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# **Glutamine induces heat shock protein and protects against Escherichia coli LPS-induced vascular hyporeactivity in rats**

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## Abstract

**Background:** Vascular hyporeactivity is an important problem associated with sepsis. Although the mechanism involves inflammatory pathway activation, specific therapeutic approaches are not defined. Glutamine (Gln) has been shown to provide some anti-inflammatory effects and improve outcomes in sepsis. Here we tested the hypothesis that glutamine could reduce Escherichia coli LPS-induced vascular hyporeactivity and evaluated the role of heat shock protein 70 (HSP70) induction in this process.

**Methods:** Twenty four male Sprague-Dawley (SD) rats were divided into control, LPS shock and alanyl-glutamine dipeptide+LPS shock (Ala-gln+LPS) groups. 6hr post administration of LPS, phenylephrine (PE, 0.5~2.5 $\mu$ g/kg) was applied intravenously to all groups and the percentage increase in MAP was detected, respectively. The concentration-response curve of PE was obtained in tension experiments, and the average values of PE E<sub>max</sub> and EC<sub>50</sub> were calculated. The plasma concentrations of malondialdehyde (MDA), TNF- $\alpha$  and IL-6 were detected in all groups. The expression of HSP70 from heart, liver, lung and aorta were also assayed in all groups.

**Results:** The maximal percentage increase in MAP induced by PE was significantly reduced to 12.7% in the LPS shock group ( $P < 0.05$ ) and restored to 15.6% in the Ala-gln+LPS group ( $P < 0.05$ ), while then control group was 24.7%. The average values of PE E<sub>max</sub> and EC<sub>50</sub> were significantly impaired in the LPS shock group ( $P < 0.05$ ), but partially restored in the Ala-gln+LPS group ( $P < 0.05$ ). The expression of HSP70 from the heart, aorta, lung and liver were much higher in the Ala-gln+LPS group than those in the LPS shock group, respectively ( $P < 0.05$ ). The plasma concentrations of TNF- $\alpha$ , IL-6 and

MDA were much lower in the Ala-gln+LPS group than those in LPS shock group.

**Conclusion:** Glutamine effectively improves vascular reactivity by inducing the expression of HSP 70, reducing inflammatory cytokine release and peroxide biosynthesis in LPS shock rats. These results suggest that glutamine has a potentially beneficial therapeutic effect for septic shock patients.

## Introduction

Septic shock is a complex pathophysiological state and despite considerable therapeutic advances, it remains a major therapeutic challenge with high incidence of mortality [1]. Vascular hyporeactivity to catecholamine vasoconstrictors is a characteristic feature during septic shock, which plays a key role in this pathological process and results in arterial hypotension, multiple organ dysfunction and death. The underlying mechanism of impaired vasopressor responsiveness in septic shock is not completely understood but likely involves activation of inflammatory pathways [2].

The therapeutic approaches for the treatment of vascular hyporeactivity in septic shock have included using large dose vasoactive agent, nitric oxide synthases inhibitors [3], guanylate cyclase inhibitor [4], low dose corticosteroids [5] and antioxidant therapy [6]. These have been experimentally used in clinical and animal studies, but their value in therapeutics are not proven. Thus, the precise mechanisms of cardiovascular dysfunction during sepsis warrant further study and the new therapeutic approaches should be explored.

Heat-shock proteins (HSPs) are self-protective proteins that maintain cell homeostasis against various forms of stress as an adaptive response [7]. These proteins are induced by a wide variety of stressors and have broad cytoprotective functions. The 70-kDa family of HSP (HSP70), in particular, plays a vital role in cellular protection and has been detected in various tissues subject to stress [8, 9]. Heat stress, gene transfer, and some small molecule agents have been reported to induce HSP70 expression [10–12] although potential clinical value of these approaches have not been defined.

Glutamine, a nonessential amino acid, has been demonstrated to attenuate proinflammatory cytokine release [13] and lung metabolic dysfunction in animal models of endotoxin shock through enhanced HSP expression [14]. No previous studies have evaluated the impact of Gln administration on sepsis related vascular hyporeactivity. In this study, we examined the hypothesis that pretreatment of glutamine can induce HSP70 expression and improve vascular reactivity in a relevant rat model of LPS-induced sepsis.

