Amino Acids (2012) 43:1485–1498
DOI 10.1007/s00726-012-1221-2

ORIGINAL ARTICLE

Decreased glutamate, glutamine and citrulline concentrations in plasma and muscle in endotoxemia cannot be reversed by glutamate or glutamine supplementation: a primary intestinal defect?

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Received: 31 October 2011 / Accepted: 12 January 2012 / Published online: 28 January 2012

Abstract Endotoxemia affects intestinal physiology. A decrease of circulating citrulline concentration is considered as a reflection of the intestinal function. Citrulline can be produced in enterocytes notably from glutamate and glutamine. The aim of this work was to determine if glutamate, glutamine and citrulline concentrations in blood, intestine and muscle are decreased by endotoxemia, and if supplementation with glutamate or glutamine can restore normal concentrations. We induced endotoxemia in rats by an intraperitoneal injection of 0.3 mg kg^{-1} lipopolysaccharide (LPS). This led to a rapid anorexia, negative nitrogen balance and a transient increase of the circulating level of IL-6 and TNF-α. When compared with the values measured in pair fed (PF) animals, almost all circulating amino acids (AA) including citrulline decreased, suggesting a decrease of intestinal function. However, at D2 after LPS injection, most circulating AA concentrations were closed to the values recorded in the PF group. At that time, among AA, only glutamate, glutamine and citrulline were decreased in gastrocnemius muscle without change in intestinal mucosa. A supplementation with 4% monosodium glutamate (MSG) or an isomolar amount of glutamine failed to restore glutamate, glutamine and citrulline concentrations in plasma and muscle. However, MSG supplementation led to an accumulation of glutamate in the intestinal mucosa. In conclusion, endotoxemia rapidly but transiently decreased the circulating concentrations of almost all AA and more durably of glutamate, glutamine and citrulline in muscle. Supplementation with glutamate or glutamine failed to restore glutamate, glutamine and citrulline concentrations in plasma and muscles. The implication of a loss of the intestinal capacity for AA absorption and/or metabolism in endotoxemia (as judged from decreased citrulline plasma concentration) for explaining such results are discussed.

Keywords Endotoxemia · Citrulline · Monosodium glutamate · Glutamine · Intestine

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Introduction

Endotoxemia results from massive transfer of endotoxin (or more currently called bacterial lipopolysaccharide LPS from Gram-negative bacteria) from the intestinal lumen to the bloodstream. It is well known that endotoxemia provokes a systemic inflammatory reaction with increased concentration of pro-inflammatory cytokines, anorexia and a catabolic state resulting in negative nitrogen balance

(Austgen et al. 1992; Biolo et al. 1997; Karinch et al. 2001). Endotoxemia severely affects nitrogen metabolism in the body notably at the intestinal level. Indeed, several studies have reported that endotoxemia is characterized by decreased amino acid (AA) intestinal absorption (Abad et al. 2001; Gardiner and Barbul 1993; Gardiner et al. 1995a, b; Salloum et al. 1991). In addition, endotoxemia affects the morphology of the intestinal mucosa (Chambon-Savanovitch et al. 1999; Crouser et al. 2000), increases the intestinal permeability (Hietbrink et al. 2009) and decreases mucosal oxygen consumption (King et al. 1999).

In the meantime, endotoxemia is associated with an increase of AA release from muscles (Vesali et al. 2005). Among AA, one of the most important AA released by skeletal muscles into the bloodstream is glutamine, synthesized from glutamate and ammonia (Chakrabarti 1998). This AA is believed to be taken up by other cells including hepatocytes (Inoue et al. 1993) and enterocytes of the small intestine. Enterocytes use glutamine as a major oxidative substrate for ATP production (Darcy-Vrillon et al. 1994). In fact, the energy need of the intestinal epithelial cells is high (Duee et al. 1995), because, the epithelium is renewed within a few days in mammals (Potten and Allen 1977), thus, requiring ATP in anabolic pathways. ATP synthesis is also required for sodium export to the bloodstream at the expense of the Na/K ATPase activity (Buttgereit and Brand 1995). In the enterocytes, glutamine from either luminal or blood plasma origin is converted into glutamate and ammonium in the mitochondria through the catalytic activity of glutaminase (Pinkus and Windmueller 1977). Then, glutamate is converted to α-ketoglutarate which enters the tricarboxylic cycle, allowing production of reduced coenzymes (NADH, $FADH_2$) used by the mitochondria for ATP synthesis. In addition, glutamine metabolism in enterocytes leads to citrulline synthesis through the stepwise conversion of glutamate into glutamyl-semialdehyde and ornithine (Windmueller and Spaeth 1975). However, glutamine is not the only precursor for enterocyte citrulline synthesis, because, arginine and proline can also produce citrulline (Dillon et al. 1999; Guihot et al. 1997). Glutamate represents another ATP-producing substrate for enterocytes as well as a precursor for citrulline synthesis (Blachier et al. 2009). In addition, glutamate is a precursor of *N*-acetylglutamate in enterocyte mitochondria, and this metabolite is known to act as an activator of the carbamoylphosphate synthetase I activity which catalyzes the first metabolic step involved in citrulline biosynthesis (Geng et al. 2011). Glutamate is often used in its monosodium form (MSG) in human diet due to its flavor-enhancing property (Beyreuther et al. 2007).

Citrulline, which is not present in alimentary and endogenous proteins, is thus produced in enterocytes from four AA. Citrulline is released in the portal vein with limited hepatic uptake (van de Poll et al. 2007); appears in the peripheral blood and can be then partly used by the kidney for the de novo arginine synthesis (Dhanakoti et al. 1990). From a convergent series of experimental and clinical studies, plasma citrulline concentration has been proposed as a reliable parameter for estimating the intestine functional capacity (Bailly-Botuha et al. 2009; Chen et al. 1996; Crenn et al. 2000, 2003, 2008; Jianfeng et al. 2005; Lutgens et al. 2003, 2004; Picot et al. 2010; Santarpia et al. 2008; van Vliet et al. 2009; Wakabayashi et al. 1995). The relationship between plasma citrulline concentration and enterocyte function has been demonstrated in rodents after surgical resection of small bowel (Lutgens et al. 2003; Wakabayashi et al. 1995) or in mice after a single homogenous total-body irradiation which provokes functional disorder (Chen et al. 1996). More recently, studies have been conducted in patients with small-bowel enterocyte dysfunction, i.e., after ionizing radiation (Lutgens et al. 2004), after massive resection (Bailly-Botuha et al. 2009; Crenn et al. 2003, 2008; Jianfeng et al. 2005; Rhoads et al. 2005; Santarpia et al. 2008), after intestinal surgery (Crenn et al. 2000; Picot et al. 2010) and after chemotherapy (van Vliet et al. 2009). In addition, serum citrulline has been shown to correlate with enteral tolerance and bowel length in infants with short-bowel syndrome (Rhoads et al. 2005). All these studies led to propose that plasma citrulline concentration may prove to be a simple and reliable marker of reduced anatomic enterocyte mass, of villous atrophy and subsequently of intestinal functional failure, defined as "the reduction in the functioning gut mass below the minimal amount necessary for the absorption of nutrients". Plasma citrulline concentration was indeed correlated to the severity and extent of villous atrophy and thus represents a candidate marker for the effects of bowel rehabilitation therapies on gut.

In that overall context, we used the experimental model of rats treated with a relatively low-dose of LPS [i.e., 0.3 mg kg^{-1} body weight (BW)], which provokes a non-lethal endotoxemia, to test if AA (including citrulline) concentrations in blood and tissues are decreased by endotoxemia; and if supplementation with glutamate or glutamine can restore normal concentrations after different periods of time following LPS injection.

Materials and methods

Animals

Experiments were carried out in accordance with the guidelines of the French Committee for Animal Care and the European Convention on Vertebrate Animals Used for Experimentation, under European council directive (86/609/EEC). Wistar male rats (Harlan, Horst, The

Netherlands) weighing 325–350 g at the beginning of the experiment (D-7), were housed in individual stainless steel cages in ventilated room with controlled temperature (22 ± 1°C) with a 12:12-h light–dark cycle (light from 8:00 p.m. to 8:00 a.m.).

Dietary supplementation and experimental protocol

The rats were fed a normoenergetic diet (experimental diet, ED) providing 15% of energy as protein, 30% as fat and 55% as carbohydrate (Table 1) during 7 days for acclimatization, before starting endotoxemia and dietary supplementation. All diets were moistened (powdered diet/water: 1:2) to prevent spillage. A feeding pattern, designed to accustom the rats to eat in a short time a standard meal, was applied: a first meal (3 g dry matter) was given between 08:00 and 09:00 a.m. and then removed. Rats had then ad libitum access to food between 10:00 a.m. and 06:00 p.m. The rats and their food consumption were weighed each day. The animals were allowed to have free access to water throughout the experimental period. Four days (D-4) before endotoxemia induction and dietary supplementation, basal blood samples were collected from tail vein. Blood samples were centrifuged for 15 min at 1,500 g, and the plasma was frozen at −20°C for subsequent analyses. At D0, rats were randomly assigned to one of the five groups (Table 2). Each group received, 5 min before their first meal, an intraperitoneal injection of saline solution (C and PF groups) or injection of endotoxin (0.3 mg kg^{-1} body weight lipopolysaccharide LPS) from *Escherichia coli* (serotype 0127:B8, Sigma-Aldrich) in saline buffer (LPS, LPS-MSG and LPS-GLN groups). Then rats were placed in metabolic cages to collect urine and feces. At 08.00 h a.m., rats received their first meal (3 g dry matter) composed with ED and AA supplementation: monosodium glutamate 4% w/w (LPS-MSG group) or glutamine 3.46% w/w (isomolar equivalent; LPS-GLN group). The content of glutamate and glutamine in the ED is 2.8% w/w. All animals received the same amount of food before the blood sampling. Two hours after this first small meal, blood samples were collected from tail vein, centrifuged and frozen for subsequent analysis. Then, except for animals in the PF group (which received the same amount of food similar to animals in the LPS-MSG group), rats had free access to food supplemented or not with appropriate AA until 06:00 p.m. At D1, the same protocol was used without injection of saline solution or LPS. At D2, the rats were weighed and euthanatized 2 h after ingestion of the test-meal (3 g dry matter). To determine tissue protein synthesis rate, rats were injected subcutaneously with 300 µmol kg^{-1} BW of a flooding dose of L-[1-^{13}C]-valine (50 mol%, Cambridge Isotope Laboratories, Andover, MA, USA) 20 min before the organ collection following the method described by Mosoni et al. (1996). Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1} BW) (Sanofi Synthélabo Santé Animale, Libourne, France). After incision of the abdomen, blood was collected quickly from the portal vein and vena cava and the animals were killed by rupture of the caudal vena cava and aorta. Blood samples were centrifuged and the plasma was frozen at −20°C for subsequent analyses. Tissues were promptly removed, rinsed, weighed and frozen until analysis. Intestinal mucosa was scraped from the underlying musculature with a glass miscroscope slide and was immediately frozen in liquid nitrogen. Epididymal and subcutaneous adipose tissue and carcass (muscles with skeleton, without head, paws and tail) were removed and weighed.

Table 1 Composition of the experimental diet

Total milk protein (g kg^{-1})	140
Starch (g kg^{-1})	538.1
Sucrose (g kg^{-1})	87.6
Cellulose (g kg^{-1})	50
Soy oil (g kg^{-1})	137
Minerals (AIN-93 M) (g kg^{-1})	35
Vitamins (AIN-93 M) (g kg^{-1})	10
Choline (g kg^{-1})	2.3
Total protein (% of energy)	15
Total carbohydrates (% of energy)	55
Total fat (% of energy)	30
Metabolizable energy (kJ g^{-1})	16.8

Table 2 Groups of rats and treatment

Groups		Injection at J0	Dietary supplementation
1	Control (C)	Saline solution	Experimental diet (ED)
2	LPS	LPS	ED
3	LPS-MSG	LPS	ED + monosodium glutamate
4	LPS-GLN	LPS	ED + glutamine
5	Pair fed (PF)	Saline solution	ED

Hormones, cytokines and amino acid concentrations

Plasma insulin, TNF-α, and IL-6 concentrations were analyzed in duplicates, at D-4 (4 days before LPS injection, basal), at D0 (2 h after LPS injection) and at D1 (1 day after LPS injection) using a rat serum adipokine immunoassay (Millipore) on a Bioplex 200 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Plasma insulin and corticosterone were analyzed in duplicates within the same days using a rat endocrine immunoassay

and a rat stress hormone panel (Millipore), respectively, on the same equipment.

AA concentrations were determined in plasma (at D-4, D0, D1 and D2) after precipitation with 5% trichloroacetic acid, centrifugation and supernatant collection. For tissues (at D2), the same method was used, but in this case, precipitation with 10% trichloroacetic acid was followed by sonication. The supernatants were transferred to 10 kDa cut-off filter (Microcon Ultracel YM-10 Regenerated Cellulose 10,000 MWCO) and centrifuged for 5,000 g at 4°C during 40 min. AA were quantified in the filtrate using an automatic AA analyzer (L-8800, Hitachi, Tokyo, Japan) with ninhydrin reactant post-column reaction as previously described (Meyer 1957).

Determination of protein-synthesis rate and protein content in tissues and nitrogen balance

The ^{13}C-valine enrichment in free and protein-bound AA of tissues was measured at D2 by GC–MS (Hewlett-Packard 6890 N) and by GC-C-IRMS (HP5890/Isoprime, VG Instruments, Manchester, UK) using a 50 m apolar column (HP5MS, Hewlett-Packard) after sample preparation as previously described (Chevalier et al. 2009). Fractional synthesis rates (FSR, %/d) of tissue proteins were calculated as $FSR = E_{bound\ val}/(E_{free\ val} \times t) \times 100$, where, $E_{bound\ val}$ and $E_{free\ val}$ are protein-bound and free ^{13}C-valine enrichments in tissues. Total nitrogen was measured in tissues (at D2) by isotopic ratio mass spectrometry (IRMS, Isoprime, GV Instruments, Manchester, UK) coupled to elemental analyzer (EA, Euro Elemental Analyser 3000, EuroVector, Redavalle, Italy) after freeze–drying. Nitrogen balance was determined at D0 and D1 using total nitrogen determination in urine and feces.

Statistics

Data are expressed as means ± standard deviation (SD). The effect of the treatment (Tt) was analyzed using one-way ANOVA (SAS 9.1, SAS Institute, Cary, USA) for body composition, protein content in tissues, protein synthesis rate and AA concentration in portal blood, vena cava and tissues. Post-hoc Tukey tests for multiple comparisons were performed to enable pairwise comparisons. Differences between groups for all other results were analyzed using mixed models for repeated-measure analysis, with the treatment and the time as independent, fixed factors and rats as a random factor (version 9.1; SAS Institute Inc, Cary, NC, USA). For each variable, the most appropriate matrix of covariant structures for random statements was selected. Post-hoc tests were performed using contrast analysis. Differences were considered significant at $P < 0.05$.

Results

Effects of LPS on hormone and cytokine concentration

As indicated in Fig. 1, injection of 0.3 mg LPS per kg BW resulted at D0 (i.e., 2 h after LPS injection) in a sharp and transient increase of plasma IL-6 (Fig. 1a), TNF-α (Fig. 1b) and corticosterone (Fig. 1d) concentrations when compared with C and PF rats. At D1, these latter concentrations were not different between the control, LPS and PF groups (Fig. 1). Insulinemia was also increased after LPS injection at D0 when compared with control, but not when compared with PF animals (Fig. 1c).

Effects of LPS on food intake and body weight

The BW measured 4 days before LPS injection and at D0 (the day of injection) were not significantly different between the experimental groups (Fig. 2a). Not surprisingly, the BW decreased significantly for all the groups except C group. The supplementation with MSG or glutamine did not modify the rat BW. At D0, after injection of LPS 0.3 mg kg^{-1}, food intake was markedly decreased (Fig. 2b). Food intake progressively increased from D0 to D1 after LPS treatment. Accordingly, MSG or glutamine supplementation did not modify food intake after LPS injection.

In preliminary experiments, we found that food intake and BW were similarly affected following injection with either 0.3 or a higher dose, i.e., 1.0 mg LPS per kg BW ($n = 8$ per group, data not shown).

Effects of LPS on tissue masses, protein contents and tissue total protein contents

As shown in Table 3, LPS injection resulted in a significant decrease of the masses of gastrocnemius muscle (−8% vs. PF) and an increase of the spleen mass (+38% vs. PF). In contrast, the masses of the intestinal mucosa and of kidney remained unaffected by LPS treatment (Table 3). Adipose tissue (epididymal and subcutaneous mass), carcass, liver, stomach, caecum and colon were all unaffected by LPS treatment (data not shown).

The kidney and spleen protein contents were significantly decreased (−6 and −9%, respectively, vs. PF) after LPS injection (Table 3) with no effect of LPS on intestinal mucosa, total muscles and thymus. Plasma protein content was also not affected (data not shown). However, the total amount of proteins in thymus of LPS-treated rats was significantly decreased (−46% vs. PF) (Table 3) and was increased in spleen (+31% vs. PF) after LPS injection. But this treatment had no effect on the total amount of proteins in the intestinal mucosa and kidneys. MSG or glutamine

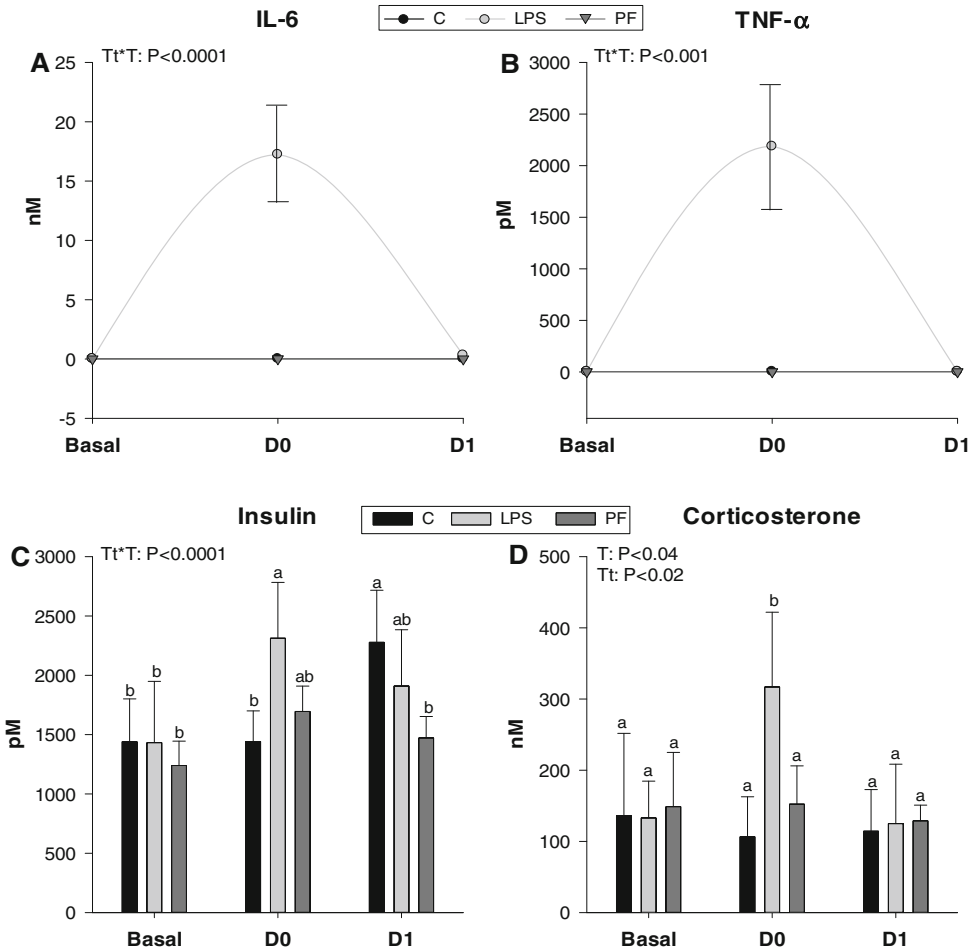

Fig. 1 Effects of LPS injection on IL-6 (**a**), TNF-α (**b**), insulin (**c**) and corticosterone (**d**) plasma concentrations in rats. Results are expressed as means ± SD. *Basal values* indicate plasma concentrations 4 days before LPS injection; *D0* indicates plasma concentrations 2 h after LPS injection and *D1* indicates plasma concentrations 1 day after LPS injection. The main statistical effects (T: time, Tt: treatment and their interaction T*Tt) from a mixed model for repeated measures over time are reported for each variable when $P < 0.05$. *Bars with different letters* are significantly different ($P < 0.05$ or less), $n = 8$ in each group

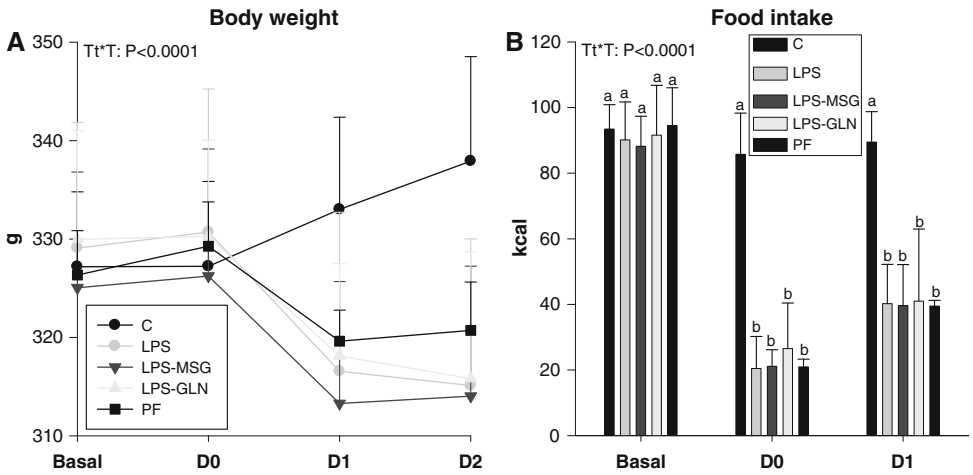

Fig. 2 Effects of LPS injection with or without monosodium glutamate (LPS-MSG) or glutamine (LPS-GLN) supplementation on body weight (**a**) and 24 h food intake (**b**) in rats. Results are expressed as means ± SD. *Basal values* are measured 4 days before LPS injection; *D0* indicates the day of LPS injection, *D1* indicates values 1 day after LPS injection and *D2* indicates values 2 days after LPS injection. The main statistical effects (T: time, Tt: treatment and their interaction T*Tt) from a mixed model for repeated measures over time are reported for each variable when $P < 0.05$. *Bars with different letters* are significantly different ($P < 0.05$ or less), $n = 8$ in each group

supplementation did not modify the effects of LPS injection on any of the parameters measured (data not shown).

Effects of LPS on nitrogen balance and protein fractional synthesis rate in tissues

As indicated in Fig. 3a, the injection of LPS resulted at D0 in a negative nitrogen balance similar to PF group. At D1, nitrogen balance was positive for C and PF groups while it was still negative for the LPS group. No effect of MSG or GLN supplementation on the LPS-induced negative nitrogen balance was recorded.

Urea concentration in blood plasma from the tail vein was not significantly different at basal time and at D0 when comparing the different groups of animals (data not shown). At D1, there was a significant increase of urea concentration in plasma from tail vein in the LPS group (Tt*T: $P < 0.0001$). At D2, in the LPS group, urea concentration was increased in the plasma recovered from the vena cava ($P < 0.0001$) and portal vein ($P < 0.0001$). At D0, urea concentrations in urine were not significantly different between groups (data not shown). At D1, urinary urea concentrations were 265.2 ± 136.8, 844.1 ± 433.4 and 392.6 ± 226.1 mM for C, LPS and PF groups, respectively. At that time, there was a significant difference between C and LPS groups ($P < 0.002$) and LPS and PF groups ($P < 0.02$) for urinary urea concentration. However, no significant difference was recorded at D2 between the different experimental groups. Nitrogen contents in feces were not significantly different between groups (data not shown).

LPS treatment resulted 2 days after injection in a 26% decreased of protein fractional synthesis rate in gastrocnemius muscle ($P < 0.02$ vs. C) with a value in PF rats between the values recorded in C and LPS groups (Fig. 3b). MSG or GLN supplementation of LPS-treated rats failed to restore muscle protein fractional synthesis rate. The protein fractional synthesis rate in the intestinal mucosa was unaffected by the LPS injection (Fig. 3c).

Effects of LPS on circulating amino acids concentrations: effects of MSG and glutamine supplementation

Lipopolysaccharide injection resulted in a rapid decrease of almost all AA in the tail vein plasma. Thus, glutamate, glutamine and AA related to their metabolism (citrulline, alanine, ornithine and proline) were significantly decreased at D0 2 h after LPS injection when compared with values measured in the PF group (Fig. 4). A significant decrease of concentrations in the tail-vein plasma was also observed for tryptophan, serine, histidine, arginine lysine, threonine, leucine, isoleucine, valine, asparagine, phenylalanine, tyrosine and methionine ($P < 0.05$ or less) at D0 (data not shown). One day after LPS injection (D1), only some AA concentrations remained significantly modified when compared with PF rats, i.e., ornithine (Fig. 4e), proline (Fig. 4f) and asparagine ($P < 0.05$ or less, data not shown). At D1, in the LPS group, the average citrulline concentration was lower when compared with PF groups, but the difference between groups was not significant ($P = 0.0628$). When the rats were supplemented with MSG or glutamine, these AA

Table 3 Effects of LPS injection on tissue masses, relative tissue protein content and total protein content in tissues at D2 (2 days after LPS injection) in rats

	C	LPS	PF	Stat effect[1]
Tissue mass (g)				
Intestinal mucosa	1.84 ± 0.63	1.68 ± 0.53	1.93 ± 0.21	NS
Kidney	1.94 ± 0.09	2.13 ± 0.16	1.89 ± 0.13	NS
Gastrocnemius muscle	0.62 ± 0.03^{a}	0.56 ± 0.02^{b}	0.61 ± 0.04^{a}	$P < 0.001$
Spleen	0.68 ± 0.11^{a}	0.98 ± 0.16^{b}	0.60 ± 0.09^{a}	$P < 0.0001$
Thymus	0.50 ± 0.08^{a}	0.37 ± 0.09^{b}	0.46 ± 0.08^{ab}	$P < 0.02$
Protein content (g/100 g)				
Intestinal mucosa	6.21 ± 0.77	5.45 ± 1.31	5.11 ± 0.49	NS
Kidney	18.94 ± 0.73^{a}	17.75 ± 0.70^{b}	18.88 ± 0.74^{a}	$P < 0.002$
Total muscle	20.65 ± 1.23	19.85 ± 1.46	21.11 ± 1.19	NS
Spleen	18.33 ± 0.59^{a}	16.80 ± 1.28^{b}	18.49 ± 0.82^{a}	$P < 0.001$
Thymus	25.79 ± 5.10	20.00 ± 3.48	25.17 ± 4.74	NS
Total protein content (g)				
Intestinal mucosa	0.11 ± 0.04	0.09 ± 0.04	0.11 ± 0.02	NS
Kidney	0.37 ± 0.02	0.39 ± 0.04	0.36 ± 0.03	NS
Total muscle	31.4 ± 2.4^{a}	28.2 ± 2.5^{b}	30.5 ± 1.6^{ab}	$P < 0.02$
Spleen	0.12 ± 0.01^{a}	0.16 ± 0.03^{b}	0.11 ± 0.02^{a}	$P < 0.0001$
Thymus	0.13 ± 0.04^{a}	0.07 ± 0.02^{b}	0.12 ± 0.04^{a}	$P < 0.005$

Values are expressed as mean ± SD. $n = 8$ in each group. Means in a row with different letters are significantly different ($P < 0.05$ or less, post hoc Tukey's test)

C control, *PF* pair fed, *NS* not significant

[1] One-way ANOVA

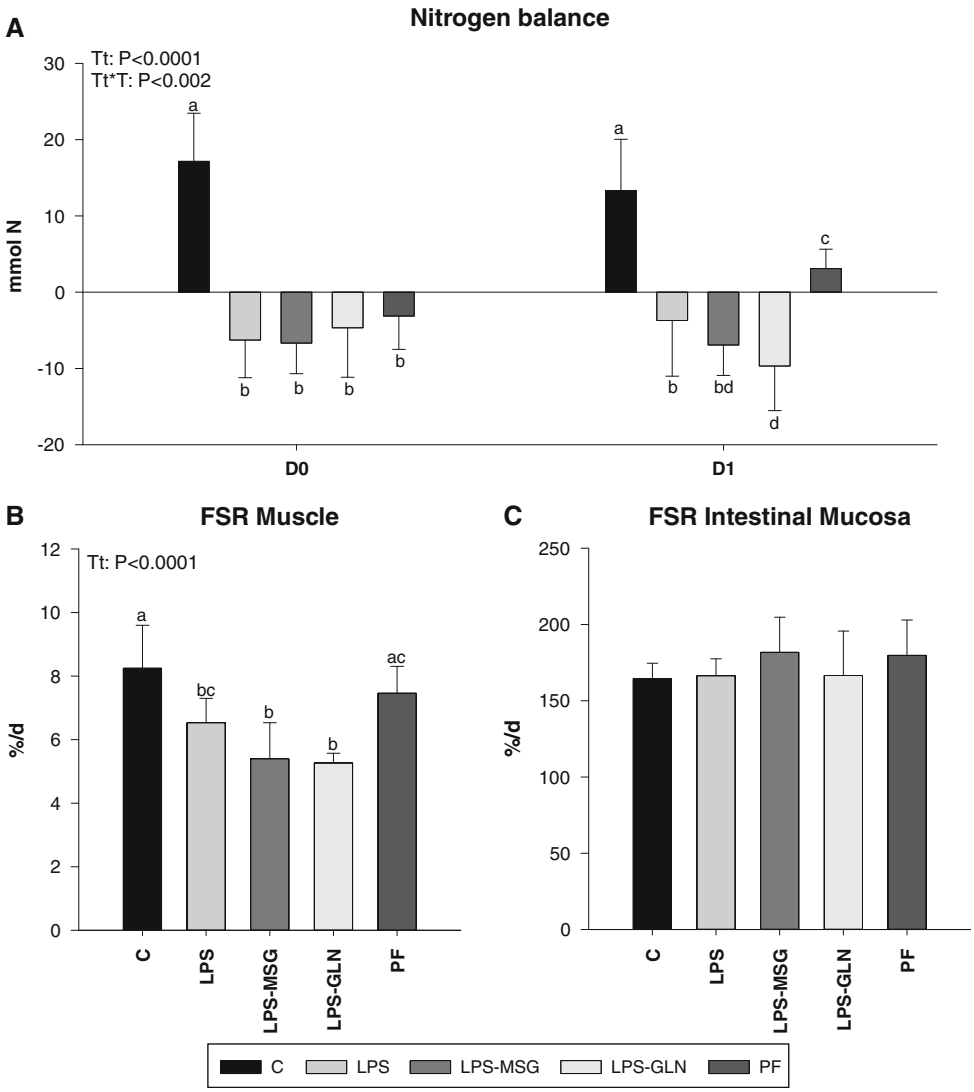

Fig. 3 Effects of LPS injection with or without monosodium glutamate (LPS-MSG) or glutamine (LPS-GLN) supplementation on the nitrogen balance (**a**) and protein fractional synthesis rate in muscle (**b**) and intestinal mucosa (**c**) in rats. Results are expressed as means ± SD. Nitrogen balance was determined on a 24-h period. *D0* indicates the day of LPS injection and *D1* indicates 1 day after LPS injection. Protein fractional synthesis rates (FSR) were measured at D2 (2 days after LPS injection). For nitrogen balance, the main statistical effects (T: time, Tt: treatment and their interaction T*Tt) from a mixed model for repeated measures over time are reported for each variable when $P < 0.05$. For fractional synthesis rate, we used one-way ANOVA. *Bars with different letters* are significantly different ($P < 0.05$ or less, post hoc Tukey's test), $n = 8$ in each group

failed to restore normal glutamate, glutamine or any of other related AA (Fig. 4).

When AA concentrations were measured in vena cava plasma 2 days after LPS injection (i.e., at D2), glutamine was the only AA whose concentration was significantly modified when compared with the PF group (Fig. 5b) with no effect of MSG or glutamine supplementation. In the portal blood plasma at D2 (2 days after LPS injection), among AA related to glutamine metabolism, LPS injection decreased significantly circulating citrulline concentration when compared to control groups (Table 4). However, citrulline concentrations in the LPS and PF group were not significantly different ($P = 0.0697$). MSG or GLN supplementation in LPS-treated rats did not significantly increase citrulline concentration in the portal blood plasma measured at D2 (Table 4). In addition, LPS injection was found to decrease glutamine concentration in the portal blood plasma when compared with values measured in pair-fed animals without any effect of MSG or GLN supplementation on this parameter.

Effect of LPS on tissue amino acid concentrations: effects of MSG and glutamine

In LPS group, when AA concentrations were measured in the intestinal mucosa at D2, no detectable change was recorded when compared with C and PF rats (Table 5). MSG but not glutamine supplementation resulted in 56% increase of glutamate concentration ($P < 0.02$ LPS-MSG vs. LPS) in the intestinal mucosa (Table 5). Others AA related to glutamate metabolism (glutamine, citrulline, alanine, ornithine and proline) were not modified in the intestinal mucosa by MSG supplementation.

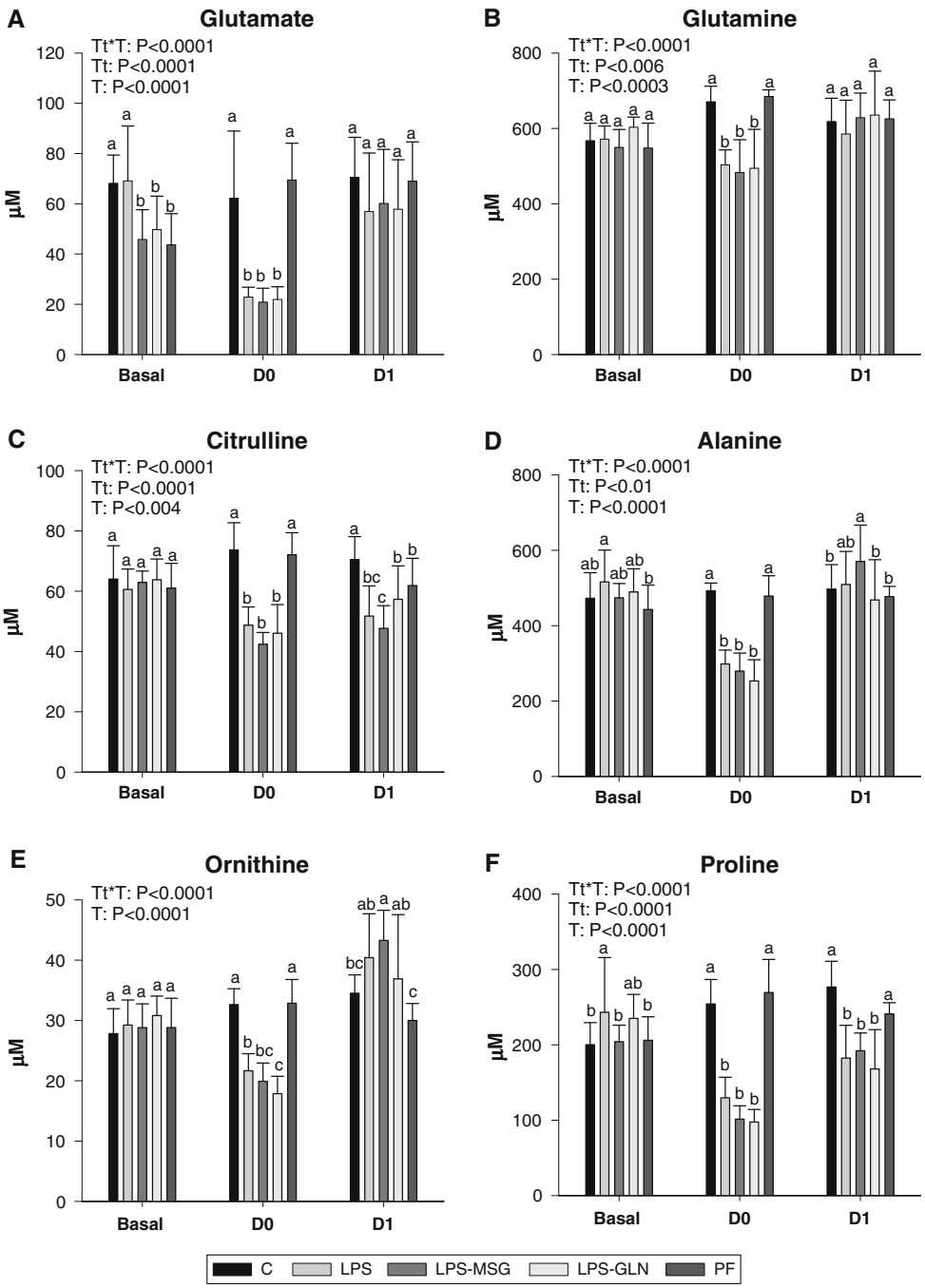

Fig. 4 Effects of LPS injection with or without monosodium glutamate (LPS-MSG) or glutamine (LPS-GLN) supplementation on glutamate (**a**), glutamine (**b**) and related amino acid concentrations (**c**, **d**, **e** and **f**) in plasma tail vein in rats. Results are expressed as means ± SD. *Basal values* indicate plasma concentrations 4 days before LPS injection; *D0* indicates plasma concentrations 2 h after LPS injection and *D1* indicates plasma concentrations 1 day after LPS injection. The main statistical effects (T: time, Tt: treatment and their interaction T*Tt) from a mixed model for repeated measures over time are reported for each variable when $P < 0.05$. *Bars with different letters* are significantly different ($P < 0.05$ or less), $n = 8$ in each group

In gastrocnemius muscle, glutamate, glutamine and citrulline (but not other related AA i.e., alanine, ornithine and proline) were significantly decreased 2 days after LPS injection when compared with both C and PF groups (Fig. 6). MSG and glutamine supplementation failed to restore glutamate, glutamine and citrulline concentration in the gastrocnemius muscle (Fig. 6). In the liver, no AA was significantly decreased at D2 after LPS injection when compared with control rats (data not shown).

Discussion

Our data clearly indicate that the dose of 0.3 mg LPS per kg BW is able to provoke classical sign of endotoxemia, i.e., anorexia, negative nitrogen balance, increased concentrations of pro-inflammatory cytokines, decreased gastrocnemius muscle mass and increased spleen weight. This dose, which is between 10 and 30-fold lower than the dose of LPS currently used in rats for inducing endotoxemia (Chambon-Savanovitch et al. 1999; Crouser et al. 2000;

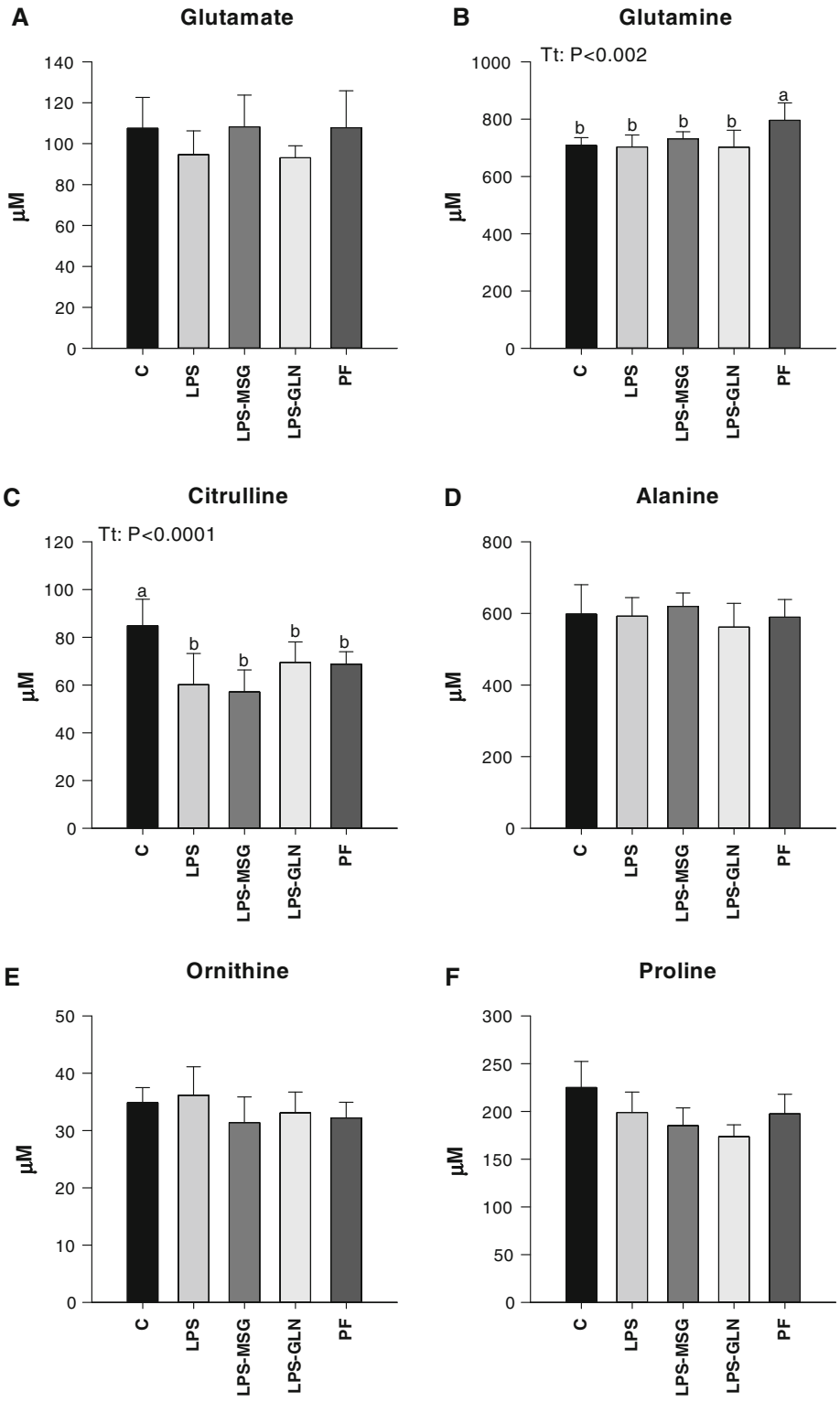

Fig. 5 Effects of LPS injection with or without monosodium glutamate (LPS-MSG) or glutamine (LPS-GLN) supplementation on glutamate (**a**), glutamine (**b**) and related amino acid concentrations (**c**, **d**, **e** and **f**) in plasma vena cava in rats. Results are expressed as means ± SD. AA concentrations were determined at D2 (2 days after LPS injection). The main statistical effect (Tt: treatment) from one-way ANOVA are reported for each variable when $P < 0.05$. *Bars with different letters* are significantly different ($P < 0.05$ or less, post hoc Tukey's test), $n = 8$ in each group

Kessel et al. 2008; Sukhotnik et al. 2007; Uehara et al. 2005), was without severe toxicity, since, it did not provoke any mortality among animals. Under such experimental conditions, almost all circulating AA concentrations were rapidly decreased when compared with values recorded in PF animals, but for most of them, returned back to control values at D1. Among AA related to glutamine and glutamate metabolism, only ornithine and proline remain modified at D1 when compared with the values measured in PF animals. At D2, glutamine was the only

Table 4 Effects of LPS injection on circulating amino acid concentration (in μM) in portal blood plasma at D2 (2 days after LPS injection) in rats

	C	LPS	LPS-MSG	LPS-GLN	PF	Stat effect[1]
Glutamate	126 ± 18	108 ± 19	134 ± 22	118 ± 27	117 ± 22	NS
Glutamine	564 ± 44^{a}	510 ± 34^{b}	529 ± 27^{b}	519 ± 58^{b}	565 ± 32^{a}	$P < 0.05$
Citrulline	111 ± 9^{a}	85 ± 10^{bc}	81 ± 11^{b}	97 ± 27^{abc}	105 ± 8^{ac}	$P < 0.002$
Alanine	683 ± 41^{ab}	732 ± 103^{ab}	803 ± 98^{a}	655 ± 103^{b}	760 ± 95^{ab}	$P < 0.03$
Ornithine	36 ± 3	35 ± 4	32 ± 2	35 ± 6	32 ± 3	NS
Proline	250 ± 38^{a}	224 ± 27^{ab}	205 ± 12^{b}	196 ± 29^{b}	232 ± 18^{ab}	$P < 0.003$

Values are expressed as mean ± SD. $n = 8$ in each group. Means in a row with different letters are significantly different ($P < 0.05$ or less, post hoc Tukey's test)

C control, *MSG* monosodium glutamate, *GLN* glutamine, *PF* pair fed, *NS* not significant

[1] One-way ANOVA

AA which remained significantly decreased when compared with PF animals.

From converging studies suggesting that citrulline plasma concentration is a good indicator of enterocyte function (Jianfeng et al. 2005), our data reinforce the view that the intestinal functions are rapidly but transiently altered in endotoxemia. Indeed, since the intestinal mucosa weight, protein content and protein synthesis rate were all unaffected at D2 after LPS injection, this indicates that the small intestine can probably rapidly recover after LPS injection in our experimental model. Since, the intestine is the main site for citrulline synthesis from glutamine, arginine, proline and glutamic acid, we propose that the decreased circulating concentration of citrulline (an AA not present in proteins) recorded 2 h after LPS injection is resulting from decreased luminal uptake and/or from decreased conversion in enterocytes of these AA precursors into citrulline.

Although the liver is known to be able to extract some citrulline (Beyreuther et al. 2007), an increased hepatic uptake of citrulline after LPS injection appears unlikely to explain the rapid decrease in concentration of circulating citrulline since urea concentration in urine was not modified 2 hours after LPS injection.

In gastrocnemius muscle, and in line with the previously published data (Biolo et al. 1997; Vesali et al. 2005), we found that endotoxemia was concomitant with decreased mass at D2 after LPS injection. The fact that these events were associated with decreased concentration of glutamine reinforces the view that glutamine concentration in muscles is an important parameter in determining the capacity for protein synthesis as already proposed (Jepson et al. 1988; MacLennan et al. 1987). We tested dietary supplements of glutamine or glutamate (in the form of MSG) to know if these AA are able to restore normal glutamine, glutamate and related AA concentrations in blood plasma and in muscles. In fact, chronic MSG supplementation has already been shown to increase glutamine plasma concentration in healthy rats (Boutry et al. 2011). In addition in isolated enterocytes, glutamate is able to decrease glutamine catabolism through inhibition of the glutaminase activity (Blachier et al. 1999). However in our experimental model, we did not observe a rapid increase of the circulating concentration of glutamine, glutamate and of other related AA after either MSG or glutamine supplementation. Since endotoxemia is known to decrease intestinal glutamine absorption (Salloum et al. 1991), and to a lesser extent

Table 5 Effects of LPS injection on amino acid concentration (in nmol/g of tissue) in intestinal mucosa at D2 (2 days after LPS injection) in rats

	C	LPS	LPS-MSG	LPS-GLN	PF	Stat effect[1]
Glutamate	2.22 ± 0.65^{a}	2.11 ± 0.89^{a}	3.29 ± 0.93^{b}	2.19 ± 0.52^{a}	2.07 ± 0.29^{a}	$P < 0.006$
Glutamine	0.90 ± 0.30	0.83 ± 0.43	1.01 ± 0.33	0.98 ± 0.24	0.93 ± 0.19	NS
Citrulline	0.13 ± 0.02	0.14 ± 0.07	0.14 ± 0.05	0.14 ± 0.03	0.14 ± 0.03	NS
Alanine	2.65 ± 0.80	2.37 ± 1.04	2.87 ± 0.95	2.58 ± 0.57	2.54 ± 0.52	NS
Ornithine	0.05 ± 0.01	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	NS
Proline	0.80 ± 0.20	0.75 ± 0.31	0.82 ± 0.22	0.82 ± 0.21	0.85 ± 0.17	NS

Values are expressed as mean ± SD. $n = 8$ in each group. Means in a row with different letters are significantly different ($P < 0.05$ or less, post hoc Tukey's test)

C control, *MSG* monosodium glutamate, *GLN* glutamine, *PF* pair fed, *NS* not significant

[1] One-way ANOVA

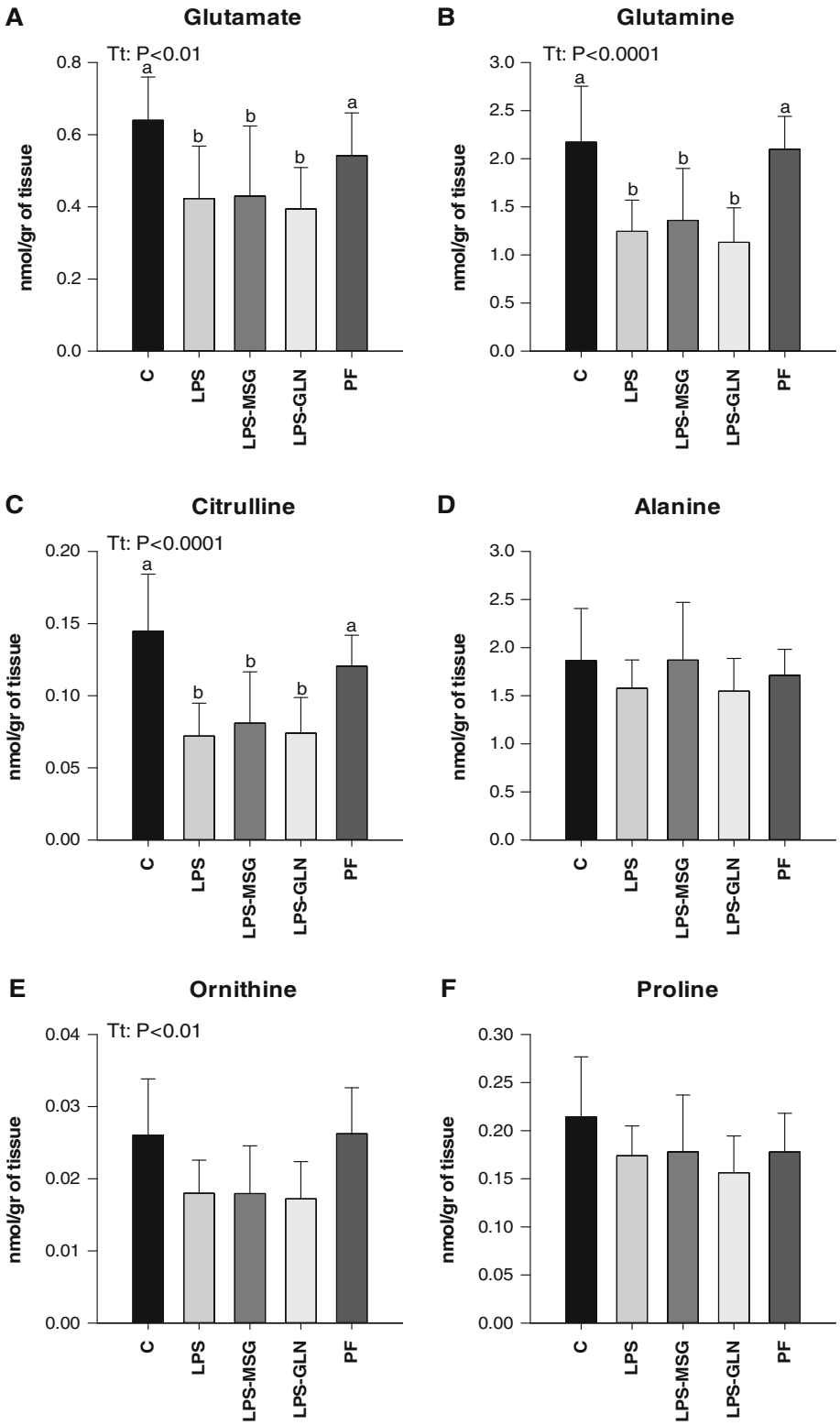

Fig. 6 Effects of LPS injection with or without monosodium glutamate (LPS-MSG) or glutamine (LPS-GLN) supplementation on glutamate (**a**), glutamine (**b**) and related amino acid concentrations (**c**, **d**, **e** and **f**) in gastrocnemius muscle in rats. Results are expressed as means ± SD. AA concentrations were determined at D2 (2 days after LPS injection). The main statistical effect (Tt: treatment) from one-way ANOVA are reported for each variable when $P < 0.05$. *Bars with different letters* are significantly different ($P < 0.05$ or less, post hoc Tukey's test), $n = 8$ in each group

glutamate absorption (Gardiner et al. 1995a), it may partly explain why glutamine and MSG failed to increase glutamine and glutamate circulating concentrations after LPS injection. Furthermore, high-enterocyte capacity for glutamine and glutamate utilization (Blachier et al. 1999), increase utilization of AA in the liver for selected protein biosynthesis (Biolo et al. 1997; Castell et al. 1989), and increase of AA deamination (documented in this study from increased plasma and urinary urea concentration 1 day after LPS treatment) likely also partly explain the

ineffectiveness of oral supplementation with glutamate or glutamine for restoring normal concentrations of glutamine in gastrocnemius muscle.

Our results, showing that glutamine is unable to restore the normal circulating concentration of citrulline shortly after LPS injection (and thus, presumably normal enterocyte function), are in contrast with several studies which indicated that glutamine supplementation decreases intestinal mucosal injury caused by endotoxemia (Haynes et al. 2009; Kessel et al. 2008; Sukhotnik et al. 2007; Uehara et al. 2005). However, it is worth to note that in these studies, glutamine supplementation was given as a pretreatment before endotoxin administration (prevention strategy) at difference with the protocol used in our study where oral supplementation was given immediately after LPS treatment (curative strategy). In addition, in the piglet model, it has been shown that oral supplementation with glutamine ameliorated the intestinal injury before LPS injection (Haynes et al. 2009) indicating that both time of glutamine supplementation and age of animals may be important parameters for explaining the effects of this AA.

In the meantime, the intestinal mucosa was able to accumulate glutamate after MSG supplementation. This preserved capacity of the intestinal mucosa of endotoxemic animals for glutamate accumulation, although impairing the capacity of supplemental MSG to rapidly restore the normal glutamate circulating concentration, may correspond to the fact that glutamate is a major excitatory neurotransmitter in the central nervous system (Moult 2009). Indeed, even if it is generally considered that except in case of impaired blood/brain barrier function, virtually no glutamate is transported from blood to brain (Beyreuther et al. 2007), one has to recall the results obtained by Olney and Sharpe (1969) more than 40 years ago. These authors found that the subcutaneous injection of large dose of MSG provoked neuronal lesions in the brain of young monkeys. Interestingly, when the MSG was given through gastric canulae (thus allowing intestinal metabolism and accumulation), such lesions were not observed (Reynolds et al. 1971); reinforcing the view that intestine play a major role in regulating the concentration of circulating glutamate. In other words, the high capacity of the small intestine mucosa for glutamate catabolism and accumulation (and capacity of the liver to synthesize glutamine from glutamate, Brosnan and Brosnan 2009) likely corresponds to both the supply of an important fuel for enterocytes and a way to limit the entry of this AA in the peripheral blood (Hinoi et al. 2004). In our study, glutamate concentration measured in the portal vein 2 days after LPS injection was not increased after MSG supplementation in endotoxemic animals confirming extensive intestinal catabolism and accumulation. The capacity of the intestine to accumulate glutamate is in line with the measurement of relatively high concentration of glutamate in enterocytes and intestinal mucosa in both healthy and endotoxemic animals (Blachier et al. 1991; van der Hulst et al. 1996, and this study).

In summary, the present study indicates that mild non lethal endotoxemia is associated with rapid decrease in concentration of circulating AA including citrulline. The decreased circulating concentration of this latter AA is considered to be associated with a decrease of the enterocyte function. Our data indicate that oral supplementation of endotoxemic animals with either glutamine or MSG is ineffective in an attempt to counteract the rapid decrease of glutamine, glutamate and citrulline plasma concentrations and, later on concentrations of these AA in muscle (and then presumably muscle protein synthesis). From this conclusion, it would appear more strategic in endotoxemia, to use glutamine or glutamate as a parenteral supplement for restoring normal glutamine concentration in muscles as long as the small intestine enterocyte absorptive function is altered.

Acknowledgments We gratefully thank Catherine Luengo and Véronique Mathé for expert technical assistance. We wish to thank Ms. T. Kuwahara for her excellent technical assistance with the amino acid analysis. The project was supported by funds from Ajinomoto Co. (Japan) and by grants from the National Institute for Agronomic Research (INRA, France).

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