Glutamine induces heat shock protein and protects against endotoxin shock in the rat

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Divisions of ¹Clinical Pharmacology and ²Pediatrics, Department of Anesthesia and Critical Care, ³Department of Pediatrics, and ⁴The Martin Boyer Laboratories, Department of Medicine, The University of Chicago, Chicago, Illinois 60637

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Wischmeyer, Paul E., Madelyn Kahana, Rachel Wolfson, Hongyu Ren, Mark M. Musch, and Eugene B. Chang. Glutamine induces heat shock protein and protects against endotoxin shock in the rat. J Appl Physiol 90: 2403–2410, 2001.—Enhanced expression of heat shock protein (HSP) has been shown to be protective against laboratory models of septic shock. Induction of HSPs to improve outcome in human disease has not been exploited because laboratory induction agents are themselves toxic and not clinically relevant. In this study, we demonstrate that a single dose of intravenous glutamine causes a rapid and significant increase in HSP25 and HSP72 expression in multiple organs of the unstressed Sprague-Dawley rat. With the utilization of a fluid-resuscitated rat model of endotoxemia, mortality was dramatically reduced by glutamine administration concomitant with the endotoxin injury. Endotoxin-treated animals given glutamine exhibited dramatic increases in tissue HSP expression and marked reduction of end-organ damage. These data suggest glutamine may protect against mortality and attenuate end-organ injury in endotoxemic shock via enhanced HSP expression. Furthermore, glutamine confers protection when administered at the initiation of sepsis, rather than as pretreatment. Thus glutamine appears to be a clinically viable enhancer of HSP expression and may prove beneficial in the therapy of sepsis and sepsis-induced organ injury.

Keywords: amino acid; organ injury; sepsis; animal model; stress proteins

SEPSIS, ISCHEMIA-REPERFUSION injury, and multiorgan dysfunction are responsible for significant human morbidity and mortality despite the use of antibiotics, vasoactive agents, and anti-cytokine therapies (1, 5, 6, 9, 13). These injuries appear to be mediated in part by the response of the immune system to an infectious agent, ischemic insult, or other severe stress (5, 6, 23). A potential approach to this problem is to make use of a natural defense mechanism called “the stress response,” which is characterized by a transient down-regulated synthesis of nonvital cellular proteins and increased production of endogenous protective factors known as heat shock proteins (HSPs) that act to allow proper refolding of proteins denatured by the incipient injury. Two members of the HSP family thought to play a vital role in cellular protection are HSP72 and HSP25/27 (HSP25 in rats and HSP27 in humans) (3, 10, 26). Previous studies in laboratory models of sepsis, ischemia-reperfusion, and acute lung injury demonstrate that these injuries can be significantly attenuated or prevented via enhanced expression of HSP72 and/or HSP25/27 (3, 10, 23, 26). Additionally, HSP expression has been shown to attenuate plasma concentrations of interleukin-1β and tumor necrosis factor-α in both in vitro and in vivo models (8, 25, 27). This attenuation appears to correlate with improved survival from a septic insult (8). The induction of HSPs to improve outcome in human disease has not been exploited because induction agents utilized in the laboratory are themselves toxic and not clinically relevant.

Our laboratory has previously shown that glutamine (Gln), a nonessential amino acid, enhances cell survival in vitro against a variety of stressful stimuli through the induction of HSP72 (20, 31). Traditionally considered a nonessential amino acid, Gln now appears to be a conditionally essential nutrient during serious injury or illness (17). In good health, Gln is the most abundant amino acid in plasma and skeletal muscle, but circulating and tissue concentrations fall precipitously after injury, surgery, or infection. (2, 22). Extensive research in animal models of sepsis and other severe injury states demonstrates that Gln supplementation improves survival, enhances immune function, decreases bacteremia, enhances gut barrier function, and inhibits gut mucosal atrophy (4, 21, 29, 33, 34). Clinical trials in human subjects have demonstrated that Gln decreases infectious complications, shortens hospital stay, and decreases hospital costs in a number of patient populations (12, 14–16, 34).

On the basis of our previous findings that Gln was a potent inducer of HSP in vitro (31), we hypothesized that Gln would enhance HSP expression in an unstressed in vivo rat model. The findings of this study confirm that Gln is indeed a potent in vivo enhancer of
HSP (both HSP 25 and 72) in multiple tissues of the unstressed rat. On the basis of this finding, we further hypothesized that Glu would be protective against a rat model of lipopolysaccharide (LPS)-induced sepsis. The results of this study confirm that Glu is indeed protective against endotoxin-induced shock injury and that the mechanism of this protection may involve enhanced HSP expression.

