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Insight into the involvement of Kupffer cell-derived mediators in the hepatoprotective effect of glycine upon inflammation: study on rat precision-cut liver slices

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Abstract. *Objective and design:* To investigate the role of inflammatory mediators in the hepatoprotective effect of glycine against lipopolysaccharide (LPS)-induced liver injury in rats.
Material or subjects: Male Wistar rats were used (N = 4 or 5 per group). Precision-cut liver slices (PCLS) were prepared for in vitro studies.
Treatment: Glycine (10 mM) and LPS (10 μg/ml) were added to the incubation medium of PCLS obtained 3 h after LPS intraperitoneal (i.p.) administration (10 mg/kg) or saline injection to rats. Glycine effects were also investigated in vivo by treating rats with a diet containing glycine (5%) during 3 days.
Methods: Tissue injury was assessed by measuring adenosine triphosphate (ATP) and glycogen contents of liver tissue as well as by measuring aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) activity in the medium (in vitro) or in the serum (in vivo). Tumor necrosis factor-alpha (TNF-α), prostaglandin E_2 (PGE_2) and NOx (reflecting nitric oxide production) were measured in the incubation medium or in the serum. Histological detection of both ED-2 and peroxidase activity were used as Kupffer cell markers. Student *t* test or two-way ANOVA were used for statistic analysis.
Results: Glycine added to the culture medium increased both ATP and glycogen contents of PCLS from LPS-treated rats, reduced the production of TNF-α and NOx whereas PGE_2 secretion by PCLS increased. In contrast to the in vitro effect of glycine, we observed that a glycine-enriched diet decreased PGE_2 secretion in the serum after LPS challenge.
Conclusion: The effect of glycine on LPS-induced mediator secretion is different considering in vitro or in vivo situations. Interestingly, glycine in vitro is able to prevent energy status depletion of PCLS occurring upon inflammation, a phenomenon probably linked to change in inflammatory mediator secretion pattern by hepatic immune cells, namely Kupffer cells.

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Key words: Kupffer cells – LPS – Glycine – Precision-cut liver slices – Prostaglandin E_2

Introduction

Gram-negative sepsis remains an important cause of morbidity and mortality in intensive care units despite recent advances in critical care [1]. The arrays of pathophysiologic features that accompany Gram-negative bacterial sepsis appear to be qualitatively similar to those encountered after LPS insult [1–3]. LPS, a component of the Gram-negative bacterial cell wall, is responsible for initiating a series of a highly complex cascading events leading to damage in multiple organs, including the liver. Although liver failure is generally thought to be a late complication after pulmonary and renal failure during sepsis, some studies indicate that hepatocellular function is depressed early after the onset of sepsis and persists during the late stage of sepsis [4]. The injection of high doses of LPS to rats can lead to death within 24 h or, in surviving animals, induce an acute-phase response in the liver accompanied by several metabolic changes such as depletion of both hepatic glycogen and ATP content [5–6].

Kupffer cells, the resident macrophages in the liver, represent 80–90% of fixed macrophages of the whole body and play a major role in removal of LPS from the bloodstream [7]. It is well known that LPS activates macrophages -in particular Kupffer cells- to release inflammatory mediators such as proinflammatory cytokines (TNF-α) and reactive intermediates ($O_2^{\cdot-}$, $NO^{\cdot}$), which may participate in the mechanism of septic choc [7–10]. Macrophages are also able to secrete immunosuppressive mediators, such as interleukin 10 or PGE_2. The latter can protect against LPS-induced liver injury, namely by down-regulating the production of pro-inflammatory cytokines [4, 11].

Glycine, a nonessential amino acid, has been shown to have beneficial effects on the liver under various patho-physiological conditions. Previous studies reported protection by

glycine against hepatocyte alterations induced by hypoxia and ischemia and various cytotoxic substances [12–14]. It also prevents reperfusion injury in a low-flow, reflow liver perfusion model in the rat and improves graft survival after liver transplantation [15–16]. Interestingly, a diet enriched with glycine minimizes LPS-induced liver and lung injury and reduces mortality due to LPS in the rat [17].

