or iNOS in the absence of hemorrhage (Fig. 2, A and B, respectively). It also

did not inhibit the increase in KLF6 protein or iNOS observed after hemorrhage (Fig. 2, A and B, respectively).

GA induces HSP70i but not HSP90. Because GA has been shown to induce HSP70i in rat lung (27), we sought to determine whether GA induces HSP70i in mouse jejunum. Western blot data in Fig. 3 show that hemorrhage by itself considerably decreased HSP90 protein levels 6 h later. GA significantly upregulated HSP70i expression (Fig. 3A) but not HSP90 (Fig. 3B) in both sham-treated and hemorrhagic mice.

Fig. 3. GA induces HSP70i overexpression. Mice were pretreated with GA (1 μ g/g body wt) or CAPE (30 μ g/g body wt) in 10% DMSO saline by intraperitoneal injection 16 h before hemorrhage ($n = 3-5$). HSP70i (A) and HSP90 (B) in lysates of mouse jejunum were detected 6 h after hemorrhage using immunoblotting analysis, quantitated densitometrically, and normalized with actin band. A: representative Western blot of jejunum HSP70i. $*P < 0.05$ vs. sham, HEMO, GA + HEMO, CAPE, and CAPE + HEMO; $**P < 0.05$ vs. sham, HEMO, GA, CAPE, and CAPE + HEMO; $***P < 0.05$ vs. sham, HEMO, GA, GA + HEMO, and CAPE + HEMO determined by two-way ANOVA and Studentized range test. B: representative Western blot of jejunum HSP90. $*P < 0.05$ vs. sham, GA, GA + HEMO, CAPE, and CAPE + HEMO determined by two-way ANOVA and Studentized range test.



Treatment with CAPE slightly increased the level of HSP70i (Fig. 3A) but did not change the level of HSP90 (Fig. 3B). These results with GA and CAPE suggest that the GA inhibition of KLF6 and iNOS expression is specific and probably mediated through its ability to upregulate HSP70i (27).

HSP70i forms complex with KLF6. Because HSP70 has been shown to form complexes with calmodulin (13), $\text{Na}^+/\text{Ca}^{2+}$ exchanger, and other biomolecules (16), we asked whether HSP70i can form a complex with KLF6. After immunoprecipitation of jejunum lysates with anti-HSP70 antibody, immunoblotting of the precipitate with an anti-KLF6 antibody demonstrated a KLF6 band (Fig. 4A), indicating that HSP70i can form a complex with KLF6. Even though HSP70 can some-

times bind nonspecifically to other proteins, we believe the binding of HSP70 to KLF6 observed in our system was specific. As a test of the capacity of HSP70 to bind nonspecifically to other proteins, we immunoprecipitated lysates with HSP70 antibody and probed immunoblots of the precipitate with antibodies for two unrelated proteins (i.e., p53 and Bcl-2). Neither of the proteins was detected in the precipitate (data not shown). The fact that KLF6 was detected in the precipitate is therefore consistent with the idea that the HSP70 specifically couples to KLF6.

The observation of an HSP70-KLF6 complex was further verified by immunoprecipitation with anti-KLF6 antibody and immunoblotting with anti-HSP70 antibody. An HSP70i band was present (Fig. 4B), indicating again that HSP70i can couple with KLF6.

HSP70i forms complex with iNOS. Our data also suggest that HSP70i can form a complex with iNOS. When tissue lysates were immunoprecipitated with anti-HSP70i and then the precipitates were immunoblotted with anti-iNOS antibody, the iNOS band was detected (Fig. 4C). When the lysate was immunoprecipitated with anti-iNOS and then the precipitates were immunoblotted with anti-HSP70i antibody, the HSP70i band was detected (Fig. 4D).

Phosphorylation is not involved in GA inhibition. Because cellular responses to many extracellular signals occur through phosphorylation or dephosphorylation of intracellular proteins, we determined whether GA exerted its inhibition through changes in protein phosphorylation. Because heat shock has been shown to increase tyrosine phosphorylation of constitutive NOS (15), we directed our attention specifically at KLF6 or iNOS phosphorylation. Blots were stripped and exposed to anti-phosphotyrosine antibody or anti-phosphothreonine antibody. Results indicate that hemorrhage did not induce KLF6 or iNOS phosphorylation at tyrosine or threonine residues (data not shown).

