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Intravenous free and dipeptide-bound glutamine maintains intestinal microcirculation in experimental endotoxemia

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ABSTRACT

Objective: The administration of glutamine (Gln), which is depleted in critical illness, is associated with an improvement of gut metabolism, structure, and function. The aim of the present study was to evaluate the effects of intravenous Gln and its galenic formulation, L-alanyl-L-glutamine dipeptide (AlaGln), on the intestinal microcirculation during experimental endotoxemia using intravital fluorescence microscopy. Gln or AlaGln administration was performed as pretreatment or post-treatment, respectively. To identify further the underlying mechanisms, amino acid levels were studied.
Methods: Sixty male Lewis rats were randomly divided into six groups ($n = 10$/group): control, LPS (lipopolysaccharide 5 mg/kg intravenously), Gln/LPS (LPS animals pretreated with Gln 0.75 g/kg Gln intravenously), AlaGln/LPS (LPS animals pretreated with AlaGln intravenously, 0.75 g/kg Gln content), LPS/Gln (LPS animals post-treated with Gln 0.75 g/kg intravenously), and LPS/AlaGln (LPS animals post-treated with AlaGln intravenously, 0.75 g/kg Gln content). Two hours after the endotoxin challenge, the microcirculation of the terminal ileum was studied using intravital fluorescence microscopy. Blood samples were drawn at the beginning, during, and the end of the experiment to determine the amino acid levels.
Results: The Gln and AlaGln pre- and post-treatment, respectively, prevented the LPS-induced decrease in the functional capillary density of the intestinal muscular and mucosal layers ($P < 0.05$). The number of adherent leukocytes in the submucosal venules was significantly attenuated after the Gln and AlaGln pre- and post-treatment ($P < 0.05$).
Conclusion: The Gln and AlaGln administrations improved the intestinal microcirculation by increasing the functional capillary density of the intestinal wall and decreasing the submucosal leukocyte activation.

Introduction

According to the "gut insult" hypothesis [1], the loss of mucosal integrity is a driving force in the pathogenesis of sepsis. Early in the course of sepsis, a splanchnic hypoperfusion initiates and further amplifies the impairment of the gut barrier function and thus reinforces the translocation of bacteria and their toxins [2,3]. There is a growing body of evidence that the amino acid glutamine (Gln) plays a pivotal role in this pathophysiologic process [4,5]. Gln acts as the principal metabolic fuel for lymphocytes, macrophages, fibroblasts, and small intestinal enterocytes. Furthermore, Gln is involved in the synthesis of the intracellular antioxidant glutathione [6]. Consequently, Gln deficiency may cause a decreased radical defense, a decreased immune response, delayed wound healing, intestinal hyperpermeability, and, hence,

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bacterial translocation [6]. In line with this hypothesis, Gln supplementation in deprived animals increases the immune and gut barrier functions, decreases bacteremia, and inhibits gut mucosal atrophy [7–9].

The depletion of endogenous Gln stores recently has been shown in septic patients. Extra- and intracellular Gln levels can decrease up to 60% [10,11], probably owing to an insufficient endogenous synthesis along the Gln synthetase pathway and/or drastically increased Gln demands of consuming organs such as immune cells and enterocytes [12]. Gln supplementation, preferably by the parenteral route, can counteract the loss of Gln and prevent or decrease the intensity of the increase in intestinal permeability in critically ill patients [4–6,13]. Thus, Gln is considered a conditionally essential amino acid and should be administered (grade A recommendation) when parenteral nutrition is indicated in intensive care patients [14]. Because of its unfavorable chemical properties, free Gln cannot be integrated in ready-to-use solutions. Instead, the dipeptide L-alanyl-L-glutamine (AlaGln) is applied as a safe and efficient Gln source [14,15].

The decisive metabolic link between Gln and gut protection during sepsis is still not known. We hypothesized that sufficient Gln availability would contribute to maintain the intestinal microcirculation, thereby supporting gut barrier function at the time of a bacterial attack. Therefore, the aim of this study was to evaluate the effects of intravenous Gln and AlaGln supplied before and after an endotoxin challenge, respectively, on the intestinal macro- and microcirculation. To identify further the underlying mechanisms, amino acid levels were studied.