The purpose of this study was to analyse the mechanism by which glycine can protect against LPS-induced tissue damage. We propose an in vitro approach, by using the model of precision-cut liver slices (PCLS) in culture. This original model allows maintaining liver architecture as well as cellular heterogeneity and interactions. Moreover, we have recently shown that PCLS is convenient model to assess the role of Kupffer cell-derived mediators in hepatic metabolism [18–20]. In the present study, we wanted to determine whether glycine, present in the incubation medium of PCLS obtained from LPS-treated rats, could affect ATP and/or glycogen content as well as lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in the incubation medium. Those parameters are known to be markers of PCLS viability on one hand [21], and to be altered by LPS, on the other hand [5–6, 22]. We analysed 3 key inflammatory mediators, i.e. TNF-α, NOx (reflecting NO$^{\cdot}$ release) and PGE_2 in the incubation medium of PCLS in order to assess their role in the glycine effects. Furthermore, we compared results obtained in vitro with the effect of glycine given in the diet during 3 days before LPS administration in rats in order to provide further insight into the putative usefulness of glycine in therapeutical approaches in septic shock. TNF-α, PGE_2 and NOx were measured in the serum. Furthermore, we performed immuno-histochemical detection of ED2, a membrane antigen exclusively present in Kupffer cells in the liver tissue [23]. The histochemical detection of endogenous peroxidase activity, responsible for oxygen radical formation, was assessed since it is considered as a relevant marker for the reticuloendothelial system and phagocytes such as Kupffer cells [24].

Materials and methods

Chemicals

Most chemicals of the purest grade available were purchased from Sigma (St Louis, MO) or Roche Diagnostics Belgium. William's medium E (containing 0.67 mM of glycine), L-glutamine, penicillin and streptomycin were purchased from Invitrogen (Belgium).

Animals

Male Wistar rats weighing 240–280 g (Harlan, The Netherlands) were housed in individual cages in a room with temperature control and an automatic 12 h light:dark cycle. After one week of adaptation, rats were used for experiments. Rat surgical procedures were carried out under pentobarbital (60 mg/kg) anaesthesia. All rats received care in compliance with institution's guidelines from the National Academy of Sciences (NIH publication 86–23; http://www.nih.gov). Experiments were approved by the Ethic Committee of the University Catholic of Louvain, Brussels, Belgium.

Study on precision-cut liver slices (PCLS)

PCLS were prepared from control rats (n = 5) or 3 h after LPS (Sigma, St Louis, MO) injection to rats (10 mg/kg i.p., n = 5). Perfusion in situ of the liver of rats was performed with ice-cold Krebs-Ringer solution prior to liver removal. Tissue cores (diameter 10 mm) were prepared from the freshly excised liver and PCLS (about 220 µm thickness) were rapidly prepared in oxygenated ice-cold Krebs-Ringer buffer using a Krumdiek slicer according to a procedure previously described [18, 25]. After 30 min of preincubation at 4 °C in William's E medium (containing 0.67 mM of glycine) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 100 nM insulin, 0.3% bovine serum albumin (BSA) (Invitrogen, Belgium), PCLS were transferred into fresh medium and incubation lasted for 4 h in the presence or in the absence of 10 mM glycine (Sigma, St Louis, MO); when PCLS were prepared from control rats, they were additionally incubated in the presence or in the absence of LPS (10 µg/ml). Two vials containing 2 slices (2 ml medium per slice) were used for each condition; they were saturated with 95% O_2–5% CO_2 and placed in a shaking water bath at 37 °C.

In vivo study

Rats were fed a powdered AO4 standard diet (UAR, France) containing either 5% glycine or 5% caseine (control diet) during 3 days. 3 h after LPS injection to rats (10 mg/kg i.p.). Blood was collected from the vena cava without anticoagulant and centrifuged 2 min at 13 000 g in order to obtain serum; aliquots of serum were stored at –80°C for further analysis. Liver pieces were stored at –80 °C for further histological analysis.

Enzyme activities

LDH, AST and ALT activities were measured in frozen aliquots of serum or incubation medium by standardised enzymatic procedures using kits from Elitec (France).

Glycogen content

Liver pieces or PCLS were sonicated in 1M KOH. They were further heated at 100 °C for 10 min. After neutralization with acetic acid and centrifugation, the supernatant was incubated in the presence of α-amylo glucosidase (Sigma, St Louis, MO) in pH 5 acetate buffer; the glucose produced was quantified by an enzymatic reaction as previously described [26].