DISCUSSION

Multiple organ failure is known to occur because of hemorrhagic shock. For this reason, we sought to develop profiles of changes in expression of several stress-related proteins thought to be involved in this process by testing six major organs from a mouse hemorrhage model.

Hemorrhage induced sequential increases in the levels of stress-related proteins c-Jun, KLF6, iNOS and then HSP70i and HIF-1 α . iNOS has previously been shown to be overexpressed after hemorrhagic shock in rodent lung (8, 27), human liver (4), and murine tissues (4, 28). Warke et al. (36) 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 GenBank, we identified four CACCC sites on the mouse iNOS promoter (−253 to −257, −818 to −822, −856 to −860, and −1,556 to −1,600) 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 −1,225 to −1,231) that probably also bind c-Jun and/or c-Fos. In our study, no c-Fos was detected with immunoblotting analysis. Because iNOS promoters include two AP-1 sites and are involved in processes such as cell injury, wound repair, em-

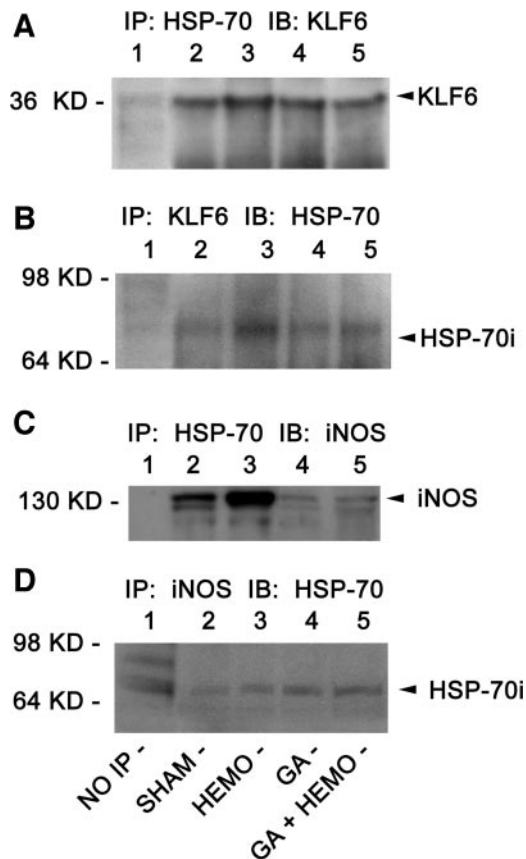


Fig. 4. GA-induced increases in HSP70i lead to formation of KLF6-iNOS complex. Mice were pretreated with GA (1 $\mu\text{g/g}$ body wt) in 10% DMSO saline by intraperitoneal injection 16 h before hemorrhage ($n = 3-5$). A: representative Western blot of jejunum HSP70i complex formation with KLF6. Lysates of mouse jejunum were immunoprecipitated (IP) with anti-HSP70i antibody, and KLF6 was then detected with anti-KLF6 antibody using immunoblot (IB) analysis. B: representative Western blot of jejunum KLF6 complex formation with HSP70i. Lysates of mouse jejunum were immunoprecipitated with anti-HSP70i antibody, and KLF6 was then detected with anti-KLF6 antibody using immunoblot analysis. C: representative Western blot of jejunum HSP70i complex formation with iNOS. Lysates of mouse jejunum were immunoprecipitated with anti-HSP70i antibody, and iNOS was then detected with anti-iNOS antibody using immunoblot analysis. D: representative Western blot of jejunum KLF6 complex formation with HSP70i. Lysates of mouse jejunum were immunoprecipitated with anti-iNOS antibody, and HSP70i was then detected with anti-HSP70 antibody using immunoblot analysis. NO IP, normal mouse serum with no antibody added to do immunoprecipitation (lane 1); sham, mouse received same handling and procedures as hemorrhagic mouse but no hemorrhage (lane 2); HEMO, hemorrhage (lane 3); GA, lane 4; GA + HEMO, GA given before hemorrhage (lane 5).