METHODS

Animals. This project was approved by the Animal Care and Use Committee of the University of Chicago. Male Sprague-Dawley rats (250–350 g) were purchased from Harlan (Madison, WI). All animals were housed at an ambient temperature of 22°C, placed on a 12:12-light-dark cycle, and given water ad libitum. Experiments were conducted between 0700 and 1700. For all manipulations, the animals were first induced with isoflurane gas anesthesia and then given intraperitoneal ketamine (85 mg/kg)-xylazine (12 mg/kg). Induction of anesthesia was performed with isoflurane preinduction to minimize stress to the animal.

Glu administration. Glu (Ajinomoto, Raleigh, NC) was prepared as a 3% solution dissolved in lactated Ringer (LR) immediately before use. Glu solutions were filtered with a 0.45-μm filter before administration. Glu solution or LR control was administered via the lateral tail vein of anesthetized rats by using a syringe pump at a rate of 0.5 ml/min (per Animal Care and Use Committee recommendation). Glu doses ranged from 0.15 to 0.75 g/kg. Time of administration varied from 10 to 20 min depending on weight of rat and dose of Glu given.

HSP detection. After completion of treatment and/or injury regimens, multiple tissues were harvested from killed rats and then immediately frozen in liquid nitrogen before processing. Tissues to be processed were then placed in homogenization buffer (10 mM Tris, 5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and aprotonin, and 50 U/ml DNase and RNase, pH 7.4). Tissues were mechanically disrupted after placement in homogenization buffer. Samples were analyzed by SDS-PAGE and Western blotting performed using 1× Towbin buffer (25 mM Tris and 192 mM glycine with 15% vol/vol methanol, pH 8.8) in a standard fashion (19). Blots were blocked with 5% Bovine (5% wt/vol; nonfat dry milk in phosphate-buffered saline with 0.2% vol/vol Nonidet P-40). For HSP72 detection, blots were then incubated with a specific mouse monoclonal antibody, C92 (StressGen, Victoria, BC, Canada). Blots were then washed, incubated with horseradish peroxidase-conjugated secondary anti-mouse antibody, and developed using an enhanced chemiluminescence system. For HSC73 measurements, the aforementioned Western blot technique was applied utilizing anti-HSC73, a specific rat monoclonal antibody to constitutive HSC73 (1B5; StressGen). HSP25 was detected using the aforementioned Western blot technique by utilizing a rabbit polyclonal anti-HSP25 (StressGen).

Induction of sepsis via LPS injection. Sepsis was induced via intravenous injection of Escherichia coli endotoxin (serotype O55:B5, Sigma Chemical, St. Louis, MO) dissolved in pyrogen-free saline. Survival studies were performed as follows. On the day of experiment, rats were anesthetized, weighed, and randomized in a blinded fashion to receive either 0.75 g/kg Glu (n = 6) or LR (n = 6) infusion as previously described. Immediately after the Glu infusion, 5 mg/kg of LPS were administered via the lateral tail vein. To approximate the clinical situation, animals were resuscitated such that both groups of animals received a total of 25 ml/kg of crystalloid fluid concomitantly with the LPS injury. This intervention has been shown to convert classic LPS-induced hypodynamic shock to a hyperdynamic shock that more closely resembles human sepsis (9). All rats in both groups were allowed to awaken from anesthesia, and survival was monitored in the awake rat periodically for 48 h. During this time, food was placed in the bottom of cage to facilitate the animals’ access to nutrition. In a second group of animals, sepsis was induced as described above and rats randomized to either Glu (n = 6) or LR alone (n = 6). Rats were then killed with pentobarbital sodium (120 mg/kg) 6 h after Glu or LPS administration. Organs (heart, lung, liver, kidney, ileum, and proximal colon) were then removed, fixed in phosphate-buffered formalin, and embedded in paraffin. Histological sections were then stained with hematoxylin and eosin and reviewed by staff pathologists who had no knowledge of the treatment groups from which the specimens were obtained.