ATP content

Liver pieces or PCLS were sonicated in 2% perchloric acid. The intracellular ATP content was measured on neutralized perchloric acid extracts by using ATP Bioluminescence Assay Kit CLS II® (Boehringer, Germany).

TNF-α, PGE_2 and NOx measurements

PGE_2 and TNF-α concentrations were measured in frozen aliquots of serum or incubation medium by immunoassay kits (PGE_2 Immunoassay, DE0100® and Quantikine rat TNF-α immunoassay, RTA00® from R&D System, UK). NOx concentration (reflecting NO$^{\cdot}$ production) was measured by the Griess reaction after nitrate reduction into nitrite [27].

ED2-staining

After air drying for 10 min, cryostat sections (10 µm) were fixed in acetone with 0.2% formaldehyde at 4°C for 2 min. After incubation in phosphate-buffered saline pH 7.4 (PBS) with 10% BSA for 30 min at room temperature, sections were incubated overnight at 4°C with mouse anti-rat ED2 antibody (DPC, Belgium) at dilution 1:400 in PBS with 1% BSA. Sections were washed thoroughly in PBS, incubated in methanol with 0.1% H_2O_2 for 10 min (in order to inhibit endogenous peroxidase activity), and then incubated for 1 h at room temperature with peroxidase-conjugated rabbit anti-mouse antibody (DakoCytomation, Denmark) diluted 1:200 in PBS with 1% BSA. After thoroughly rinsing in PBS, sections were incubated in two bath of 0.05 M Tris-HCl buffer (pH 7.4) containing 0.25% triton for 5 min. Peroxidase activity was revealed with 3,3'-diaminobenzidine-tetrahydrochloride (DAB) (Sigma, St Louis, MO) in Tris-HCl buffer containing 0.01% H_2O_2 for 10 min. After rinsing in running water, samples were counterstained with Mayer's Haemalun (VWR International, Belgium), dehydrated and mounted with DePeX mounting medium (Agar Scienific, UK). Control sections were incubated in PBS-BSA instead of primary antibody in the first step, with conjugate in the second step, and examined for non-specific staining.

Peroxidase activity in the liver

After air drying for 10 min, cryostat sections (10 µm) were incubated in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.02% H_2O_2, 0.05% DAB and 7% sucrose for 20 min at 25°C in a shaking bath [28]. After rinsing in Tris-HCl buffer, samples were counterstained with Mayer's Haemalun, dehydrated and mounted with DePeX mounting medium.

Statistical analysis

All measurements were carried out in duplicate. Values are presented either individually (with a mean value) or as the mean ± SEM. Statistical significance of observed variations was performed either by Student *t* test or two-way ANOVA with the factor glycine in vitro (gly+ and gly–) and the factor LPS (LPS+ and LPS–), using SPSS statistical program for Windows system (SPSS, Chicago, IL). When variances were heterogeneous, logarithmic transformations of individual values were used before statistical analysis. Data were considered statistically significant at the $p < 0.05$ level.

Results

Effect of glycine added in vitro on integrity of PCLS obtained from LPS-treated rats

The viability of PCLS was assessed by measuring ATP content and glycogen content of PCLS as well as by LDH, ALT and AST release in the medium after 4 h of incubation. The presence of glycine in the medium allowed maintaining higher contents of both ATP and glycogen in PCLS obtained from rats 3 h after LPS administration (Fig. 1). However, the release of LDH, ALT and AST in the medium was not affected by the presence of glycine (data not shown).

Effect of glycine added in vitro on inflammatory mediator release by PCLS

Inflammatory mediators were measured in the medium of PCLS after 4 h of incubation. TNF-α and NOx secretion by the liver tissue from LPS-treated rats was lower whereas PGE_2 release increased when glycine was present in the incubation medium (Fig. 2).

These mediators were also measured in the incubation medium of PCLS obtained from control rats (not previously injected with LPS), in the presence or in the absence of LPS challenge added to the medium (Fig. 3). Unstimulated-PCLS released significant amounts of TNF-α, NOx and PGE_2. The presence of LPS in the medium increased TNF-α release by PCLS. The presence of glycine reduced the basal and the LPS-stimulated production of TNF-α. NOx and PGE_2 production was affected neither by glycine nor by LPS in vitro.