bryogenesis, tissue differentiation, and suppression of tumorigenesis, the observation that c-Jun overexpression occurs earlier than KLF6 and iNOS overexpression suggests two hypotheses: 1) hemorrhage activates c-Jun, which upregulates KLF6 expression, leading to iNOS expression; or 2) hemorrhage activates c-Jun, which then directly binds to the iNOS promoter to induce iNOS expression. Experiments to differentiate these two possibilities are underway. c-Fos was not detected in these tissues and is therefore not discussed further.

Hemorrhage also increases levels of HSP70i and HIF-1 α protein, both of which appear after iNOS. The increase in HSP70i protein remained at a plateau up to 48 h after hemorrhage, but HIF-1 α protein continued to increase. HSP70i is known to be a cytoprotector (5, 14, 16), but its late appearance supports the idea that it is involved only in controlling injury, not in preventing it. HIF-1 is a heterodimeric protein consisting of a constitutively expressed β -subunit and an oxygen- and growth factor-regulated α -subunit. HIF-1 α is normally rapidly degraded in cells supplied with adequate oxygen. It has been shown to be overexpressed because of intratumoral hypoxia (29), chronic fetal anemia cardiac hypertrophy (22), injection of CoCl₂ (30), or treatment with pyruvate (19). It is not clear what role HIF-1 α plays in hemorrhage, because its overexpression occurs so late.

GA is an inhibitor of the ATPase activity of HSP90 (31), which has been shown to be a suppressor of caspase-3 activity and DNA cleavage in human leukemia CMK-7 cells treated with actinomycin D and colcemid (31). It is also known to reduce fluid accumulation in rat lung after hemorrhage (27) and induce degradation of HIF-1 α in prostate cancer cells (20). The GA inhibition observed in this study is probably mediated by its ability to inhibit the overexpression of KLF6 and iNOS (see schematic, Fig. 5).

CAPE is reported to be a potent inhibitor of NF- κ B (26) as well as a natural antioxidant (16). We did not find alteration of 65-kDa NF- κ B protein expression in hemorrhagic jejunum, which is in agreement with observations in another laboratory (9), where both hemorrhagic shock and resuscitation were required before increased expression of NF- κ B was observed. Therefore, even though CAPE is a potent inhibitor of NF- κ B activation (26), it showed no effects on hemorrhage-induced elevations of KLF6 and iNOS protein. Moreover, KLF6 likely plays a key role in regulating iNOS protein expression under these circumstances because the hypoxia-induced increase in KLF6 causes an upregulation of iNOS in human T cells (36). Continued investigation of the relationship between KLF6 and iNOS in hemorrhagic shock is required.

GA upregulates expression of HSP70i protein but not HSP90. It has been shown in rats subjected to both hemorrhage and resuscitation that GA treatment 24 and 48 h before hemorrhage or heat stress 16 h before hemorrhage protects against fluid accumulation in lung (27). Under those conditions, HSP70i was elevated. Experiments using HSP70 gene transfection have shown that cytokine-mediated activation of NF- κ B in cultured cells is attenuated (27). Experiments using heat stress to overexpress HSP70i also significantly attenuated cardiovascular and hepatocellular dysfunction as well as circulatory levels of tumor necrosis factor- α and IL-6 in hemorrhagic and resuscitated rat (24). Although 65-kDa NF- κ B apparently plays no role in our mouse model, HSP70i is known to protect strongly against ischemic injury (7, 12, 21, 23, 34,