Plasma Glu and metabolite levels. Animals were divided into four groups: 1) Glu (0.75 g/kg) (n = 3); 2) LR control solution (n = 3); 3) Glu (0.75 g/kg) + LPS (5 mg/kg) (n = 3); 4) LR + LPS (5 mg/kg) (n = 3). Glu or LR was administered as previously described. LPS was given immediately after Glu or LR infusion. Blood samples were then taken via a previously placed internal jugular catheter at 0, 15, 30, 60, and 240 min after infusion. Plasma Glu and glutamate levels were determined via enzymatic degradation as described by Lund (18). Plasma ammonia levels were analyzed at 240 min after Glu (0.75 g/kg) (n = 4) or LR (n = 4) infusion by the University of Chicago clinical laboratories.

Statistical analysis. Results are presented as means ± SE. Fisher’s exact test was utilized to compare survival rates between two groups of animals. HSP densitometry measurements were compared by using ANOVA and the Student’s t-test where applicable. Results were considered significant at a value of P < 0.05.

RESULTS

Glu enhances HSP expression in the unstressed rat. To examine the effect of Glu on HSP expression in the unstressed rat, a range of Glu doses from 0 to 0.75 g/kg was given as an intravenous infusion at 0.5 ml/min (average infusion time 12–15 min) via the tail vein. Multiple tissues were harvested after Glu infusion, including heart, lung, liver, kidney, colon, and ileum. Glu infusion significantly increased inducible HSP72 and HSP25 protein expression two- to threefold, appearing in <6 h in heart and lung (Figs. 1 and 2). Small increases were also seen in colon tissue after 1 h (Fig. 2). In contrast, no changes were seen in the expression of constitutive HSC73 (Fig. 2), demonstrating that Glu exerts a specific effect on inducible stress proteins, rather than increasing overall protein synthesis. No significant increases were observed in the heart, kidney, or small intestine for either HSP25 or HSP72. Time-response curves were also performed at 0.75 g/kg Glu for HSP25 and HSP72. These experiments examined multiple time points from 0 to 72 h after Glu infusion in the heart, lung, colon, liver, kidney, and ileum. There was a significant variation in rate of induction and persistence of protein among the organs tested. Notable findings include rapid induction of HSP25 in the lung (<1 h) (Fig. 2), peaking at ~6 h postinfusion, and persistence of protein to 48–72 h. In the heart,
HSP25 expression was upregulated more slowly with a peak at 12–24 h with a rapid decay after 48 h (Fig. 2). In the colon, induction of HSP25 was less, but small increases in HSP72 and HSP25 was observed after Gln infusion (Fig. 2). Again, no changes were seen in the expression of the constitutive HSC73 (Fig. 2). Furthermore, no increases were seen in HSP25 or HSP72 expression in the liver, kidney, or ileum during the time-response experiments (Fig. 2).

Gln infusion protects against lethal endotoxemia and attenuates end-organ injury. To examine the effect of Gln on protection from lethal shock, an E. coli LPS model was utilized. A dose of 5 mg/kg of E. coli LPS was administered intravenously concomitant with either Gln (0.75 g/kg) or a LR control. All rats received 25 ml/kg of intravenous LR fluid resuscitation at the time of LPS administration. The survival of the rats was then assessed over 48 h (Fig. 3). In the control rats (n = 6), survival was 33% at the end of 48 h, whereas the Gln-treated group (n = 6) demonstrated 100% survival (P < 0.03 via Fisher’s exact test). All mortality occurred in the first 18 h after LPS injury. In a separate set of rats, Gln (0.75 g/kg) (n = 6) or LR control (n = 6) was given concomitantly with the LPS injury (5 mg/kg iv). Tissue samples from the heart, lung, liver, kidney, colon, and ileum were then harvested for histological examination. Hematoxylin- and eosin-stained tissues were reviewed by blinded pathologists revealed significantly less evidence of endotoxin-induced acute lung injury in the Gln-treated animals vs. LR control animals (P < 0.05) (Fig. 4). Examination of the ileum revealed marked amelioration of endotoxin-induced intestinal damage in Gln-treated vs. LR control animals (P < 0.05) (Fig. 4). The histological examination of the remaining organs did not reveal significant sepsis-induced damage and there was no significant difference in appearance between the two groups.