Effect of a diet enriched with glycine before LPS challenge: an in vivo study

Detection of peroxidase activity in the liver tissue was performed in untreated-rats or 3 h after LPS administration to

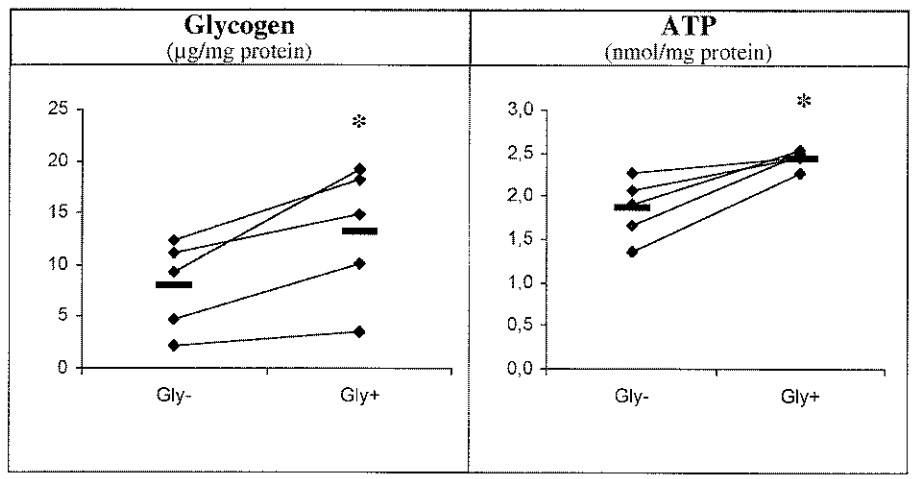

Fig. 1. Effect of glycine added to the culture medium on ATP and glycogen contents of PCLS obtained from LPS-treated rats. PCLS were prepared 3 h after LPS administration (10 mg/kg i.p.) to rats and incubated in the presence (Gly+) or in the absence (Gly–) of glycine (10 mM) for 4 h. Individual values (bar = mean value). *$p < 0.05$ (Student *t* test).

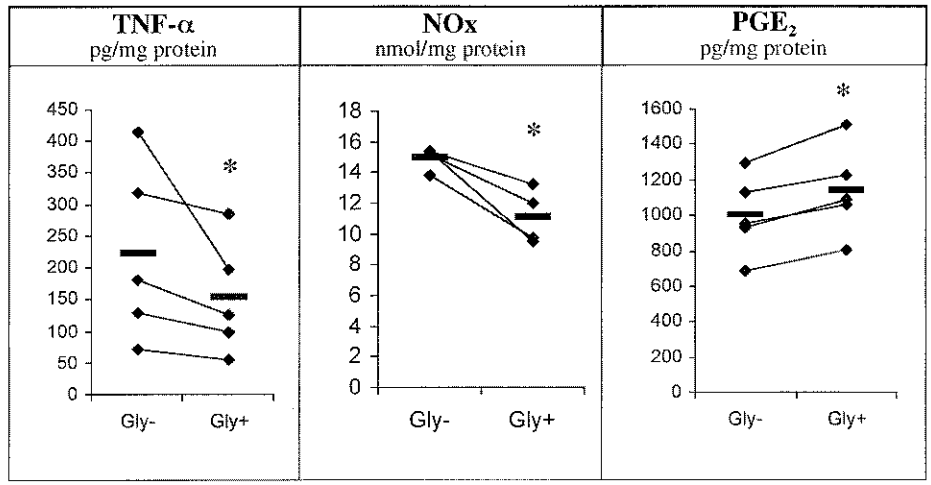

Fig. 2. Effect of glycine added to the culture medium on inflammatory mediators released by PCLS obtained from LPS-treated rats. PCLS were prepared 3 h after LPS administration (10 mg/kg i.p.) to rats and incubated in the presence (Gly+) or in the absence (Gly–) of glycine (10 mM) for 4 h. Individual values (bar = mean value). *$p < 0.05$ (Student *t* test).

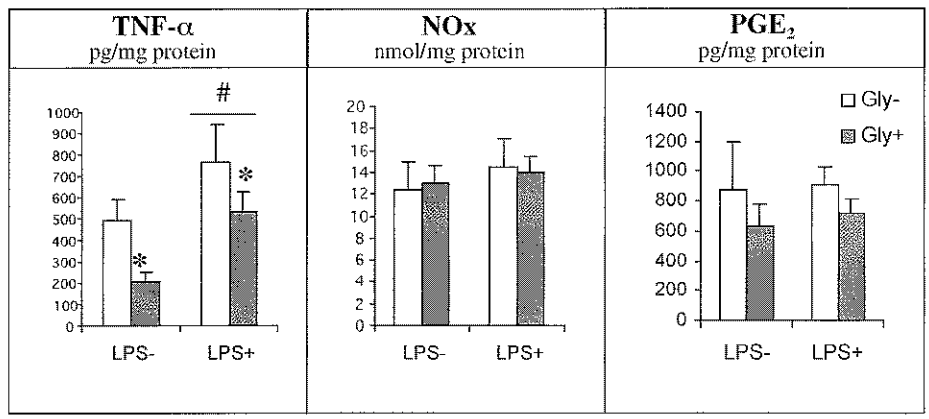

Fig. 3. Effect of glycine added to the culture medium on inflammatory mediators released by PCLS obtained from control rats. PCLS were incubated in the presence (LPS+) or in the absence (LPS–) of LPS (10 µg/ml) as well as in the presence (Gly+) or in the absence (Gly–) of glycine (10 mM) for 4 h. Values are means ± SEM ($n \geq 4$). *significant effect of glycine, #significant effect of LPS ($p < 0.05$; ANOVA).

rats that had been fed either a glycine-enriched or a casein-enriched diet (= control diet) for 3 days. Positive cells for endogenous peroxidase in control livers are mainly located in the periportal zone (fig. 4A). LPS treatment increased the number of peroxidase-positive cells near to the central vein as compared to rats injected with NaCl 0.09% (fig. 4A, B and C). Peroxidase-positive cell accumulation in the liver after LPS challenge was not affected by the glycine-enriched diet as compared to the control diet. ED2-staining was also performed in the liver in order to detect membrane antigen of fixed tissue macrophages, namely Kupffer cells (fig. 4D, E and F). Kupffer cells were present in the whole parenchyma in control livers. They were swollen after LPS administration independently of the diet as compared to NaCl-injected rats. However the distribution of ED2-staining macrophages after LPS challenge was changed, into a less dense pattern, in animals having received the supplementation of glycine (fig. 4F). Dietary glycine decreased PGE_2 levels in the serum 3 h after LPS injection as compared to control dietary treatment, without significantly affecting the secretion of TNF-α and NOx (fig. 5). In LPS-treated rats, hepatic contents in glycogen and ATP as well as the release of LDH, ALT and AST in the serum were not affected by dietary glycine (data not shown).

Discussion

The liver is thought to be a major organ in the development of multiple organ dysfunction after sepsis [29, 30]. Several studies have been reported, suggesting the beneficial effects of glycine on cellular and organ injury, as well as on survival after endotoxic shock [14, 17]. Are hepatic immune cells-derived molecules implicated in this protective effect? Our results are in favour of this hypothesis. We have used PCLS, an in vitro model in which Kupffer cells are viable and metabolically active [18, 19]. We show here that in PCLS obtained from LPS-treated rats, the content in ATP and glycogen upon

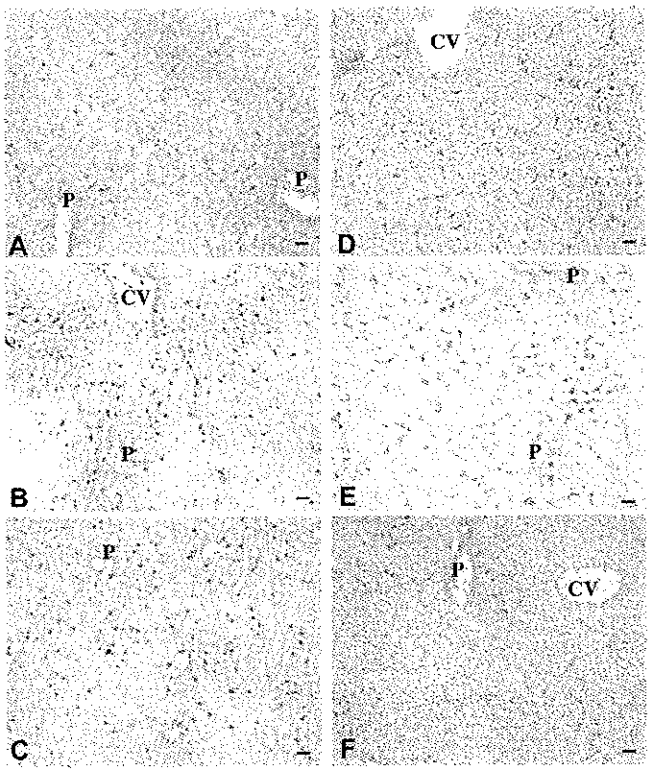

Fig. 4. Histochemical detection of endogenous peroxidase activity (A, B and C) and immuno-histochemical detection of ED2 (D, E and F) in the liver. Control rats having received a control diet (A and D); 3 h after LPS administration (10 mg/kg i.p.), in rats previously fed a diet with 5% caseine during 3 days (B and E); and, 3 h after LPS administration (10 mg/kg i.p.) in rats previously fed a diet with 5% glycine during 3 days (C and F). All ED2-positive cells are Kupffer cells. P, portal space; CV, central vein. Bar = 50 µm.

incubation is higher when incubated in the presence of glycine. Despite a lack of effect on cytosolic enzyme release (LDH, AST and ALT) in the medium, it clearly reflects an improvement of energy status of liver cells by glycine.

We have previously shown that $GdCl_3$ injection – which depleted Kupffer cells – prior to PCLS preparation allowed to maintain a higher ATP concentration in PCLS whereas TNF-α release was lower, suggesting that such a cytokine, derived from Kupffer cells, altered PCLS integrity upon incubation [18]. We show here that glycine increases ATP and glycogen content, and decreases TNF-α secretion and NOx production, when added in the culture medium of PCLS prepared from LPS-treated rats. This supports the idea that the prevention of both ATP and glycogen depletion in PCLS by glycine is, in part, mediated through inhibition of cytotoxic mediator (TNF-α) and reactive intermediates (NO$^{\cdot}$) release by Kupffer cells. To establish whether alterations of ATP and glycogen contents occurring upon LPS stimulation are primarly mediated by TNF-α secretion and/or NO$^{\cdot}$ release, the incubation of PCLS in the presence of anti-TNF-α antibodies or L-NAME (N-nitro-L-arginine methyl ester) -an inhibitor of NOS activity- should be performed.

Our study also supports a role of PGE_2 in the protective effect of glycine in vitro: PGE_2 production by PCLS obtained from LPS-treated rats increases in the presence of glycine. Indeed, it was shown, on the one hand, that PGE_2 abolishes LPS-induced TNF-α secretion by Kupffer cells in culture after 4 h of incubation [7]. On the other hand, PGE_2 has been reported to inhibit the glucagon-stimulated glycogen-phosphorylase activity (antiglycogenolytic effect) in rat hepatocytes [31].

The effect of glycine in vitro on mediator secretion was less pronounced if rats did not receive an injection of LPS prior to PCLS preparation: only TNF-α secretion was reduced independently of the presence of LPS in vitro. The different pattern in mediator secretion could be related to the accumulation and activation of leukocytes in liver tissue after LPS administration, such as neutrophils or monocytes (as suggested by the enhanced peroxidase activity in the liver). Indeed, in rat, an influx of neutrophils in the liver has been observed within 3–6 h after the administration of endotoxin from *Escherichia coli* [8]. Their functions at sites of infection include phagocytosis and production of toxic which facilitate the elimination of invading organisms or endotoxin but can also participate in severe tissue damage [32]. Froh et al. demonstrated that Kupffer cells, as well as other macrophages and leukocytes, express mRNA and protein for glycine-gated chloride channel (GlyR). However, heterogeneity of the GlyR α-subunit, existing within monocyte/

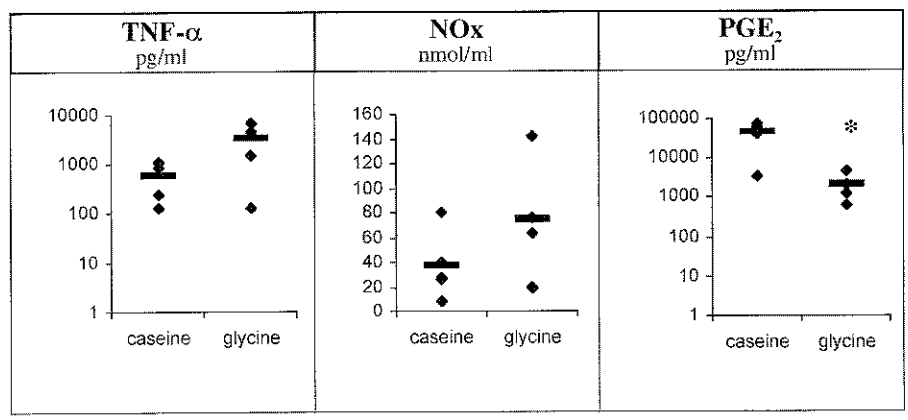

Fig. 5. Effect of a diet enriched with glycine on inflammatory mediators released in the serum 3 h after LPS administration. TNF-α, NOx and PGE_2 concentrations in the serum 3 h after LPS injection (10 mg/kg i.p.) in rats previously fed a diet with either 5% caseine or 5% glycine during 3 days before LPS challenge. Individual values (bar = mean value). *$p < 0.05$ (Student *t* test).

macrophage lineage population, possibly creates a functional diversity between these cells [33]. This phenomenon may lead to differential glycine effects on mediator secretion if we consider Kupffer cells alone in PCLS obtained from non-treated rats or both Kupffer cells and recruited-immune cells in PCLS from LPS-treated rats.

In a recent study, we have shown that the addition of fructan-type fiber in the diet of rats (oligofructose) was able to affect intra-hepatic immune cells and reduced hepatic tissue injury due to LPS [34]. Could dietary glycine supplementation have an effect on LPS-induced injury in vivo?

Ikejima et al. reported that a diet containing glycine (5% for 3 days) minimized LPS-liver injury and reduced mortality due to LPS in the rat [17]. Moreover, serum TNF-α levels were blunted by a supplementation of glycine in the diet 1 h after intravenous injection of LPS as compared to control diet. In our study, we show that dietary glycine (5% for 3 days) decreases Kupffer cells in the liver, assessed by ED2 staining at least in LPS-treated animals. However, dietary glycine failed to significantly modify serum TNF-α and NOx concentrations as well as ATP and glycogen contents in liver tissue 3 h after LPS challenge. Several hypothesis may be proposed. Our in vivo protocol differed from Ikejima et al. considering the composition of the control diet (5% caseine versus 20%, respectively), the route of LPS administration (i.p. versus i.v., respectively) and the time of experiment after LPS injection (3 h versus 24 h, respectively).

In addition, in contrast to the in vitro effect of glycine on PCLS, we observed that a glycine-enriched diet decreased PGE_2 secretion in the serum after LPS challenge. This last result suggests that the effect of glycine on LPS-induced mediator secretion is different considering in vitro or in vivo situations. In vivo, Kupffer cells are not the sole source for PGE_2 secretion; other extra-hepatic immune cell exhibit GlyR and may influence PGE_2 production [33, 35]. The dose-duration effect of glycine in vivo might also explain some discrepancies between studies. Although PCLS were incubated with 10 mM glycine which is compatible with the concentration found in plasma after the glycine-enriched diet [17], the hepatic concentration of glycine after the dietary supplementation is unknown.

In conclusion, if beneficial effects of glycine on liver function as well as on survival after endotoxic shock have been previously described in vivo, this is the first study showing that this amino acid may counteract, when added in vitro, PCLS alterations previously generated through LPS injection. This phenomenon could be related to a specific pattern of mediator secretion by immune cells present in the liver tissue, i.e. Kupffer cells.

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