Glutamine’s protection against sepsis and lung injury is dependent on heat shock protein 70 expression

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Singleton KD, Wischmeyer PE. Glutamine’s protection against sepsis and lung injury is dependent on heat shock protein 70 expression. Am J Physiol Regul Integr Comp Physiol 292: R1839–R1845, 2007. First published January 18, 2007; doi:10.1152/ajpregu.00755.2006.—Glutamine (GLN) has been shown to protect against inflammatory injury and illness in experimental and clinical settings. The mechanism of this protection is unknown; however, laboratory and clinical trial data have indicated a relationship between GLN-mediated protection and enhanced heat shock protein 70 (HSP70) expression. The aim of this study was to examine the hypothesis that GLN’s beneficial effect on survival, tissue injury, and inflammatory response after inflammatory injury is dependent on HSP70 expression. Mice with a specific deletion of the HSP70 gene underwent cecal ligation and puncture (CLP)-induced sepsis and were treated with GLN (0.75 g/kg) or a saline placebo 1 h post-CLP. Lung tissue NF-κB activation, inflammatory cytokine response, and lung injury were assessed post-CLP. Survival was assessed for 5 days post-CLP. Our results indicate that GLN administration improved survival in Hsp70+/− mice vs. Hsp70+/- mice not receiving GLN; however, GLN exerted no survival benefit in Hsp70−/− mice. This was accompanied by a significant decrease in lung injury, attenuation of NF-κB activation, and proinflammatory cytokine expression in GLN-treated Hsp70+/− mice vs. Hsp70+/− mice not receiving GLN. In the Hsp70−/− mice, GLN’s attenuation of lung injury, NF-κB activation, and proinflammatory cytokine expression was lost. These results confirm our hypothesis that HSP70 expression is required for GLN’s effects on survival, tissue injury, and the inflammatory response after global inflammatory injury.

inflammation; NF-κB; Hsp70.1 and Hsp70.3 genes; cecal ligation and puncture

ONE OF THE MOST BASIC MECHANISMS of cellular protection involves the expression of a highly conserved family of proteins known as heat shock or heat stress proteins (HSPs). The 70-kDa heat shock protein (HSP70) family is a group of proteins that are critical for protein assembly, folding, and transport (8). The mouse is known to possess two inducible HSP70 genes, Hsp70.1 and Hsp70.3. These two genes are separated by 7 kb located on chromosome 17, show 99% homology, and are both responsible for the initiation of transcription to produce HSP70 protein (12, 18).

The expression of these important inducible proteins after injury or illness can induce “stress tolerance” and protect against a stress that would otherwise be lethal. Previous data have shown that the induction of the heat stress response, specifically HSP70, can provide significant protection against many forms of in vivo and in vitro injury, including ischemia and reperfusion (19, 20, 27), lung injury (42), and sepsis (17, 32, 43). Additionally, HSP expression has been shown to attenuate NF-κB activation and proinflammatory cytokine release in models of injury (7, 11, 28, 36, 46, 53), and this attenuation appears to correlate with improved survival from sepsis (2).

Glutamine (GLN), previously considered a nonessential amino acid, is now known to be essential during critical illness and injury (15). GLN levels are known to fall precipitously after major organ ischemia stress (15). Recent clinical data have revealed that GLN deficiency at admission to the intensive care unit is a strong predictor of hospital mortality (25). GLN has been shown to improve the outcomes in various states of clinical and experimental illness and surgical intervention (5, 9, 11, 14, 23, 34, 36, 40, 41, 48); however, the mechanism of this protection has yet to be elucidated (30). Finally, as with enhanced HSP70 expression, GLN administration has been shown to attenuate NF-κB activation and proinflammatory cytokine response in cellular and animal models of illness and injury (3, 11, 35, 49).

A significant body of preexisting literature has hypothesized a relationship between HSP70 expression and GLN’s protection in both in vitro and in vivo settings (1, 11, 16, 22, 31, 33, 36, 48, 50, 54). In the clinical setting, our group (54) has demonstrated that GLN can increase serum HSP levels in critically ill patients, which correlated with improved outcomes. However, a solid mechanistic link between HSP70 expression and GLN’s protective effect against illness and injury has yet to be demonstrated. This study utilized mice with specific deletions of the Hsp70.1 and Hsp70.3 genes to examine the hypothesis that GLN’s attenuation of the inflammatory response, tissue protection, and improved survival following sepsis is mediated by HSP70 expression.