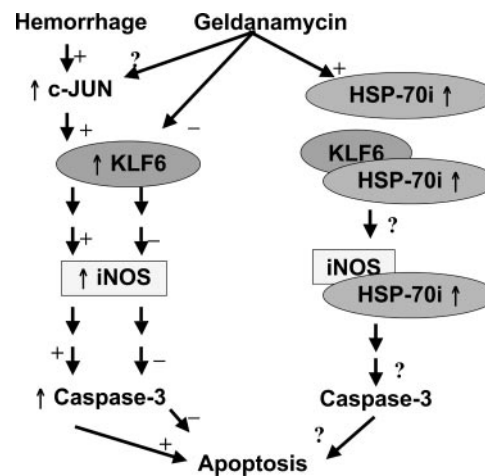


Fig. 5. Schematic representation of model for interaction between hemorrhage-induced changes and points where GA might block changes. KLF6 and HSP70i are normally associated with each other. Hemorrhage increases c-Jun that probably increases caspase-3 activity and upregulates expression of KLF6 and iNOS proteins. Treatment with GA induces increased HSP70i expression that leads to increased complex formation between HSP70i and both KLF6 and iNOS. Because GA also inhibits KLF6 expression, less KLF6 is available to bind iNOS promoter, and less iNOS is expressed, which leads to caspase-3 inhibition (17).

35) and other types of injury (2, 16, 18, 24, 35, 37, 39). Our data show that the GA-induced increase in HSP70i protein levels leads to the formation of a HSP70i-KLF6 complex that does not alter KLF6 phosphorylation. It is possible that HSP70 complexation with KLF6 leads to less KLF6 binding to the iNOS promoter and, in turn, results in decreased iNOS protein (11, 17).

Figure 5 is a schematic representation of our model for the interaction between hemorrhage-induced changes, indicating points where GA might block these changes. On the basis of our data, KLF6 and HSP70i are first normally associated with each other in some way. After hemorrhage, increases in c-Jun protein upregulate expression of KLF6 and iNOS proteins and, somehow, caspase-3 activity. Treatment with GA induces increased HSP70i protein expression that results in increased formation of complexes with KLF6, thereby limiting the levels of free KLF6. As a result, less KLF6 is available to bind to the iNOS promoter, and less iNOS is expressed. Caspase-3 activity is inhibited directly or indirectly (through other intermediate proteins such as survivin). Further studies are needed to understand the underlying mechanism in detail.

In summary, this is the first report to show the time response of hemorrhage-induced increases in expression of the stress-related proteins c-Jun, KLF6, iNOS, HSP70i, and HIF-1 α . Our observations show that treatment with GA, but not CAPE, inhibits KLF6 and iNOS protein expression by increasing HSP70i, which forms complexes with both KLF6 and iNOS. Our results, taken together with findings from other laboratories, indicate that GA may be therapeutically useful before surgery or as an additive to resuscitation fluids to reduce hemorrhage-induced injury. More studies with GA are needed, especially those that can better define dose- and time-response relationships after hemorrhage.

ACKNOWLEDGMENTS

The authors thank Dr. David E. McClain for editorial assistance and R. Lee Collins for graphic support.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army, the Uniformed Services University of the Health Sciences, or Department of Defense.

GRANTS

DOD RADII STO R supported this work.