Gln enhances HSP expression in LPS-stressed rats. In order for the effect of Gln on HSP expression to be protective against endotoxemia, tissue levels of the protective HSPs (i.e., HSP27 and HSP72) must be rapidly unregulated in the injured animal. To study the effect of Gln on HSP expression in the LPS-stressed rat, we examined two groups of LPS-injured animals utilizing the aforementioned 5 mg/kg LPS dose given concomitantly with either Gln (0.75 g/kg) (n = 5) or an LR-infused controls (n = 6). Animals were killed 6 h after LPS injury, and multiple tissues were harvested and processed for Western blot evaluation of HSP content. Gln treatment significantly enhanced expression of inducible HSP72 in heart tissue (P < 0.005), lung tissue (P < 0.001), colon tissue (P < 0.001), and kidney

Fig. 1. Dose-response effect of glutamine (Gln) on heat shock protein (HSP) 72 expression in heart and lung tissue in unstressed rats. Rats were treated with varying concentrations of Gln given over 12–15 min. Tissues were then harvested 4 h after Gln administration, and HSP72 expression was analyzed by Western blot as described in METHODS. Image shown is representative of those obtained in 4 different experiments. Graph represents percent increase in HSP72 over control. All Gln doses [0.15 g/kg (open bars), 0.45 g/kg (gray bars), and 0.75 g/kg (black bars)] significantly increased HSP72 concentrations (measured by densitometry) over control.

*P < 0.001, **P < 0.01, ***P < 0.05 compared with control by ANOVA.

Fig. 2. Time course effect of Gln on HSP25 expression in multiple tissues of rat. Rats were treated with 0.75 g/kg Gln given over 12–15 min. Tissues were harvested at various time points after Gln infusion. HSP25 and constitutive HSC73 expressions were analyzed by Western blot as described in METHODS. Image shown is representative of those obtained in 3 experiments.
No changes were observed in constitutive HSP73 in any organs tested. HSP25 expression was significantly enhanced by Gln treatment in heart tissue ($P < 0.016$), liver tissue ($P < 0.007$), and colon tissue ($P < 0.05$) (Fig. 6). Significant changes in HSP25 or HSP72 were not observed in the other organs tested.

**Gln infusion increases plasma Gln levels.** Gln (0.75 g/kg) infusion rapidly increased Gln plasma levels in the presence and absence of LPS injury (Fig. 7). Plasma Gln levels were significantly increased at 15, 30, and 60 min after Gln infusion vs. the LR control ($P < 0.05$). This increase occurred in the presence and the absence of LPS injury. Gln levels at 15 and 30 min after infusion were slightly lower in the Gln-treated, LPS-injured animals vs. animals not receiving LPS, although this difference was not statistically significant. Plasma glutamate was not significantly increased in the Gln-treated animals at any of the time points tested (Fig. 7). Plasma ammonia was not significantly increased by Gln infusion at 240 min postinfusion (Table 1).

**DISCUSSION**

The results of this study provide conclusive proof that Gln is a potent, clinically relevant enhancer of tissue ($P < 0.000004$) (Fig. 5).
HSP expression in both the stressed and unstressed animal. Unlike previous inducers of the heat shock response, Gln exerts its effects on HSP expression without any detectable harmful effects on the organism. Furthermore, this study demonstrates that a single dose of intravenous Gln given concomitantly with an endotoxin injury can prevent mortality and markedly reduce end-organ histological damage. The ability of Gln to protect against an endotoxin injury when given at the onset of injury, rather than as pretreatment, is vital to the potential application of Gln to human illness, because protective agents that must be given as pretreatment are limited in their clinical utility. Because many patients presenting in septic shock often do not receive therapy until they severely ill, the next question to be addressed is whether Gln given after a septic injury can afford protection to the host. LPS models are not the optimal model with which to address this question because the initial injury and rise in cytokines occurs in <2 h. To
address this question of the efficacy of Gln after a septic injury, we are planning to study a cecal ligation and puncture model of septic shock because this model has a slower onset and appears to be a more clinically relevant method with which to address this important question. Previous studies in rat models of HSP induction to protect against septic shock have utilized sodium arsenite or heat as an inducer for the stress response. Sodium arsenite is known to be quite toxic, with a previous experiment showing a 20% mortality rate from the arsenite alone (24). HSP expression has

Table 1. Effect of Gln administration on ammonia release

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<th>LR</th>
<th>Glutamine</th>
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<tr>
<td>Preinfusion</td>
<td>40.35 ± 3.8</td>
<td>49.6 ± 10.9</td>
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<tr>
<td>Postinfusion</td>
<td>38.59 ± 3.7</td>
<td>55.6 ± 13.0</td>
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Values are means ± SE given in μmol/l. Rats were given glutamine (Gln; 0.75 g/kg) (n = 4) or lactated Ringer (LR) alone (n = 4), and plasma ammonia was measured 4 h postinfusion.
also been induced by measures that increase core body temperature (8). However, these measures are clinically impractical, because they would be poorly tolerated by patients and would have detrimental effects on many cellular functions (10). In contrast, our findings indicate Gln is a nontoxic agent that induces organ-specific HSP expression in the stressed and unstressed rat, particularly in vital organs such as the lung, kidney, heart, and colon. This enhancement occurs at doses of Gln that appear to be safe for human administration. Normal human plasma Gln levels are ~0.4–0.7 mmol/l, and baseline Gln values in our animals closely approximated observed human normals. Previous human studies of intravenous Gln administration reveal no significant elevations of its toxic metabolites, ammonia and glutamate, in patients who do not have significant renal or hepatic dysfunction (11). We have administered Gln as a continuous infusion (0.57 g·kg⁻¹·day⁻¹) to critically ill burn patients with significant renal and liver dysfunction without significant elevations of ammonia, with the exception of patients with complete liver and/or renal failure, who are intolerant of any significant protein load. The dose used in this study (0.15–0.75 g·kg⁻¹·dose⁻¹) is given as a single short infusion, which has not yet been tested in humans. We are planning to test single-dose infusions of Gln in humans in the near future. Before this trial, we administered Gln to animals at the dose used in this study without any notable toxicity (i.e., seizures, abnormal behavior, decreased feeding, weight loss) up to a week after Gln infusion (Wischmeyer, unpublished observations). Furthermore, we demonstrate that Gln administration at 0.75 g/kg does not lead to significant increases in the accumulation of its toxic metabolites glutamate and ammonia. This was true in the presence and absence of LPS-injury.

Gln, which is now thought to be essential in times of stress, has been shown to be vital to the function of multiple organs, particularly during injury or illness. It is known that Gln is translocated out of lung and skeletal muscle in response to many forms of injury and stress (17). Increased Gln utilization by the gut, inflammatory cells, and the kidney has also been shown during serious injury (19, 28, 30). Explanations for this phenomenon have focused on the need for metabolic substrate by stressed organs (20, 28, 30) and the role of Gln as a precursor for glutathione synthesis (11). It is possible that protective effects of Gln against an endotoxic insult may be mediated by one or both of these effects. However, our findings define a new and potentially vital mechanism by which stress-induced Gln translocation from lung and skeletal muscle stores may protect an organism against an injury such as sepsis. Gln may serve as a trigger for the induction of the stress response and protect the stressed host by actions such as organ-specific upregulation of HSP.

The plasma levels of Gln obtained after Gln infusion (0.75 g/kg) parallel closely the Gln doses required for maximal HSP expression and cellular protection in our in vitro research (31). Because Gln levels are significantly reduced after injury and illness (2, 22), this depletion may result in impairment in the body's ability to respond to ongoing illness and injury.

Significant increases in HSP expression can be seen in multiple tissues, with the exception of the ileum. This is notable because Gln appears to afford marked protection of this tissue on histological examination. One potential mechanism to explain this protection is previous research indicates that manipulation of stress protein (HSP) expression may attenuate the expression of the proinflammatory cytokines (8) and improve survival from a septic injury. Multiple studies have demonstrated that enhanced HSP expression is associated with significant attenuation of the proinflammatory cytokines tumor necrosis factor-α and interleukin-1β (7, 8, 32). It is possible that attenuation of Gln of small bowel injury is secondary to decreases in proinflammatory cytokine-related injury. We are currently completing a set of experiments to examine the possibility that Gln may also attenuate proinflammatory cytokine expression and whether this potential effect may be mediated via an HSP-related pathway.

In conclusion, Gln has previously been given in many forms of human illness and injury without any notable toxicity (14–16, 34). Furthermore, many of these studies have shown clinical benefit of Gln administration, particularly in patients at risk for infectious or septic morbidity and mortality (14–16, 34). This study provides potential mechanism to explain the benefit seen in these clinical trials and begins to clarify potential ambiguities in the dosing, mechanism, and appropriate patient population for the use of Gln. The results of this study imply that Gln could be utilized to induce the protective stress response and prevent end-organ injury in situations when a major clinical stress is anticipated. Specifically, before major surgical procedures (i.e., cardiac, vascular, transplantation) and at the onset of severe insults such as trauma and major burns.

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REFERENCES