MATERIALS AND METHODS

Animal preparation. The experiments described in this paper were performed in adherence to the National Institutes of Health guidelines for the use of experimental animals. Animal protocol was approved by the Animal Care and Use Committee of the University of Colorado Health Sciences Center. All experiments were also conducted and the animals cared for in accordance with the Helsinki Declaration guiding principles for research involving animals.

Animals and experimental protocol. Experiments were performed on genotypic male Hsp70−/− mice bearing the null alleles and wild-type controls (~25 g body wt). Targeted deletion of Hsp70.1 and Hsp70.3 in embryonic stem cells and generation of Hsp70−/− mice were carried out as previously described (13). Mice were purchased from the University of California at Davis Mutant Mouse Regional Resource Center (Davis, CA) and were maintained on a standard diet and water ad libitum. Animals were housed at constant temperature.

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with 10 and 14 h of light and dark exposure, respectively. Animals underwent an acclimatization period of at least 7 days before use in these experiments. Sepsis was induced by cecal ligation and puncture (CLP). After anesthesia with intraperitoneal injection of ketamine (40 mg/kg) and xylazine (6 mg/kg), a 1-cm incision was made in the abdominal wall and the cecum was carefully extruded. Approximately 25% of the cecum was then ligated just below the ileocecal valve to avoid bowel obstruction. The cecum was punctured twice by a sterile 22-gauge needle and was squeezed to extrude fecal material into the peritoneal cavity. The muscle and skin layers of the abdomen were then closed. All of the above manipulations were performed by the same surgeon to ensure consistency. This length of abdomen were then closed. All of the above manipulations were opened and the cecum manipulated; however, no CLP was performed, given fluid resuscitation subcutaneously (40 ml/kg) during the post-operative period. A sham group of animals for each of the knockouts and wild-type groups was also included in which the abdomen was opened and the cecum manipulated; however, no CLP was performed, and the abdomen was closed.

Survival studies. Four groups of animals [Hsp70−/− (n = 25), Hsp70+/+ (n = 40), Hsp70−/− + GLN (n = 25), and Hsp70+/+ + GLN (n = 45)] underwent CLP procedure as described above and received an intravenous injection via lateral tail vein of 0.75 g/kg GLN (given as alanyl-glutamine) or Ringer lactate 1 h after the CLP procedure. Four groups of sham animals, which received anesthesia, had an abdominal incision created, the cecum manipulated (without CLP), and the abdomen closed. Animals were observed at regular intervals for occurrence of mortality over the subsequent 5 days post-CLP. Moribund animals (defined as bradycardia to a heart rate <40 beats/min, severe lethargy, and unresponsive to painful stimulation) were killed with a lethal dose of ketamine-xylazine as defined by the University of Colorado Animal Care Committee. Animals were checked at regular intervals for pulse (via chest palpation) and moribund status.

Tissue collection. In a separate set of animals (n = 4/time point), Hsp70−/− and Hsp70+/+ mice given GLN or Ringer lactate underwent CLP, and lung tissue was collected at 1, 2, 6, and 24 h after CLP. All tissues were removed and immediately frozen in liquid nitrogen and stored at −80°C.

TNF-α and IL-6 detection. TNF-α and IL-6 concentrations from lung tissue were measured by ELISA. Lung tissue samples were removed from animals and weighed (so that each was consistently 250 g). Lung homogenate was then centrifuged for 15 min at 8,000 × g at 4°C. The supernatant was then aliquoted into microtiter wells and a 1:100 dilution was used for IL-6 and 1:10 dilution for TNF-α. The plates were developed with a chemiluminescent substrate and read in a UVP chemiluminescent darkroom system (UVP, Upland, CA). An internal positive control of TNF-α-activated nuclear extract was used as a reference point for maximal signal. “Cold competition” for this assay was performed by adding wild-type NF-κB competitor consensus binding element to wells. This reduced the signal level to near zero. A mutated NF-κB binding sequence had no effect on signal, ensuring signal specificity.