REFERENCES

- Baue AE, Durham R, and Faist E. Systemic inflammatory response syndrome (MODS), multiple organ dysfunction syndrome (MODS), multiple organ failure (MOF): are we winning the battle? *Shock* 10: 79–89, 1998.
- Bellmann K, Wenz A, Radons J, Burkart V, Kleemann R, and Kolb H. Heat shock induces resistance in rat pancreatic islet cells against nitric oxide, oxygen radicals and streptozotocin in vitro. *J Clin Invest* 95: 2840–2845, 1995.
- Chabrier PE, Demerle-Pallardy C, and Auguet M. Nitric oxide synthases: targets for therapeutic strategies in neurological diseases. *Cell Mol Life Sci* 55: 1029–1035, 1999.
- Collins JL, Vodovotz Y, Hierholzer C, Villavicencio T, Liu S, Alber S, Gallo D, Stolz DB, Watkins SC, Godfrey A, Gooding W, Kelly E, Peitzman AB, and Billiar TR. Characterization of the expression of inducible nitric oxide synthase in rat and human liver during hemorrhagic shock. *Shock* 9: 117–122, 2003.
- De Maio A. Heat shock proteins: facts, thoughts, and dreams. *Shock* 11: 1–12, 1999.
- Esposito E, Rotilio D, Di Matteo V, Di Giulio C, Cacchio M, and Algeri S. A review of specific dietary antioxidants and the effects on biochemical mechanisms related to neurodegenerative processes. *Neurobiol Aging* 23: 719–735, 2002.
- Fleming SD, Starnes BW, Kiang JG, Stojadinovic A, Tsokos GC, and Shea-Donohue T. Heat stress protection against mesenteric I/R-induced alterations in intestinal mucosa in rats. *J Appl Physiol* 92: 2600–2607, 2002.
- Hierholzer C and Billiar TR. Molecular mechanisms in the early phase of hemorrhagic shock. *Langenbecks Arch Surg* 386: 302–308, 2001.
- Hierholzer C, Billiar TR, and Tweardy DJ. Requirements for NF- κ B activation in hemorrhagic shock. *Arch Orthop Trauma Surg* 122: 44–47, 2002.
- Hierholzer C, Harbrecht B, Menezes JM, Kane J, MacMicking J, and Nathan CF. Essential role of induced nitric oxide in the initiation of the inflammatory responses after hemorrhagic shock. *J Exp Med* 187: 917–928, 1998.
- Hill MM, Adrain C, and Martin SJ. Portrait of a killer: the mitochondrial apoptosome emerges from the shadow. *Mol Interv* 3: 19–25, 2003.
- Hiratsuka M, Mora BN, Yano M, Mohanakumar T, and Patterson GA. Gene transfer of heat shock protein 70 protects lung grafts from ischemia-reperfusion injury. *Ann Thorac Surg* 67: 1421–1427, 1999.
- Kiang JG, Carr FE, Burns MR, and McClain DE. HSP-72 synthesis is promoted by increase in $[Ca^{2+}]_i$ or activation of G proteins but not pH_i or cAMP. *Am J Physiol Cell Physiol* 267: C104–C114, 1994.
- Kiang JG and McClain DE. Heat stress. In: *Combat Medicine Basic and Clinical Research in Military, Trauma, and Emergency Medicine*, edited by Tsokos GC and Atkins JL. Clifton, NJ: Humana, 2003, p. 83–101.
- Kiang JG, McClain DE, Warke VG, Krishnan S, and Tsokos GC. Constitutive NO synthase regulates the Na^+/Ca^{2+} exchanger in human T cells: role of $[Ca^{2+}]_i$ and tyrosine phosphorylation. *J Cell Biochem* 89: 1030–1043, 2003.
- Kiang JG and Tsokos GC. Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacol Ther* 80: 183–201, 1998.
- Kiang JG, Warke VG, and Tsokos GC. NaCN-induced chemical hypoxia is associated with altered gene expression. *Mol Cell Biochem* 245: 211–216, 2003.
- Klosterhalfen B, Hauptmann S, Tietze L, Tons C, Winkeltau G, Kupper W, and Kirkpatrick CJ. The influence of heat shock protein 70 induction on hemodynamic variables in a porcine model of recurrent endotoxemia. *Shock* 7: 358–363, 1997.
- Lu H, Forbes RA, and Verma A. Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. *J Biol Chem* 277: 23111–23115, 2002.
- Mabjeesh NJ, Post DE, Willard MT, Kaur B, Van Meir EG, Simons JW, and Zhong H. Geldanamycin induces degradation of hypoxia-inducible factor 1 α protein via the proteosome pathway in prostate cancer cells. *Cancer Res* 62: 2478–2482, 2002.