## **Materials and Methods**

### **Animals**

The study was approved by the Ethical Committee of Animal Research at the College of Medicine, Southeast University, Nanjing, China. Twenty four healthy male SD rats weighing 250 ~ 300g were randomly divided into three groups as the following: a control group, intravenous infusion of 5-7 ml lactated Ringer solution (LR) (n=8); LPS shock group, intravenous infusion of 5-7 ml LR until 1 hour before intravenous administration of LPS (Sigma Chemical, St. Louis, MO, USA) 10mg/kg (n=8); Ala-gln+LPS group, intravenous infusion of 5-7 ml of 4% Ala-gln until 1 hour before intravenous administration of LPS (n=8). All fluids were infused by micropump at a rate of 5-7 ml/h.

### **Gln administration**

Gln was administered as 20% Ala-gln (Fresenius Kabi Co. Austria), diluted into 4% solution with LR for intravenous infusion since Ala-gln must be diluted five times for intravenous administration in clinical application. 5-7 ml 4% Ala-gln was administered to yield  $0.75 \text{ g}\cdot\text{kg}^{-1}\cdot\text{dose}^{-1}$  Gln. Ala-gln solution or LR vehicle was administered via femoral vein injection.

### **Measurement of MAP**

All rats were anesthetized with sodium pentobarbital 40mg/kg i.p, and a supplemental dose of 20 mg/kg was added if necessary. The rats were allowed to keep spontaneous breathing. A catheter was placed in the femoral artery and connected to the pressure transducers for recording MAP, and other one was placed in the femoral vein as a route for drug administration. MAP of the rats were decreased after administration of LPS in LPS

group and Ala-gln+LPS group, the percentage of MAP decrease by 25-30% of baseline level was regarded as endotoxin shock [15].

Six hours post administration of LPS, PE (Shanghai Harvest Pharmaceutical Co., Ltd., China) with 0.5, 1.0, 2.0, and 2.5µg/kg was applied every 20 min via the femoral vein; the percentage of increase in MAP was recorded in each group.

### **Isolated vascular function**

All rats were anesthetized by sodium pentobarbital and killed by decapitation after measuring levels of MAP. Thoracic aorta was rapidly isolated and prepared with endothelium intact. Vascular segments (3-4 mm) were suspended by stainless steel hooks in 10-ml tissue baths containing Krebs' buffer at 37°C, oxygenated by constant bubbling of a 95/5% O<sub>2</sub>/CO<sub>2</sub> mixture and incubated for 90 min. Tension data were collected with Biologic Signal Collecting System (Nanjing Medical University, Nanjing, China). After five washes, concentration-effect data were obtained by cumulative addition of PE ( $1 \times 10^{-9}$  to  $1 \times 10^{-4}$  M, Sigma Chemical, St. Louis, MO, USA).

### **HSP70 protein expression detection with western blot analysis**

Six hours post injection LPS, the heart, liver, lung and aorta were harvested and immediately frozen in liquid nitrogen and stored at -80°C until analysis. Tissues were homogenized in buffer (10 mM Tris, 5 mM EDTA, 2% Triton X-100, 0.2mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin and aprotinin) and mechanically disrupted. Samples were analyzed by SDS-PAGE using a transfer buffer (25 mM/L Tris, 192 mM/L glycine and 20% methanol) in a wet-transfer apparatus. Blots were blocked with 5% nonfat dry milk in phosphate-buffered saline with 0.1% Tweens-20. Then incubated with

mouse-anti-rat monoclonal HSP70 antibody (Sigma Chemical, St. Louis, MO, USA). After repeated washing, rabbit-anti-mouse secondary antibody (horseradish peroxidase conjugated) incubation was performed, developed with chemiluminescence system and followed with film exposure thinken

### **The plasma TNF- $\alpha$ , IL-6 and MDA detection**

The arterial blood sample (1.5 ml) was collected at 90 min (for TNF- $\alpha$  detection, it was thought to achieve plasma peak within 2 hr post LPS injection[16]) and 6 h (for plasma IL-6 and MDA detection) post administration of LPS from all groups, respectively. Blood was then centrifuged for 8 minutes at 3500g and 4°C and supernatant was collected. The plasma IL-6 and TNF- $\alpha$  were analyzed utilizing ELISA kits (Shanghai Hua Sen Science & Technology CO. Ltd., Shanghai, China). Results were then obtained using a microplate reader (BIO-RAD, 680, Japan). The content of plasma malondialdehyde (MDA) levels was measured with thibabaturic acid reaction (TBA reaction).

## Statistical Analysis

Concentration-response data are fitted to a sigmoidal  $E_{\max}$  model using GraphPad Prism Software (San Diego, CA, USA.). The values of PE  $E_{\max}$  and  $EC_{50}$  were determined for each treatment group. Data are presented as mean  $\pm$  SD. Statistical analyses were performed using one-way analyses of variance. All analysis was performed using the Statistical Software Package (SPSS Version 11.5). Statistical significance was assigned at  $p < 0.05$ .

## Results

### MAP changes to phenylephrine

There were no significant differences in baseline levels of MAP between all groups, but MAP reduced to 75-70% of baseline level 6h after administration of LPS. PE produced dose-dependent increase in MAP in all groups, but the maximal percentage of increase in MAP significantly decreased to 12.7% in LPS shock group ( $P < 0.05$ ) and restored to 15.6% in Ala-gln+LPS group, respectively, when maximal percentage of increase in control group was 24.7% ( $P < 0.05$ ; fig.1).

### Isolated vascular response to phenylephrine

In the vascular tension experiments, each tissue developed the tension to PE in a concentration –dependent way. The average values of PE  $EC_{50}$  and  $E_{max}$  were  $8.55 \pm 0.08$  nmol/L and  $1.86 \pm 0.05$  g in control group (Tab.1). PE  $E_{max}$  significantly reduced to 51% ( $0.95 \pm 0.01$  g) in LPS shock group and restored to 68% ( $1.27 \pm 0.03$ g) in Ala-gln+LPS group, when PE  $E_{max}$  in control group was taken as 100% ( $P < 0.05$ ; fig. 2, Tab.1). Likewise, fitted PE  $EC_{50}$  significantly increased to  $13.49 \pm 0.06$  nmol/L in LPS shock group and reversed to  $10.15 \pm 0.04$  nmol/L in Ala-gln+LPS group ( $P < 0.05$ ; Tab.1).

### Gln enhances HSP70 expression

The analyses from western blot shown that HSP70 expression in heart tissue (fig.3 A), aorta tissue (fig.3 B), lung tissue (fig.3 C) and liver tissue (fig.3 D) were little in control group, but markedly stronger expressions in LPS shock group ( $P < 0.05$ ; fig. 3 A-D). The expressions of HSP70 were much higher than those in LPS shock group alone from four tissues in Ala-gln+LPS group ( $P < 0.05$ ; fig. 3 A-D).

### **Gln decrease plasma concentration of TNF- $\alpha$ ,IL-6 and MDA**

The plasma TNF- $\alpha$ , IL-6 and MDA levels were low in control group ( $49.7\pm 12.2$  pg/ml,  $23.5\pm 9.2$  pg/ml,  $4.66\pm 0.55$  mol/ml). It significant increased in LPS shock group ( $293.1\pm 52.2$  pg/ml,  $296.2\pm 60.2$  pg/ml,  $9.71\pm 0.87$  mol/ml;  $P<0.01$ , Tab.2). Pretreatment with Ala-gln significant decreased plasma concentration of TNF- $\alpha$ , IL-6 and MDA compared with LPS shock group ( $131.8\pm 27.7$  pg/ml,  $204.1\pm 42.2$  pg/ml,  $5.89\pm 0.58$  mol/ml;  $P<0.05$ , Tab.2).

## Discussion

The results of this study demonstrate that pretreatment of Ala-gln significantly improved vascular response to catecholamine vasoconstrictors and that the effect of Ala-gln is associated with its capacity to induce HSP70 expression, and attenuate release of proinflammatory cytokines and oxidizing species production after septic shock.

HSP70 is the most important protein in HSP family that generates protective effect against the injuries in the presence of various stresses [16]. 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 [17]. HSP expression has also been induced by measures that increase core body temperature [18]. However, these measures are clinically impractical, because they would be poorly tolerated by patients and would have detrimental effects on many cellular functions [19]. Gln, may have therapeutic value in safely and effectively enhancing the expression of HSP and increase the survival from septic shock [20]. Therefore, we chose Ala-gln as a HSP expression inducer in this study.

In present study, we designed pretreatment of Gln 0.75g/kg 1 hour before LPS injection, and performed vascular functional test after 6 hours administering LPS. The dose of Gln dipeptide utilized in this study was based on our previous data which indicating that maximal HSP70 mRNA expression in rats occurs at 6~12 hr after intravenous 0.75-g/kg dose Gln [21]. This dose also has been demonstrated safely to induce HSP70 expression

in sepsis rats [22]. One limitation of this study seems to be that we chose the pretreatment of Ala-gln rather than do it at the onset of injury or post a septic injury as some excellent experiment design which were more get close to the clinical utility[13,20,22]. This was done because we consider that Ala-gln induced maximal HSP70 expression occurs at 6 hr after it was used, but on the other hand, administration of LPS fast result in hypotension at 3<sup>rd</sup>-4<sup>th</sup> hr in endotoxemia rat [23]. If Gln injection time is same with or post LPS application, maybe could not exactly evaluate Gln's therapeutic effects on vascular hyporeactivity in endotoxic shock because maximal HSP expression dose not obtained.

A potential limitation of this study is that we chose to utilize a vehicle-based control rather than an iso-nitrogenous amino acid control. Previous studies have shown that alanine does not lead to significant enhancement of HSP-70 both in animals [24] and patients [25], suggesting that the pharmacological effects we have observed are related to the Gln treatment.

Ala-gln must be diluted five times for intravenous administration according to its description, therefore, there is an excessive amount of fluid infusion in present study. This acute volume overloading results in slight increase in blood pressure and heart rate , but dose not lead rat death. The effects of volume overloading on the measurement of MAP should be considered. However, the volume of infusion in every administration was strictly controlled to be the same in three groups according to rat's body weight, and the MAP still decreased after administration of LPS. This indicates that although volume overloading could temporarily change the hemodynamics in rat, the change of MAP could be still considered as a sensitive parameter reflecting vascular response to agonists in present

study. Furthermore, total 3 ml blood was drawn at 90 min and 6 hr before starting the experiment may partially reduce the influence come from the hypervolemia.

Endotoxin shock is characterized by a marked oxidant stress [26] and by a rapid production of different cytokines [27]. NF-[kappa] B is a transcription factor which plays a central role in the modulation of the inflammatory and immune responses and induces the expression of many genes of inducible nitric oxide synthase, cytokines tissue factor, and adhesion molecules involved in the pathogenesis of endotoxin shock [28]. The results of our study showed that the plasma TNF- $\alpha$  , IL-6 and MDA levels in Ala-gln+LPS group were lower more than those in LPS shock group, which indicated that to inhibit proinflammatory cytokine release and peroxide production may be also attributed to the protective effects of Gln on LPS-induced vascular hyporeactivity. The mechanism involving it may be: ① Glutamine inhibits the expression of the inflammatory cytokines directly [22]; ② Gln-induced HSP70 expression further enhanced this effect [29].

The data provided in this study demonstrate two discrete mechanistic effects produced by Ala-gln, namely reduced LPS-induced cytokine presence in plasma and increased HSP70 in multiple tissues. The actual upstream mechanisms responsible for these changes are not clear, but they may be discretely regulated and influenced by Ala-gln. We hypothesize that this dual effect of decreased cytokine-induced vascular injury/action and increased vascular cell survival may be critical for the improved outcomes we observed. Further studies to define the molecular pathways responsible for these discrete actions are clearly warranted, as is further investigation of Gln as a modulator of sepsis-related cardiovascular outcomes.

**Conclusion:**

Glutamine has been well demonstrated to protect against organs dysfunction in animal experiments and in critically ill patients by inducing HSP70 expression, attenuating sepsis-induced metabolic dysfunction, reducing inflammatory cytokine release and peroxide production. Here, we further demonstrated that administration of 0.75g/kg dose Gln could protect against vascular hyporeactivity in endotoxic shock rats. Thus, the Ala-gln could be utilized to induce the protective stress response and prevent end-organ injury, and possibly decrease mortality from sepsis and improve outcomes in critical ill patients.

## **Key messages**

- Alanyl-glutamine improves vascular hyporeactivity in endotoxic shock rat.
- The protective role of alanyl-glutamine on vascular reactivity comes from inducing HSP70 expression, and reducing inflammatory cytokine release and peroxide biosynthesis.
- These results suggest that alanyl-glutamine have potential beneficial therapeutic effects in sepsis.

## **Abbreviations**

EC<sub>50</sub> = Median effective dose; E<sub>max</sub> = Maximum efficacy; Gln = Glutamine

HSP = Heat shock protein; IL-6 = Interleukin-6; LPS = lipopolysaccharide; LR = Lactated

Ringer; MAP = mean arterial pressure; MDA = Malondialdehyde; PE = Phenylephrine; SD =

Sprague-Dawley; TNF- $\alpha$  = Tumor necrosis factor- $\alpha$ .

## **Competing interests**

The authors declare that they have no competing interests.

## **Authors' contributions**

LJ carried out the design of the study, established the experimental setup, drafted the manuscript and participate part of the animal experiments. QW carried out the in vivo and in vitro animal experiment, blood analysis, and performed the statistical analysis . FW carried out the HSP70 protein expression detection.

All authors read and approved the final manuscript.

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### Figure legends:

**Fig.1** The percentage of increase in MAP induced by phenylephrine in different group rats. The maximal percentage of increase in MAP significantly decreased to 12.7% in LPS shock group ( $P < 0.05$ ) and restored to 15.6% in Ala-gln+LPS group, when maximum percentage of increase in control group was 24.7% ( $n=8$ , Mean  $\pm$  SD). \*  $P < 0.05$  vs. Ala-gln+LPS group; #  $P < 0.05$  vs. control group.

**Fig.2** The concentration-response curves of phenylephrine in aortic rings from different group rats (Mean  $\pm$  SD). PE  $E_{max}$  significantly reduced to 51% in LPS shock group and restored to 68 % in Ala-gln+LPS group ( $P < 0.05$ ), when PE  $E_{max}$  in control group was taken as 100%. \* $P < 0.05$  vs Ala-gln+LPS group ; # $P < 0.05$  vs control group.

**Fig.3** Effects of glutamine (Ala-gln) on HSP70 expression in heart, aorta, lung and liver in endotoxin rats. HSP70 expression were analyzed by Western blot analyze. Relative density is refer to the ratio between HSP70 and GAPDH. The expression of HSP70 was significantly increased after LPS injection compared with control group in heart (A), aorta (B), lung (C) and liver (D). (\* $P < 0.05$ ;  $n = 5$ ). The expressions of HSP70 were much higher than those in LPS shock group alone from four tissues in Ala-gln+LPS group (#  $P < 0.05$ ;  $n=5$ ).

**Table 1: E<sub>max</sub> and EC<sub>50</sub> value of aorta rings to phenylephrine in different group rat**

Group	E <sub>max</sub> (g)	EC <sub>50</sub> (nmol/l)
Control	1.86±0.04	8.55±0.08
LPS shock	0.95±0.01*	13.49±0.06*
Ala-gln+LPS	1.27± 0.02*#	10.15±0.04*#

Data are shown as mean ± SD, n=8 in each group. \*P < 0.05 vs control group;  
# P < 0.05 vs LPS shock group.

E<sub>max</sub> = maximum efficacy; EC<sub>50</sub> = median effective dose; Ala-gln = alanyl-glutamine dipeptide; LPS = lipopolysaccharide.

**Table 2: Plasma concentration of TNF-α, IL-6 and MDA in the different group rat**

Group	TNF-α ( pg/ml )	IL-6 ( pg/ml )	MDA ( mol/ml )
Control	49.7±12.2	23.5±9.2	4.66±0.55
LPS shock	293.1±52.2*	296.2±60.2*	9.71±0.87*
Ala-gln+LPS	131.8±27.7*#	204.1±42.2*#	5.89±0.58*#

Data are shown as mean SD, n=8 in each group. \* P < 0.05 vs Gln group; # P < 0.05 vs control group.

Ala-gln = alanyl-glutamine dipeptide; IL-6 = Interleukin-6; LPS = lipopolysaccharide;  
MDA = Malondialdehyde; TNF-α = Tumor necrosis factor-α

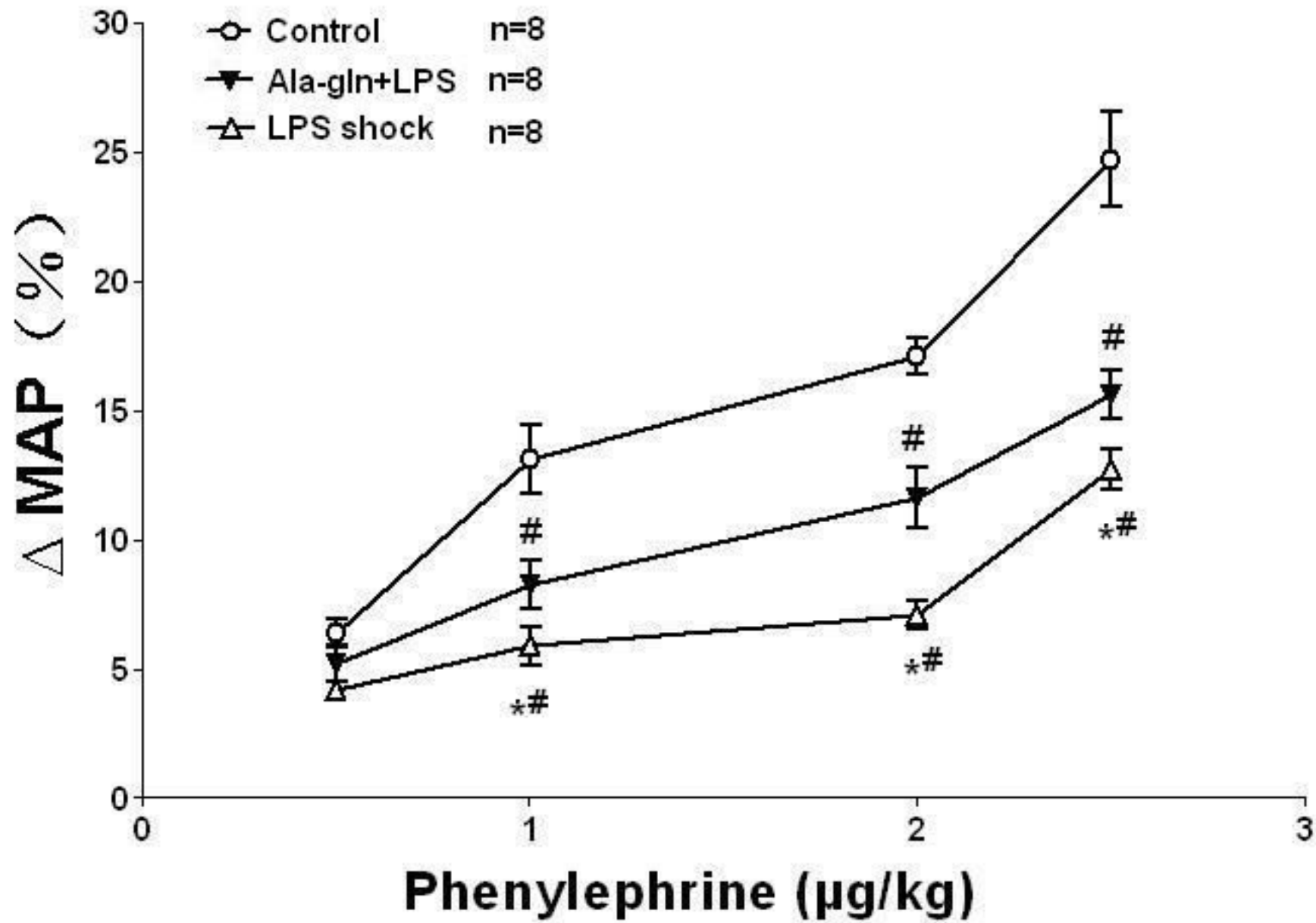


Figure 1

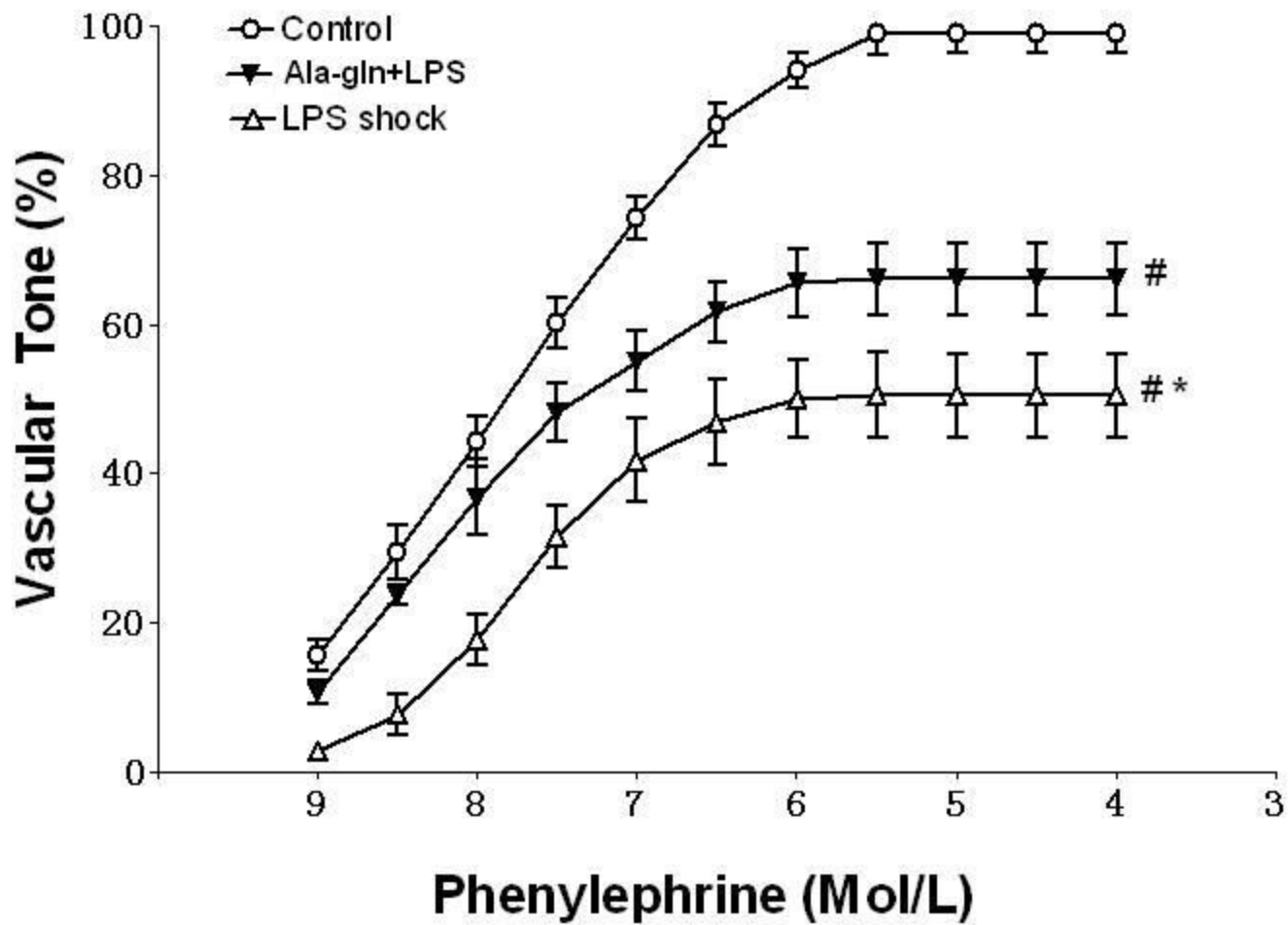


Figure 2

Control LPS Ala+LPS

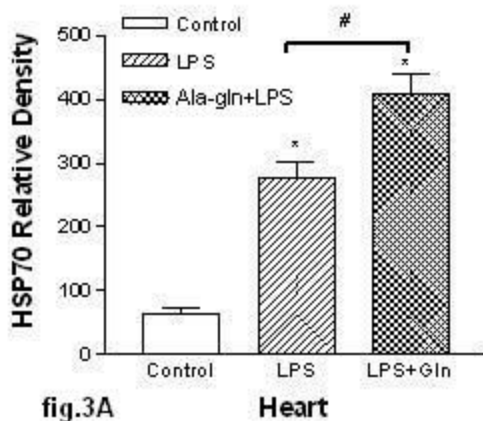
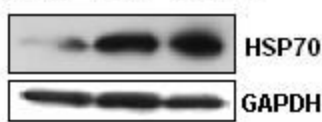


fig.3A

Heart

Control LPS Ala+LPS

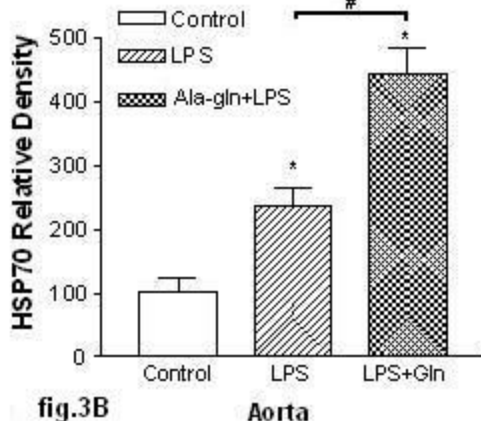
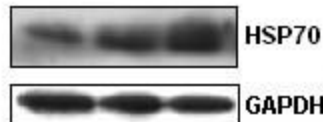


fig.3B

Aorta

Control LPS Ala+LPS

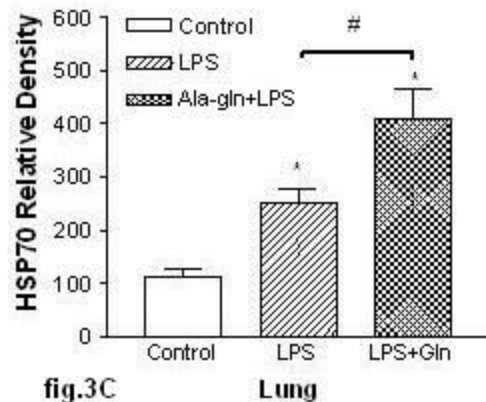
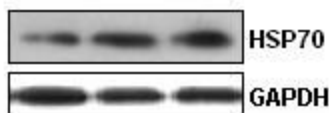


fig.3C

Lung

Control LPS Ala+LPS

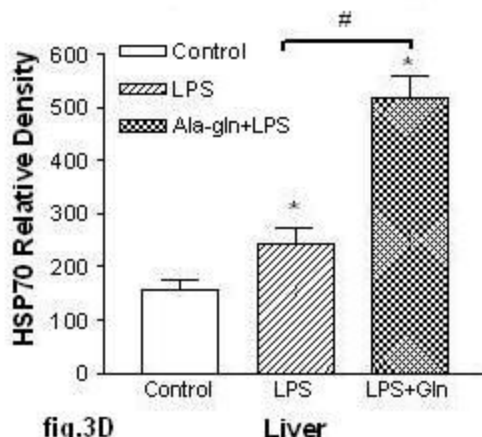
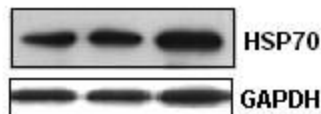


fig.3D

Liver