Lung histology. In a separate set of animals (n = 4/group) the aforementioned groups (Hsp70−/− + GLN, Hsp70+/+ + GLN, Hsp70−/− sham, Hsp70+/+ + GLN, and Hsp70+/+ + GLN) underwent CLP: 24 h after CLP, both lungs were harvested for assessment of lung pathology. The chest cavity was opened, and mainstem bronchi were loosely ligated. Tracheotomy was performed, and a 22-gauge cannula was inserted into the trachea. A second 22-gauge cannula was inserted into the left ventricle, and 10% buffered formalin was injected into the lung to inflate it. As a result, residual blood was removed from lung. All lungs were fixed under standardized levels of inflation (20 cmH2O pressure). Once the lungs were fully filled with formalin, the mainstem bronchi were ligated. Lungs were then removed and immediately fixed in 10% PBS-buffered formaldehyde for 24 h, rinsed in PBS for 1 h, and then stored in 70% ethanol. Lungs were embedded in paraffin and sectioned at 5 μm. For histology, the sections were deparaffinized in water and stained with hematoxylin and eosin. Digital images were captured in a blinded manner with a Qimaging Ratiga 1300 color digital camera and Olympus BX51 microscope (Olympus America, Melville, NY). Ten histological images were collected for each animal and evaluated blindly by a pathologist using a systematic scoring system (4).

Statistical analysis. Results are presented averages ± SE. Lung tissue cytokines, Western blot analysis, NF-κB activation, and histology were compared by Student’s t-test or ANOVA followed by Student-Newman Keuls’s test where applicable. Cox regression analysis with 95% confidence intervals (CIs) calculated for hazard ratios (HRs) was utilized to compare survival data. Results were considered significant at a P value < 0.05.

RESULTS

Effect of GLN on HSP70 expression in Hsp70.1 and Hsp70.3 knockout and wild-type mice. To ensure that the production of HSP70 protein was suppressed in Hsp70−/− + GLN mice, Western blot analysis was conducted to detect protein synthesis (Fig. 1A). All Hsp70−/− + GLN mice (including sham mice) expressed little to no HSP70, whereas the Hsp70+/+ + GLN mice expressed increased levels of HSP70 expression (P < 0.001 vs. Hsp70−/−) over time. The Hsp70+/+ sham + GLN group expressed a basal amount of HSP70, with a dramatic increase of expression after CLP-induced sepsis by 24 h (P < 0.01 vs. Hsp70+/+). A separate evaluation of the effects of GLN on HSP70 expression in the Hsp70+/+ mice was carried out. GLN administration to Hsp70+/+ mice led to enhanced HSP70 expression at all time points measured after CLP vs. Hsp70+/+ mice not receiving GLN (Fig. 1B) (P <
0.01 at each time point post-CLP). All results are representative of experiments carried out in triplicate.

GLN-mediated attenuation of pulmonary NF-κB after sepsis is dependent on HSP70 expression. To determine the effect of GLN supplementation on NF-κB activation following sepsis, nuclear extracts from Hsp70−/−/H11002, Hsp70−/−/H11001, Hsp70−/−/H11002/H11001, and Hsp70−/−/H11001/GLN (n = 4/group) lung tissues were assayed for specific nuclear p65 binding activity at 1, 2, 6, and 24 h sepsis by using a transcription factor assay kit as previously described. Figure 2 shows that sepsis-induced activation of NF-κB signaling was decreased significantly at all time points after sepsis in the Hsp70−/−/H11002/GLN mice (P < 0.01 vs. all groups). When GLN was administered to Hsp70−/− mice, no attenuation of NF-κB signaling was observed. This technique is similar to an EMSA; addition of wild-type NF-κB competitor duplex highly suppressed detection of the signal. Incubation with a mutated NF-κB p65 probe showed no effect, validating the signal specificity of these experiments. An internal positive control of TNF-α-activated nuclear extract was used as a reference point for maximal signal.

GLN-mediated attenuation of proinflammatory cytokine release after sepsis is dependent on HSP70 expression. To determine the effect of GLN supplementation on proinflammatory cytokine release after CLP-induced polymicrobial sepsis, we studied four groups of animals: Hsp70−/−/H11002, Hsp70−/−/H11001, Hsp70−/−/H11002/H11001, and Hsp70−/−/H11001/GLN (n = 4/group) at 1, 2, 6, and 24 h post-CLP procedure. Sham groups for each condition were also studied, and TNF-α expression and IL-6 expression were undetectable in these groups. GLN administration significantly decreased lung TNF-α expression at 1, 2, 6,
and 24 h and lung IL-6 expression at 6 and 24 h [post-CLP in Hsp70+/+ + GLN vs. all groups, P < 0.01 (Fig. 3, A and B)]. Hsp70+/+ without GLN had a significant attenuation in TNF-α at 6 and 24 h vs. Hsp70−/− and Hsp70+/+ + GLN (P < 0.05; Fig. 3A). Hsp70+/+ mice given GLN had an average lung IL-6 level at 6 h of 32.64 ± 8 pg/ml (mean ± SE), whereas Hsp70−/− mice given saline control had an average IL-6 level of 1.726 ± 28 pg/ml (means ± SE). When GLN was administered to Hsp70−/− mice, no attenuation of TNF-α or IL-6 expression occurred.

