Alanyl-Glutamine Dipeptide Does Not Affect Hemodynamics despite a Greater Increase in Myocardial Heat Shock Protein 72 Immunoreactivity in Endotoxemic Sheep

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ABSTRACT The possible beneficial effect of supplemental glutamine (Gln) in critically ill patients has been suggested to be mediated by the induction of the cytoprotective heat shock proteins (HSP)32 and HSP72. There is evidence that HSP72 and HSP32 have opposite effects on the hemodynamic situation during endotoxemia. Therefore, the effect of Gln supplementation on the cardiovascular system is not clear. We investigated the effect of alanyl-Gln (Ala-Gln) dipeptide on cardiovascular function in healthy and endotoxemic sheep. Ten sheep catheterized for chronic studies received Ala-Gln 700 mg/(kg · d) [equal to 470 mg/(kg · d)Gln] on 4 consecutive days, and 10 sheep received NaCl (9 g/L) as the control solution. On d 4, four sheep of each group were killed and myocardial samples were taken for immunohistochemistry. The remaining sheep received a continuous infusion of endotoxin [Salmonella typhosa, 10 ng/(kg · min)]. Hemodynamic parameters were measured before application of Ala-Gln or the control solution, and during endotoxemia. Myocardial HSP72 immunoreactivity was determined by immunohistochemistry. After 24 h of endotoxemia, the sheep exhibited a hyperdynamic circulation. No difference was found in the hemodynamic parameters between treatment and control group. Ala-Gln treated sheep had a greater increase in myocardial HSP72 immunoreactivity compared with controls after (P < 0.05) but not before endotoxemia. In summary, Ala-Gln increased HSP72 immunoreactivity after endotoxemia, but did not alter hemodynamic parameters. Thus, Ala-Gln supplementation does not seem to aggravate the hyperdynamic circulation in endotoxemic shock. J. Nutr. 131: 1433–1437, 2001.

KEY WORDS: cardiac myocytes, endotoxemia, glutamine, heat shock protein 72, sheep

Glutamine (Gln) is a conditionally essential amino acid. During catabolic illnesses such as severe sepsis, Gln utilization increases substantially, especially in the gastrointestinal tract, resulting in decreased plasma levels and intracellular concentration, despite compensatory release of Gln from muscles (Furst 1983, Noguchi et al. 1997). Gln becomes essential under these circumstances (Lacey and Wilmore 1990, Souba et al. 1990). In its native form, Gln is relatively unstable and poorly soluble for addition to existing preparations for parenteral nutrition. The synthetic Gln-containing dipeptide, alanyl-Gln (Ala-Gln), is stable, highly soluble and readily hydrolyzed into equimolar amount of Ala and Gln after intravenous administration. Therefore, Ala-Gln is often used as a suitable source of free Gln in parenteral nutrition (Furst et al. 1990).

Glutamine seems to have beneficial effects in critical illness. In one study of critically ill patients, Gln-supplemented total parenteral nutrition reduced the length of stay in the intensive care unit and improved long-term survival (Griffiths et al. 1997). In patients with operative trauma, parenteral supplementation of Gln also shortened hospitalization and improved nitrogen economy (Schulzki et al. 1999). The incidences of pneumonia, sepsis and bacteremia were significantly lower in severely injured patients receiving Gln-enriched enteral nutrition compared with those receiving conventional nutrition (Houdijk et al. 1998).

It has been suggested that the protective effects of Gln are mediated at least in part by the induction of heat shock proteins (HSP) (Chow and Zhang 1998, Musch et al. 1998, Wischmeyer et al. 1997). Gln has been shown to enhance the synthesis of the inducible isoform of HSP70, HSP72, in intestinal cells (Musch et al. 1998, Wischmeyer et al. 1997) and in other cell types (Cai et al. 1991, Nissim et al. 1993, Sanders and Kon 1991) and of HSP32 (Tamaki et al. 1999), which is the inducible form of heme oxygenase (HO-1). But because HSP72 and HSP32 are thought to have different effects on hemodynamics, it is not clear which effect Gln supplementation exerts on the cardiovascular system. Induction of HSP72 restored blood pressure in hyperdynamic endotoxemia in different animal models (Hauser et al. 1996, Klosterhalfen et al. 1997). The inducible isoform HO-1, the heat shock protein (HSP32), also is hypothesized to have an influence on hemodynamic changes during endotoxemia, probably by its product...
carbon monoxide (CO). CO activates soluble guanylate cyclase, resulting in elevated intracellular cGMP and leading to smooth muscle relaxation and vasodilation (Maines 1997). In septic shock, the induction of HO-1 and increased production of CO are thought to contribute to the hyperdynamic circulation (Yet et al. 1997). Thus, an additional increase in CO due to the induction of HO-1 by Gln might lead to further vasodilation and aggravate the hyperdynamic situation. We investigated the effects of Gln supplementation on HSP expression and cardiovascular function in a model of hyperdynamic endotoxemic sheep. HSP72 served as a marker of heat shock response.

MATERIALS AND METHODS

After approval by the Government Animal Research Committee, the experiment was carried out in adult chronically instrumented ewes (n = 20; 41 ± 3 kg) (Reckert, Nordwalde, Germany).

Preparation. Under ketamine anesthesia (10–15 mg/kg Ketanest 50, Parke-Davis GmbH Berlin, Freiburg, Germany), the sheep were catheterized with an indwelling pulmonary artery catheter inserted percutaneously through an introducer sheath via the jugular vein (8.5F Catheter Introducer Set, pvb Medizintechnik, Kirchseeon, Germany; 7.5F, Edwards Swan Ganz, Edwards Critical Care Division, Irvine, CA) and with a femoral-arterial catheter (18-gauge, Leader Cath, Vygon, Aachen, Germany). During instrumentation, anesthesia was maintained with repeated intravenous injections of propofol (2 mg/kg, Disoprivan, Zeneca, Schwestingen, Germany), when necessary. After 24 h of recovery, the catheters were connected to pressure transducers (DTEX Druckwandler Kit, Ohmeda GmbH and KG, Erlangen, Germany) and a monitor (Hellige Servomed, Hellige, Freiburg, Germany). A continuous intravenous infusion of Ringer’s lactate [2 mL/(kg·h)] was started. All measurements were performed in awake sheep, which were held in metabolic cages with free access to water and food (hay, oats and concentrated feed) throughout the experiment.

Experimental protocol. After baseline measurements (BL1), 10 sheep received 700 mg/kg Ala-Gln (Dipeptiven, Fresenius, Germany) intravenously over 30 min (equal to 470 mg/kg Gln) in 3.5 mL/kg Dipeptiven solution. Dipeptiven, a commercially available solution, consists of 20 mg Ala-Gln (8.2 g l-alanine, 13.45 g l-glutamine) dissolved in 100 mL H2O. Ten sheep received 3.5 mL/kg NaCl (9 g/L), intravenously over 30 min. Ala-Gln and NaCl were given on 4 consecutive days. On d 4 after baseline measurements (BL2), 1 h after Ala-Gln or NaCl administration, four of the sheep treated with Ala-Gln and four who received NaCl were anesthetized with propofol (4 mg/kg Disoprivan, Zeneca, Schwestingen, Germany) and killed with a lethal dose of potassium chloride. The other 12 sheep received a continuous intravenous infusion of Salmonella typhosa endotoxin [10 ng/(kg·min), Sigma Chemical, Deisenhofen, Germany] for the next 24 h. The dosage of endotoxin was chosen on the basis of several previous studies in which this dosage resulted in a hyperdynamic circulation in our animal model (Bone et al. 1993, Bode et al. 1996, Meyer et al. 1996). The baseline endogenous Ringer’s lactate [2 mL/(kg·h)] was increased according to central venous pressure, to maintain adequate intravascular volume. Cardio-pulmonary data were obtained after 4, 8, 12 and 24 h of endotoxemia (Gravenstein et al. 1997). Cardiac output measurements were performed by thermodilution technique (Shoemaker and Parsa 1995), using the average of three injections of cold (2–5°C) saline solution (9520 A cardiac output computer, Edwards Laboratories, Irvine, CA). At the end of the experiment all sheep were anesthetized with propofol (4 mg/kg) and killed with a lethal dose of potassium chloride.

Histology and immunohistochemistry. Frozen 5-μm cross sections of the left ventricle were mounted on silan-coated glass slides and fixed in 4°C cold acetone for 90 s. The monoclonal mouse antibody to the inducible form of the 70-kDa HSP family, HSP72 (SPA-810, IgG1 isotype, StressGen Biotechnologies, Victoria, Canada) was applied in a humidified chamber for 45 min at room temperature at 1:400 dilution in 6 g/L bovine serum albumin, fol-

lowed by a rabbit anti-mouse bridging antibody (1:30 in PBS, 30 min at room temperature; Dako, Hamburg, Germany) and a monoclonal mouse alkaline phosphatase anti-rabbit immunoglobulin (Ig) complex (IgG1 isotype; 1:100 in RPMI, 60 min at room temperature; Dako). The enzyme reaction was developed for 25 min at room temperature in a freshly prepared fuchsin solution containing naph-thol-bis-phosphate and levamisole. Finally, the sections were counterstained with hematoxylin and mounted with Kayser’s glycerine gelatin. Omission of the primary antibody served as negative control.

We compared immunohistochemical staining to look for differences in HSP72 expression. To quantify the degree of HSP72 staining, a grading system of 0–3 was used as follows: 0, no staining; 1 and 2, increasing degrees of intermediate staining; and 3, extensive staining. The slides were evaluated microscopically with a magnification of X250 by two independent observers unaware of the experimental protocol.

Data analysis. Data on hemodynamics are expressed as means ± (SEM) of n observations, where n represents the number of sheep (n = 4 for each nonendotoxemic group; n = 6 for each endotoxemic group). Statistical analysis of the hemodynamic data measured before and during endotoxemia was performed using two-way ANOVA for repeated measurements followed by a Student-Newman-Keuls test for multiple comparisons. Data for grading of immunoreactivity of HSP72 are given as median and 25th and 75th percentiles. Differences in the graded immunoreactivity of HSP72 between healthy and endotoxemic subjects were analyzed statistically using the nonparametric Mann-Whitney U test, two-tailed and one-tailed, respectively. Because there is evidence for Gln increasing the expression only of endotoxemic controls. Administration of Ala-Gln alone in healthy sheep did not result in an increase in HSP72. Despite the higher increase in HSP72 immunoreactivity during endotoxemia in Ala-Gln–treated sheep, no differences in hemody-
namic parameters were found compared with endotoxemic controls. These data must be interpreted with caution, i.e., the study was done in only a small number of sheep. The dosage of Gln chosen for this study was found to be most beneficial in humans (Weingartmann et al. 1996). But because only one dosage of Ala-Gln was investigated, the lack of a hemodynamic response to Ala-Gln supplementation in this specific experimental setting does not exclude a hemodynamic effect of Ala-Gln in a different clinical setting.

Similar to our finding of induction of HSP72 by Gln treatment only after endotoxemia, previous studies have also demonstrated that Gln treatment increased HSP72 induction only after additional exposure to oxidative stress; no induction was found with Gln treatment alone (Cai et al. 1991, Nissim et al. 1993, Sanders and Kon 1991). In contrast to these findings, an induction of HSP72 by Gln without prior stress exposure was reported in different cell types in vitro (Wischmeyer et al. 1997) and in vivo (Kojima et al. 1998) studies. The reasons for the different findings remains to be elucidated.

Endotoxemia increased HSP72 immunoreactivity in cardiomyocytes in both Ala-Gln–treated sheep and controls. Whether HSP72 is induced during sepsis without prior Gln treatment is controversial. The induction of HSP72 and HSP70 mRNA by endotoxin has been demonstrated previously in rodents (Flohe et al. 1999, Fujiwara et al. 1999, Ofenstein et al. 2000). In other studies, no increased expression of HSP72 protein was found during sepsis (Chen et al. 1999, Weiss et al. 2000). A possible explanation for these apparently contradictory findings might be the severity of sepsis induced in the different models. Schroeder and colleagues (1999) demonstrated ex vivo an impaired inducibility of HSP70 in lymphocytes of patients with severe sepsis compared with nonseptic patients. Patients with clinical signs of recovery from severe sepsis showed an increase in HSP70 expression. No induction of HSP70 during sepsis was found in animal models with a high mortality rate (Weiss et al. 2000). Because we worked with a model of chronic endotoxemia with...
a low mortality rate, the sheep were still able to express HSP72 during endotoxemia.

HSP play a major role in the pathophysiology of infection and inflammation; they are thought to protect cells from oxidative stress and are involved in the induction of immune reactions (Buchman 1994, Ribeiro et al. 1994, Villar et al. 1994). Induction of HSP72 reduced mortality rate and organ damage in septic rodents (Eaves-Pyles et al. 2000, Ribeiro et al. 1994, Villar et al. 1994). Recently, it was shown that induction of HSP72 decreased bacterial translocation in a burned mouse model with gut-derived sepsis (Eaves-Pyles et al. 2000). Furthermore, induction of HSP72 was shown to attenuate the endotoxin-induced hypotension (Hauser et al. 1996, Klosterhalfen et al. 1997). Because the expression of nitric oxide synthase (iNOS) during endotoxemia decreased with increased levels of HSP70 (Hauser et al. 1996, Lau et al. 2000), it has been postulated that HSP70 protection against endotoxin is probably mediated through modulation of iNOS activation and the subsequent decreased synthesis of nitric oxide (NO). NO is thought to play a major role in the development of the hyperdynamic circulation in sepsis. In sepsis, inflammatory mediators stimulate the inducible form of iNOS, resulting in increased production of NO. NO relaxes vascular smooth muscles, which leads to peripheral vasodilation and systemic hypotension. Thus it has been suggested that the protective effect of HSP72 in sepsis might be caused by alteration of hemodynamics via modulation of iNOS activation. In the present study, we found no alterations in cardiovascular function despite an increased myocardial HSP72 immunoreactivity after prior supplemental Ala-Gln during endotoxemia.

We demonstrated recently in postsurgical, critically ill patients that parenteral supplementation of Gln shortened hospital stay and improved nitrogen balance (Schulzki et al. 1999). In previous studies, Gln supplementation has been shown to shorten the stay in the intensive care unit and improve long-term survival in critically ill patients. In studies of septic animals, parenteral Gln reduced mortality (Ardawi 1991, Inoue et al. 1993, Naka et al. 1996). It has been suggested that HSP72 mediates the protective effect of Gln against oxidant stress (Choi and Alam 1996, Houdijk et al. 1994, Klosterhalfen et al. 1997, Kojima et al. 1998, Musch et al. 1998, Ogle et al. 1994, Ziegler and Young 1997).

In this study, we investigated the effect of Ala-Gln supplementation on myocardial HSP72 immunoreactivity and hemodynamics. Ala-Gln supplementation increased myocardial HSP72 immunoreactivity during endotoxemia, but did not

![Figure 3](https://example.com/figure3.png)

**Figure 3** Immunohistochemical staining for HSP72 in left ventricle from endotoxemic and healthy sheep treated with alanyl-glutamine (Ala-Gln) or vehicle. (A) Healthy sheep treated with vehicle, no staining; (B) healthy sheep treated with Ala-Gln, slight staining; (C) endotoxemic sheep (after 24 h of endotoxemia) treated with vehicle, intermediate staining; (D) endotoxemic sheep (after 24 h of endotoxemia) treated with Ala-Gln, extensive staining. → HSP72 staining; ↑ no HSP72 staining; magnification X200

![Figure 4](https://example.com/figure4.png)

**Figure 4** Grading of myocardial HSP72 immunoreactivity in alanyl-glutamine (Ala-Gln)–treated and untreated healthy (n = 4) and endotoxemic (n = 6) sheep. Grading of immunoreactivity: 0, no staining; 1 and 2, increasing degrees of intermediate staining; 3, extensive staining. Boxes, 25th-75th percentiles, with centerline as median; bars, 10th-90th percentiles; *P < 0.05
after cardiovascular function in this specific experimental setting. Thus Ala-Gln seems not to aggravate the hyperdynamic circulation in sepsis. However, considering the limited number of sheep included in this study as well as investigation of only one dosage of Ala-Gln, the results of this study should be interpreted with caution. Apart from hemodynamic parameters, no other clinical aspects were investigated. Because we did not show beneficial effects of Ala-Gln supplementation in this study, the experiment does not provide direct evidence that Gln supplementation results in clinical benefits in the absence of hemodynamic effects. Further studies are necessary to investigate the effect of Gln supplementation on hemodynamics and other clinical parameters.

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