Material and methods

Animals

All experimental procedures were performed according to German animal safety legislations and were approved by the local animal care committee. Sixty 60 male Lewis rats (body weight 250 ± 50 g; Charles River Laboratories, Sulzfeld, Germany) were housed in chip-bedded cages and kept under 12-h light/dark rhythmic conditions (temperature 22°C, humidity 55–60%). Standard rat chow (Altromin, Lage, Germany) and water were available ad libitum. After the experiment, all animals were sacrificed by an overdose of intravenous pentobarbital using a rapid intravenous administration.

Anesthesia and preparation

Anesthesia was induced by an intraperitoneal administration of pentobarbital 60 mg/kg (Pentobarbital Natrium, Fagron, Barsbüttel, Germany) and maintained (corneal reflex test) throughout the study period with repeated intravenous pentobarbital injections (5 mg/kg). With the animals positioned in a supine position, the neck area was shaved and disinfected. Polyethylene catheters (vein: inner diameter 0.28 mm, outer diameter 0.61 mm; artery: inner diameter 0.58 mm, outer diameter 0.96 mm; Smith Medical, Kent, UK) were introduced into the left external jugular vein and common carotid artery for fluid resuscitation for the administration of endotoxin and fluorescence dyes. All animals received a tracheostomy to permit access to the airway. The animals spontaneously breathed room air. A specially tempered microscopy bench was used to maintain a body temperature of 37 ± 0.5°C. Subsequent to shaving and disinfection of the abdominal area, a median laparotomy was performed from the xyphoid process to the symphysis 30 min before the initiation of the intravital microscopy.

Experimental protocol

The experiment started after a 15-min resting period after the surgical preparation. Animals were randomly assigned to one of six groups (n = 10/group): control, LPS (lipopolysaccharide 5 mg/kg intravenously from *Escherichia coli*, serotype O26:B6; Sigma, Steinheim, Germany), Gln/LPS (animals treated 15 min before the LPS challenge with Gln 0.75 g/kg intravenously; Sigma), AlaGln/LPS (LPS animals pretreated with AlaGln intravenously corresponding to a Gln content 0.75 g/kg; Dipeptiven, Fresenius, Bad Homburg, Germany), LPS/Gln (animals treated with Gln 0.75 g/kg intravenously 15 min after LPS administration), and LPS/AlaGln (LPS animals post-treated with AlaGln intravenously, Gln content 0.75 g/kg). The used LPS contained traces of lipoprotein (according to the manufacturer's information). The chosen Gln and AlaGln dosages are comparable to similar studies in experimental sepsis in rats [7]. Intravenous Gln and AlaGln administrations alone had no effect on the healthy microcirculation in pilot experiments.

To check the dipeptide assimilation, arterial blood samples (1 mL) were harvested in heparinized tubes from five LPS/AlaGln animals before and two times after (20 and 120 min) the dipeptide administration.

All administered fluids, including the intra-arterial flush, were calculated to guarantee that all animals received an equal volume of intravenous fluids (total volume 15 mL · kg^{-1} · h^{-1}). The duration of each experiment, including the induction of anesthesia, did not exceed 240 min.

Intravital fluorescence microscopy

Intravital fluorescence microscopy was performed using an epifluorescent microscope (Axiotech Vario, Carl Zeiss, Jena, Germany) with a light source (HBO 50, Carl Zeiss, Jena, Germany), ocular (10×), lens (20×/0.5 Achroplan, Carl Zeiss, Jena, Germany), and filter (type #20, Carl Zeiss, Jena, Germany) for the examination of rhodamine 6G and a filter (type #10, Carl Zeiss, Jena, Germany) for the examination of fluorescein isothiocyanate albumin 2 h after the onset of the experiment on a segment of the terminal ileum 1 cm proximal from the ileocecal valve, which was held in place by a supporting device [16]. A cover slip (microscope slides 76 × 26 mm; Mensel, Braunschweig, Germany) was used as a transparent cover to facilitate the microscopic evaluation of approximately 1 cm^2 of the intestinal surface. Areas of the intestine not being examined were covered with gauze and continuously superfused with isotonic saline kept at 37°C to avoid dehydration and exposure to ambient air. To avoid mechanical damage, the gut that had no direct contact to the cover slip was adapted by a hanging drop, warmed to 37°C, to the glass. To prevent tissue damage caused by the phototoxic effects of repeated long-lasting light exposure, repeated observation by intravital fluorescence microscopy on a same area of intestine was avoided. Images were taken by a black-and-white CCD video camera (BC-12, AVT-Horn, Aalen, Germany) linked to a black-and-white monitor (PM-159, Ikegami Electronics, Neuss, Germany) and video recorded simultaneously (Panasonic NV-SV120EG-S, Matsushita Audio Video, Tokyo, Japan) for offline evaluation. Within the described configurations, a total magnification of 500× on the 14-inch monitor was achieved.

Fifteen minutes before starting microscopy, all leukocyte subpopulations were stained by an intravenous injection of 200 μL of a 0.05% rhodamine 6G solution (Sigma). The microscope was then set to focus on the submucosa of the prepared intestinal section. Six visual fields containing non-branching collecting venules over a length of at least 300 μm and another six visual fields displaying postcapillary venules were observed and recorded for 30 s per field. A solution containing 200 μL of a 5% fluorescein isothiocyanate albumin (Sigma) dissolved in saline was subsequently administered intravenously to distinguish plasma from blood cells. After setting the focus, six video sequences (30 s) of random fields of the capillaries within the longitudinal muscle layer and then within the circular muscle layer were recorded. Then, a section of the intestinal lumen (2-cm length, antimesenteric) was opened using a microcautery knife to facilitate the examination of the mucosa. After flushing with warm isotonic saline (37°C), the intestine was lifted and held by the supporting device. Sections of the mucosa directly bordering the mesentery were examined to circumvent any possible influence from the microcauterization. Again, six video sequences (30 s each) of randomly chosen mucosa sections were recorded. The evaluation of all video sequences took place offline on a video monitor in a blinded fashion.

Macrocirculation

As markers of macrocirculation, blood pressure and heart rate were continuously monitored using the vein and artery catheters, respectively (Hewlett Packard, Saronno, Italy).

Microcirculation

The microcirculation was assessed by functional capillary density (FCD) and leukocyte adherence using image analysis. The FCD of the six different fields observed in the longitudinal and circular muscular layers and the mucosal layer were calculated by a morphometric determination of the length of red blood cell–perfused capillaries (centimeters per square centimeter). In the submucosal collecting and postcapillary venules, the number of firmly adhering leukocytes was analyzed. Firmly adherent leukocytes were defined as stained cells that did not move or detach from the endothelial lining within each vessel segment in an observation period of 30 s. Data are presented as the number of cells per square millimeter of endothelial surface, calculated from the diameter and length of the vessel segment studied, assuming cylindrical geometry.

Peptide/amino acid measurements

Arterial blood samples were centrifuged (2800 × *g*, 5–10 min) and the plasma obtained was deproteinized with 50 μL of sulfosalicylic acid containing norvaline 1 mmol/L as an internal standard. Peptides and amino acids were analyzed in the protein-free supernatant as described in detail previously [17].

Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat, Jandel Scientific, Erkrath, Germany). All data were expressed as group mean ± standard deviation. Mean arterial pressure, heart rate, and temperature were analyzed by a two-way analysis of variance (repeated measures in the factor of time) followed by the Scheffe test. Intravital fluorescence microscopic data were analyzed using a one-way analysis of variance followed by the Newman-Keuls multiple-comparison test. The significance level was set at $P < 0.05$.

Results

High-performance liquid chromatographic analysis of blood samples from LPS/AlaGln animals confirmed a rapid hydrolysis of the dipeptide supplied. Only traces of the dipeptide could be detected 20 min after administration. The dipeptide constituents alanine and Gln intermittently increased and approached baseline levels after 120 min (Table 1).

The two parameters of macrocirculation were altered after the LPS injection and/or the Gln treatment. Heart rate remained stable in the control group but was significantly increased in the LPS animals ($P < 0.05$ LPS versus control; Fig. 1A). The administration of Gln or AlaGln before or after the LPS application significantly decreased the LPS-induced heart rate increase. Compared with the control group, the mean arterial blood pressure decreased, with the lowest value being observed 30 min after the administration of LPS, and then gradually increased to control levels 120 min after the LPS administration (Fig. 1B). Treatment with Gln or AlaGln decreased the mean arterial blood pressure even further at 30 min but recovered to the same levels in the control and LPS groups at 120 min.

The rectal body temperature remained stable in the control group. The LPS administration resulted in an increased rectal body temperature ($P < 0.05$ LPS versus control; Fig. 1C), starting 45 min after the LPS application. The treatment with Gln or AlaGln (before or after the LPS administration) significantly

Table 1
Plasma amino acid concentrations in five animals before and after treatment with lipopolysaccharide and L-alanyl-L-glutamine

Amino acid (μmol/L)	Before		After 20 min		After 120 min	
	mean	SD	mean	SD	mean	SD
Glutamic acid	107.5	24.3	630.0	112.2	80.4	17.5
Asparagine	40.3	6.8	74.0	6.0	27.3	5.8
Serine	212.7	34.4	355.2	42.2	150.2	35.0
Glutamine	615.9	124.1	2889.6	640.5	752.4	162.1
Histidine	67.4	12.5	102.8	14.3	58.3	13.7
Glycine	254.0	55.3	312.9	47.5	182.1	47.1
Threonine	145.7	46.9	230.8	53.4	87.4	26.6
Citrulline	68.4	12.3	155.3	16.7	58.2	12.1
Arginine	119.1	29.1	156.4	21.3	65.8	14.8
Alanine	424.4	132.0	3606.9	682.7	310.1	117.6
L-Alanyl-L-glutamine	ND		11.8	14.0	ND	
Taurine	67.9	10.3	169.1	29.9	48.2	8.2
Tyrosine	92.5	17.4	114.6	10.6	49.4	5.8
Aaba	8.0	2.5	12.6	3.5	7.4	0.3
Valine	116.7	33.3	126.6	23.4	95.2	24.5
Methionine	43.7	6.4	47.2	4.0	24.0	7.0
Tryptophan	48.6	12.1	50.3	9.2	29.1	6.2
Phenylalanine	50.7	10.1	48.2	7.8	36.5	10.8
Isoleucine	52.7	15.7	55.8	11.4	45.5	12.1
Ornithine	41.6	8.4	60.0	6.6	39.3	8.2
Leucine	108.1	22.5	112.4	17.3	91.9	21.9
Lysine	295.3	103.2	356.3	91.8	195.0	56.2

ND, not detectable; Aaba, α-Aminobutyric acid

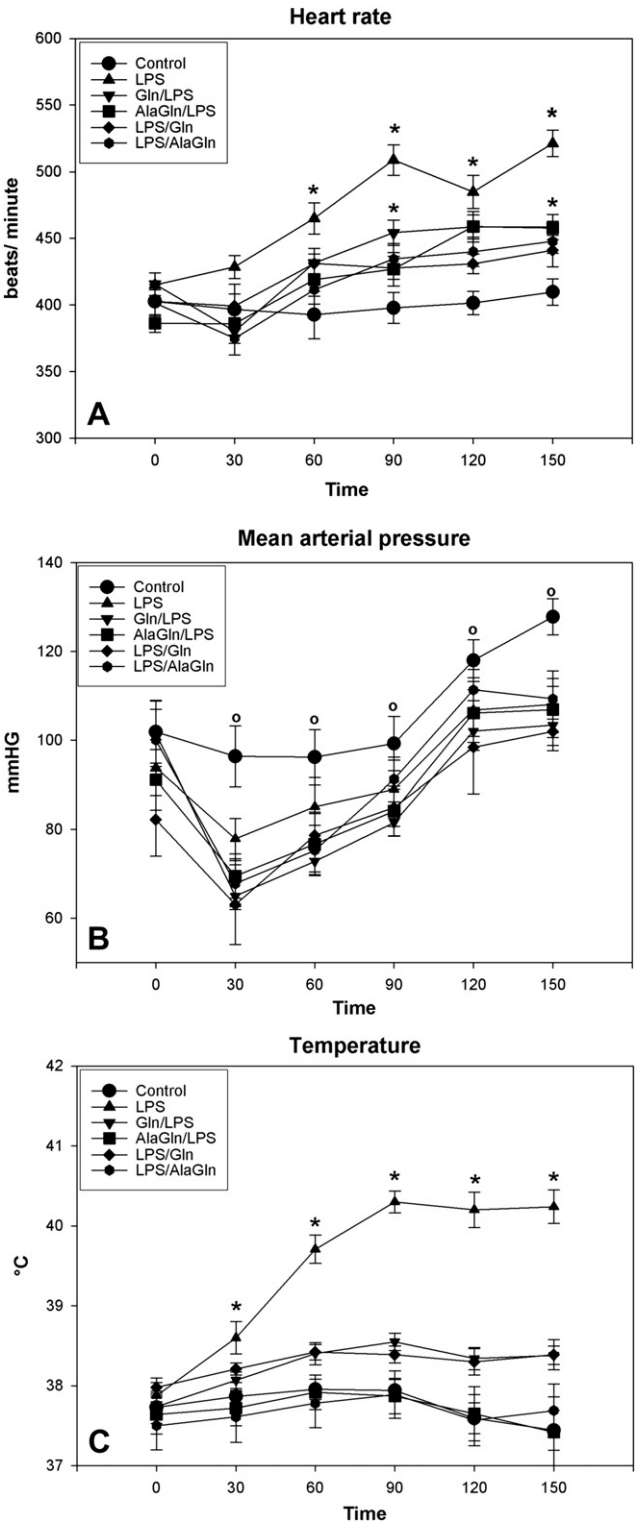

Fig. 1. Macrocirculation and rectal body temperature. (A) Heart rate (beats/min). (B) Mean arterial pressure (mmHg). (C) Rectal body temperature (°C). ° $P < 0.05$ versus LPS group; * $P < 0.05$ versus control group. AlaGln, L-alanyl-L-glutamine; Gln, glutamine; LPS, lipopolysaccharide.

decreased the rectal body temperature to the same levels of the control group.

Figure 2 shows the functional (perfused) and non-functional (not perfused) capillaries of the intestinal wall. Assuming a constant relation between the functional and non-functional capillaries in healthy animals, we calculated the sum of all capillaries in the control group. A line was drawn through the data points of the control animals that connected the perfused and non-perfused capillary axes at the sum values of the capillaries. This presentation clearly shows changes in the number of capillaries. Data points on the left (below) of the line indicate that capillaries were closed; values on the right (above) of the line indicate that additional capillaries were opened. Significances are indicated for the perfused capillaries. The significances for the non-perfused capillaries (not shown) confirmed the capillary function findings.

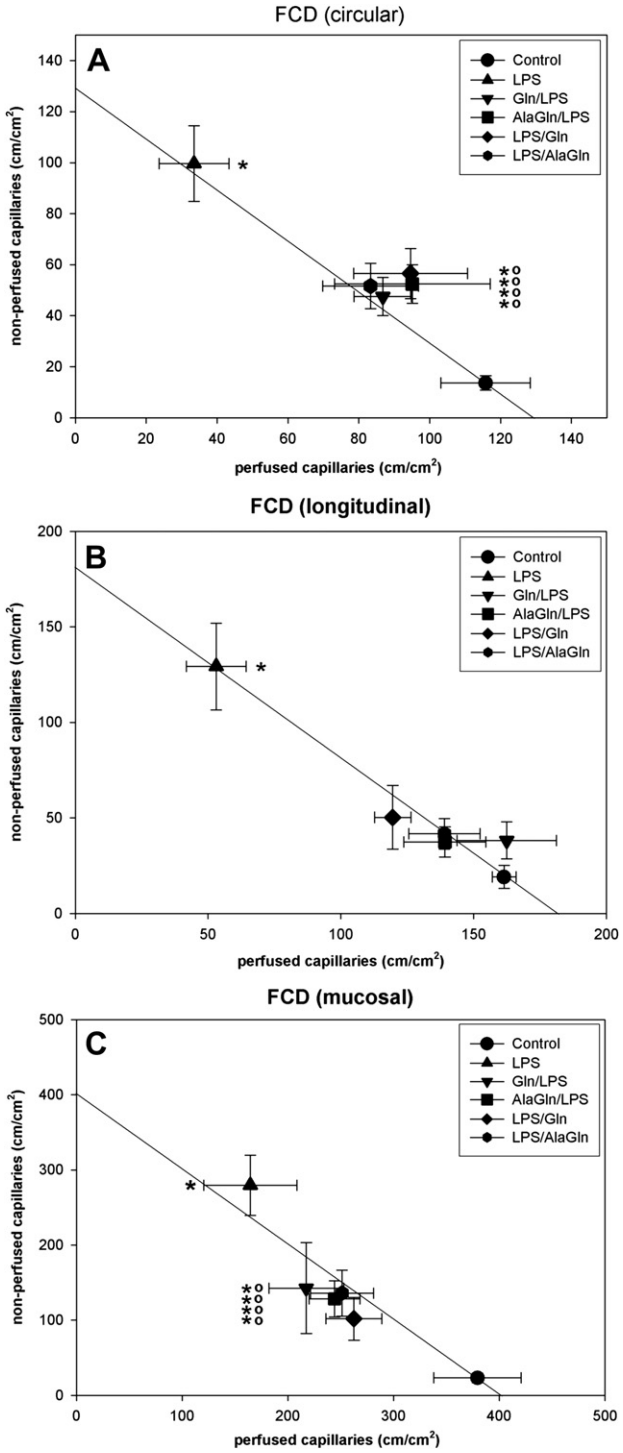

Fig. 2. Functional capillary density. (A) Circular muscular layer (cm/cm^2). (B) Longitudinal muscular layer (cm/cm^2). (C) Mucosal layer (cm/cm^2). $^{\circ}$ $P < 0.05$ versus LPS group; * $P < 0.05$ versus control group. AlaGln, L-alanyl-L-glutamine; FCD, functional capillary density; Gln, glutamine; LPS, lipopolysaccharide.

The untreated LPS animals showed a significant impaired intestinal microperfusion, with a decreased FCD and an increased number of non-functional capillaries in the muscular layers and the mucosa. Interestingly, in the mucosal layer, the data points of the untreated LPS animals were shifted to the right side of the connecting line, suggesting the opening of additional capillaries. The Gln and AlaGln administrations attenuated the endotoxin-induced FCD decreases in the circular muscle layer and the mucosa, respectively. The FCD decrease was completely reversed in the longitudinal muscle layer. The impact on the FCD remained the same when Gln or AlaGln was given before or after the LPS challenge.

In endotoxemic animals, the amount of adherent leukocytes in the submucosal collecting venules (V1 venules) was attenuated after the administration of Gln and AlaGln before or after treatment, respectively. Also, in the smaller, postcapillary venules (V3 venules), Gln and AlaGln significantly decreased the number of adherent leukocytes (Fig. 3). The pretreatment with AlaGln was most effective.

Discussion

Our experiments demonstrated that administration of free Gln or dipeptide-bound Gln before or after an LPS challenge effectively increased the FCD in all layers of the intestinal wall and decreased the interactions of leukocytes with endothelia in the intestinal venules. Most interestingly, the dipeptide AlaGln was as effective as free Gln itself. As shown in selected animals, AlaGln was rapidly metabolized, providing free Gln for the consuming cells and organs.

In several clinical studies, a dominantly parenteral administration of Gln has found been to contribute to the maintenance of the mucosal integrity and, thus, to decrease of bacterial translocation [4–6,13]. The underlying mechanisms of this Gln action are not completely understood. Recent studies have indicated that Gln can induce heat-shock protein-70 expression, attenuate sepsis-induced metabolic dysfunction, and decrease the inflammatory cytokine release and oxidative stress [18–20]. Our results confirm a further metabolic link among these previous observations. In endotoxemia, the systemic activation, adherence, and emigration of leukocytes in different organs leads to a disturbance of the microcirculation, followed by multiple-organ dysfunction syndrome and multiple organ failure [1]. An adequate Gln availability before or even after a challenge can prevent such leukocyte-endothelial cell interactions, probably by influencing cytokine biology. This interpretation is further supported by recent experimental studies. We found that Gln and AlaGln can significantly protect against vascular hyporeactivity and decrease leukocyte adhesion in the mesenteric microcirculation during experimental endotoxemia [21]. Arndt et al. [22] showed that oral Gln administration attenuates leukocyte–endothelial cell adhesion in mesenteric venules in indomethacin-induced long-lasting ileitis in the rat.

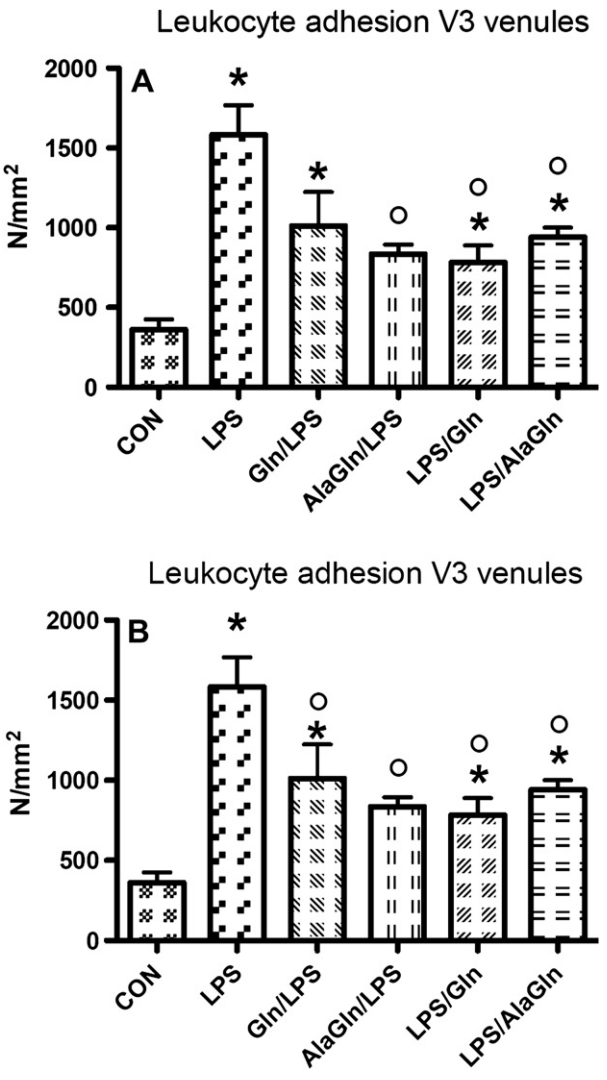

Fig. 3. Leukocyte adhesion. (A) Submucosal collecting (V1) venules (n/mm^3). (B) Submucosal postcapillary (V3) venules (n/mm^3). $^{\circ}$ $P < 0.05$ versus LPS group; * $P < 0.05$ versus control group. AlaGln, L-alanyl-L-glutamine; CON, control; Gln, glutamine; LPS, lipopolysaccharide.

The obstruction of the microvessels by adhering leukocytes is one of the leading causes for the deterioration of the capillary perfusion. In our experiments, we observed a significant improvement of the FCD in the muscular and mucosal layers of the intestinal wall. However, we cannot exclude the (beneficial) effects of the experimental treatment on other factors involved in the disturbance of microvascular perfusion, e.g., disseminated intravascular coagulation or vasoactive effects [21]. For example, Hayashi et al. [23] found that Gln attenuates inflammation by regulating nitric oxide synthase activity.

All these processes are closely associated with the inflammatory response [24,25]. In particular, the increase of the heat-shock protein response can attenuate a proinflammatory cytokine release [26] by binding to the heat-shock element present in the promoter region of interleukin-1β and most likely of other cytokines [27]. This leads to an attenuation of the plasma concentration of tumor necrosis factor-α and interleukin-1β in vitro and in vivo models [28–30].

In conclusion, the results of the present study demonstrate that Gln and AlaGln treatments have beneficial effects within the intestinal microcirculation in a rat model of experimental endotoxemia. We observed that the Gln and AlaGln administrations significantly attenuated the leukocyte adherence and improved the FCD. Clinical trials are necessary to study the impact of Gln (AlaGln) supplementation on the microcirculation in human sepsis.

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