GLN’s protection against lung injury following sepsis is dependent on HSP70 expression. All tissues were examined and scored by a blinded pathologist. There were no pathological changes in lung tissue from sham-operated animals in either wild-type or knockout groups given GLN. Pulmonary changes consistent with lung injury and the acute respiratory distress syndrome, including neutrophil accumulation, septal thickening, and hyaline membrane formation (47) (Fig. 4), were seen in Hsp70−/− + GLN mice. Low-power sections (×10) demonstrated the aforementioned changes consistent with acute respiratory distress syndrome and lung injury after CLP in the Hsp70−/− mice given GLN; however, a significant decrease in lung injury was demonstrated in the Hsp70+/+ mice given GLN (P < 0.01 vs. Hsp70−/− + GLN). A higher resolution (×40) demonstrated the lack of lung consolidation, decreased cellularity, and reduced septal edema and absence of proteinaceous exudate in the Hsp70+/+ mice given GLN.

GLN improves survival in Hsp70+/+ mice but not in Hsp70−/− mice after CLP-induced sepsis. Animals were followed for 5 days for occurrence of mortality. All mortality occurred within the first 72 h after CLP. Figure 5 depicts survival from CLP in Hsp70−/− (n = 25), Hsp70+/+ (n = 40), Hsp70−/− + GLN (n = 25), and Hsp70+/+ + GLN (n = 45). In Hsp70+/+ + GLN mice, only 9 of 45 (20%) died within 3 days. Of 40 Hsp70+/+ mice given vehicle, 18 (45%) died by 3 days (HR, 2.64; 95% CI, 1.18 to 5.88; P < 0.01). No mortalities occurred in any of the sham groups. No survival benefit was observed after GLN administration to Hsp70−/− mice (HR, 6.63; 95% CI, 2.96 to 14.96; P < 0.001) or in the Hsp70−/− mice not receiving GLN (HR, 5.56; 95% CI, 2.46 to 12.58; P < 0.001). All groups compared with Hsp70+/+ mice given GLN.

DISCUSSION

This study demonstrates for the first time that GLN-mediated attenuation of the inflammatory response, tissue injury, and improved survival following sepsis is dependent on the expression of HSP70. These results confirm hypotheses from
extensive preexisting data, which have shown a relationship between enhanced HSP70 expression and GLN-mediated protection against stress and injury both in vivo and in vitro (1, 11, 16, 22, 31, 33, 36, 48, 50, 54). We have recently shown that GLN can enhance HSP70 in critically ill patients, and this was correlated with improved clinical outcome (54). The result of this study strengthens the possibility that a key component of GLN-mediated benefit on clinical outcome is enhanced HSP expression.

Previous data have shown that GLN deficiency leads to an impaired ability to express HSP70 in both laboratory and clinical settings (1, 6, 16, 22, 24, 29, 31, 33, 36, 44, 48, 54). Clinical critical illness is known to lead to significant GLN deficiency after 24–72 h (10, 15, 25). After stress or injury, GLN is released into the bloodstream in large amounts from muscle stores. This has been hypothesized to be due to increased utilization as a metabolic fuel by lymphocytes, enterocytes, and renal cells (15). It is possible that GLN is consumed by these cells not only for metabolic needs; GLN may also serve as a stress signal that organisms use to signal the activation of protective pathways, such as the HSP70 pathway. Furthermore, recent data have revealed that entering a critical care setting with a low GLN level predicts a greater risk of mortality (25). It is possible that the increased risk conferred by low plasma GLN levels is due to the organism’s or patient’s inability to express HSP70 appropriately. Furthermore, previous data have shown that lung tissue has an impaired ability to express HSP70 after sepsis (45). This could be because, following an inflammatory stress, the lung exhibits an impaired transport of GLN (26). This impaired transport, in conjunction with rapid depletion of the circulating GLN pool, may lead to an impaired cellular ability to synthesize HSP70. Our previous data indicate that GLN supplementation after sepsis can correct this impaired HSP70 expression (36). This effect may be
mediated by recent data from our laboratory (21, 36) showing that GLN can enhance activation, nuclear translocation, promoter binding, and phosphorylation of Heat Shock Factor-1. These data are also important because we have shown in the Hsp70\(^{-/-}\) mice that the ability to make and express stress-inducible HSP70 is vital to survival following sepsis (38).