- Marber MS, Mestrlil R, Chi SH, and Sayen MR. Overexpression of the rat inducible 70-kDa stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. *J Clin Invest* 95: 1446–1456, 1995.
- Martin C, Yu AY, Jiang BH, Davis L, Kimberly D, Hohimer AR, and Semenza GL. Cardiac hypertrophy in chronically anemic fetal sheep: increased vascularization is associated with increased myocardial expression of vascular endothelial growth factor and hypoxia-inducible factor 1. *Am J Obstet Gynecol* 178: 527–534, 1998.
- Mestrlil R, Giordano FJ, Conde AG, and Dillmann WH. Adenovirus-mediated gene transfer of a heat shock protein 70 (HSP 70i) protects against simulated ischemia. *J Mol Cell Cardiol* 28: 2351–2358, 1996.
- Mizushima Y, Wang P, Jarrar D, Cioffi WG, Bland KI, and Chaudry IH. Preinduction of heat shock proteins protects cardiac and hepatic functions following trauma and hemorrhage. *Am J Physiol Regul Integr Comp Physiol* 278: R352–R359, 2000.
- Moore WM, Webber RK, Jerome GM, Tjoeng FS, Misko TP, and Currie MG. L-N^G-(1-iminoethyl)lysine: a selective inhibitor of inducible nitric oxide synthase. *J Med Chem* 37: 3886–3888, 1994.
- Natarajan K, Singh S, Burke TR Jr, Grunberger D, and Aggarwal BB. Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF- κ B. *Proc Natl Acad Sci USA* 93: 9090–9095, 1996.
- Pittet JF, Lu LN, Geiser T, Lee H, Matthey A, and Welch WJ. Stress preconditioning attenuates oxidative injury to the alveolar epithelium of the lung following haemorrhage in rats. *J Physiol* 538: 583–597, 2001.
- Rajnik M, Salkowski CA, Thomas KE, Li YY, Rollwagen FM, and Vogel SN. Induction of early inflammatory gene expression in a murine model of nonresuscitated, fixed-volume hemorrhage. *Shock* 17: 322–328, 2002.
- Semenza GL. HIF-1 and tumor progression, pathophysiology and therapeutics. *Trends Mol Med* 8: S62–S67, 2002.
- Sharp FR, Bergeron M, and Bernaudin M. Hypoxia-inducible factor in brain. *Adv Exp Med Biol* 502: 273–291, 2002.
- Shiono Y, Fugita Y, OKAS, and Yamagaki Y. ATPase inhibitors suppress actinomycin D-induced apoptosis in leukemia cells. *Anticancer Res* 22: 2907–2911, 2002.
- Song Y, Ao L, Raeburn CD, Calkins CM, Abraham E, Harken AH, and Meng X. A low level of TNF- α mediates hemorrhage-induced acute lung injury via p55 TNF receptor. *Am J Physiol Lung Cell Mol Physiol* 281: L677–L684, 2001.
- Stevenson MA and Calderwood SK. Members of the 70-kilodalton heat shock protein family contain a highly conserved calmodulin-binding domain. *Mol Cell Biol* 10: 1234–1238, 1990.
- Stojadinovic A, Kiang JG, Smallridge RC, Galloway RL, and Shea-Donohue T. Induction of heat-shock protein 72 protects against ischemia/reperfusion in rat small intestine. *Gastroenterology* 109: 505–515, 1995.
- Suzuki K, Sawa Y, Kaneda Y, Ichikawa H, Shirakura R, and Matsuda H. In vivo gene transfection with heat shock protein 70 enhances myocardial tolerance to ischemia-reperfusion injury in rat. *J Clin Invest* 99: 1645–1650, 1997.
- Warke VG, Nambiar MP, Krishnan S, Tenbrock K, Geller DA, Koritschoner NP, Atkins JL, Farber DL, and Tsokos GC. Transcriptional activation of the human inducible nitric-oxide synthase promoter by Kruppel-like factor 6. *J Biol Chem* 278: 14812–14819, 2003.
- Weiss YG, Maloyan A, Tazelaar J, Raj N, and Deutschman CS. Adenoviral transfer of HSP-70 into pulmonary epithelium ameliorates experimental acute respiratory distress syndrome. *J Clin Invest* 110: 801–806, 2002.
- Wong HR, Ryan M, Menendez IY, Denenberg A, and Wispe JR. Heat shock protein induction protects human respiratory epithelium against nitric oxide-mediate cytotoxicity. *Shock* 8: 213–218, 1997.
- Wong RH, Menendez IY, Ryan MA, Denenberg AG, and Wispe JR. Increased expression of heat shock protein-70 protects A549 cells against hypoxia. *Am J Physiol Lung Cell Mol Physiol* 275: L836–L841, 1998.