GLN effects on HSP70 may play a vital role in the regulation of the inflammatory response (7, 11, 28, 36, 46, 53). Previous data have indicated that enhanced HSP70 expression can attenuate the inflammatory response via decreased NF-\(\kappa\)B activation and proinflammatory cytokine expression (7, 53). GLN administration has also been shown to have similar effects on NF-\(\kappa\)B activation and proinflammatory cytokine expression (3, 11, 35, 49, 51). Thus we hypothesized that GLN’s effect on the inflammatory response is due to enhanced HSP expression. The data from this study confirm that GLN’s effect on the inflammatory response following sepsis is dependent on HSP70 expression.

Limitations of this study include that we only examined lung tissue and lung injury in this model. This was done because a main cause of death following CLP is acute respiratory distress syndrome (46). Furthermore, recent data from a study in which HSP-70 was transferred via an adenoviral vector into pulmonary epithelium after initiation of sepsis via CLP suggest that the known deficit in HSP-70 expression may contribute to the formation of lung injury and that the correction of this deficit can improve survival and lung injury (46). However, we have previous data indicating that GLN can enhance HSP70 and HSP25 expression in the heart, colon, and kidney following shock (48). Further studies in these mice are needed to examine the effect of HSP70 gene deletion on the expression of other HSPs and on injury models that are more specific for other organ injury. Other potentially beneficial mechanisms that GLN may affect include improved gut barrier function (39) and improved myocardial function (52) following critical illness and injury. The dose of intravenous GLN for these experiments is based on previous in vitro data indicating that maximal HSP expression occurs at a plasma GLN concentration of between 4 and 8 mM (50). Another limitation of these data is that plasma GLN levels were not analyzed. Previous data from our laboratory indicated that a single 0.75-g/kg dose of intravenous GLN to a rat after endotoxemia results in a plasma GLN level of 3–7 mM (48). This dose of GLN was found to markedly enhance HSP expression in multiple organs (including the lung), attenuate proinflammatory cytokine release, and improve survival after endotoxemic shock (48).

These results indicate that the expression of inducible HSP70 is vital to GLN’s protection against overexpression of inflammatory mediators, lung injury, and mortality following sepsis. This is the first demonstration that the protective effect of GLN in sepsis is dependent on HSP70 expression. Further experiments in other in vivo models of injury and illness are needed to confirm this mechanism in other settings. This study is clinically relevant because to date there are no practical methods to enhance the expression of HSPs in critically ill patients. These results reveal that GLN’s ability to increase the expression of inducible HSP70 may have a therapeutic potential in illnesses mediated by inflammatory cytokine release. The use of GLN as a pharmacological agent to enhance HSP70 and attenuate systemic inflammation could be applied not only for the treatment of critical illness and injury but to other inflammatory diseases such as inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis. Finally, these data indicate that future clinical trials of GLN therapy are needed to examine tissue or cellular HSP70 expression as an end point to indicate efficacy of GLN treatment.

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GRANTS

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REFERENCES

25. Plumley AG. Glutamine’s protection against cellular injury is dependent on
hsp72-mediated protection by glutamine against oxidant injury in IEC18 cells. Proc
27. Novak F, Heyland DK, Avenell A, Drover JW, Su X. Glutamine supplementation in
serious illness: a systematic review of the evidence. Crit Care Med 30:
depletion impairs cellular stress response in human leukocytes. Br J Nutr 87, Suppl. 1:
S17–S21, 2002.
29. Oudemans-van Straaten HM, Bosman RJ, Treskès M, van der Spel HJ, Zandstra DF.
Plasma glutamine depletion and patient outcome in acute ICU admissions. Intensive Care Med 27:
30. Pan M, Fischer CP, Wasa M, Bode BP, Souba WW. Characterization of glutamine and
glutamate transport in rat lung plasma membrane vesicles. J Surg Res 69: