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ORIGINAL CONTRIBUTION

Glutamine attenuates nitric oxide synthase expression and mitochondria membrane potential decrease in interleukin-1β-activated rat hepatocytes

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Abstract
Background Mitochondrial dysfunction induced by nitric oxide (NO) overproduction is involved in the pathogenesis of organ failure during many severe diseases. Recently, several experiments have reported that glutamine (Gln) modifies inducible nitric oxide synthase (iNOS) gene expression which is thought to be responsible for its beneficial effects in critical illnesses.
Aim of the study This study was designed to evaluate the effects of Gln on NO production, iNOS expression and mitochondria membrane potential ($\Delta\psi$m) variation in interleukin (IL)-1β-activated rat hepatocytes. The mechanical effects of nuclear factor kappaB (NF-κB) were also investigated.
Methods Primary cultured rat hepatocytes were isolated by an in situ collagenase perfusion method. The cultured hepatocytes were treated with IL-1β (1 nmol/l) alone or with Gln in concentrations ranging from 2 to 10 mmol/l for 24 h. The concentrations of alanine aminotransferase (ALT) and nitrite in the culture medium were detected by biochemical methods. The levels of iNOS protein and mRNA in the hepatocytes were analyzed by western blot and real-time RT-PCR. The $\Delta\psi$m of the hepatocytes was assessed with flow cytometry after incubating with fluorescent probe JC-1. Th binding activity of NF-κB in hepatocytes was investigated using electrophoretic mobility shift assay.
Results IL-1β significantly enhanced iNOS protein and mRNA level in the cultured rat hepatocytes which consisted with the increased NO production and ALT releasing in the culture medium. These effects were inhibited by the treatment of glutamine in a dose-dependent manner. The $\Delta\psi$m of hepatocytes was markedly decreased after IL-1β stimulation which was significantly attenuated by Gln at 5 and 10 mmol/l. NF-κB activity was increased by IL-1β stimulation and this effect was augmented by Gln at 5 and 10 mol/l.
Conclusions These observations indicate that Gln has a capability to protect mitochondria function in hepatocytes under inflammatory stress. Although the molecular mechanism remains to be clarified, our findings suggest this effect may be related to down-regulation of iNOS gene expression through an NF-κB independent pathway.

Keywords Glutamine · Interleukin-1β · Nitric oxide · Mitochondria membrane potential · Rat hepatocytes

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Introduction

Liver dysfunction is a common situation in the clinical course of sepsis; mechanisms by which excessive inflammatory response results in hepatocytes damage are still uncertain. Interleukin-1β (IL-1β) has been proposed to mediate the cellular and organ injury by promoting inducible nitric oxide synthase (iNOS) expression and NO production under the condition of inflammation in liver [2, 16, 21]. We previously found that stimulation of NO in cultured hepatocytes led to correlative reduction of mitochondria membrane potential ($\Delta\psi$m) and cell viability (unpublished), indicating that mitochondria dysfunction caused by NO overproduction may be the potential mechanism of inflammatory stress induced liver injury.

Pharmacologic glutamine (Gln) supplementation is known to decrease infectious complications, shorten hospital stay, and decrease hospital costs in a number of patient populations [8, 12]. Previous studies have reported that Gln inhibits NO synthesis in many cell types, including macrophages, endothelial cells, intestinal cells and hepatocytes. Recently, the regulatory potential of Gln for iNOS expression was also recognized [19, 24]. In this study, primary cultured rat hepatocytes stimulated with IL-1β were used as a model for iNOS induction and mitochondria depolarization. We tested whether the IL-1β induced iNOS gene overexpression and $\Delta\psi$m reduction could be prevented by Gln administration in vitro. The influences of Gln on the major transcriptional nuclear factor-κB (NF-κB) was also investigated because it was required for the induction of iNOS gene expression in rat hepatocytes [10, 16].

Materials and methods

Materials

Recombinant rat IL-1β was purchased from PeproTech (Princeton, USA). The IL-1β had a purity greater than 98% by SDS-PAGE gel and HPLC analyses and contained less than 0.1 ng of endotoxin per μg protein. Collagenase IV and JC-1 were purchased from Invitrogen (Carlsbad, USA). L-glutamine was purchased from Sigma (St. Louis, USA).

Hepatocyte isolation

Hepatocytes were isolated from the livers of 6-week-old Sprague–Dawley rats weighing 180–220 g (Institute of Laboratory Animal Sciences, Shanghai, P. R. China, water ad libitum) by a recirculating in situ collagenase (0.03%) perfusion method as described previously [22]. All procedures were approved by the Institutional Animal Care Committee of Southeast University. Hepatocytes were separated from nonparenchymal cells by centrifugation three times at 50*g*. Hepatocyte purity assessed by routine hematoxylin and eosin histochemical stain was >98% and cell viability consistently exceeded 90% by the trypan blue exclusion test.

Cell culture and treatment

Isolated hepatocytes were suspended in culture medium at 5–6 $\times$ 10^5 cells/ml, seeded onto plastic dishes (2 ml per dish. 35 $\times$ 10 mm.) and then cultured as monolayer in a 5% CO_2 humidified incubator at 37 °C. The culture medium used was RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 unit/ml), streptomycin (0.1 mg/ml) and insulin (0.16 unit/ml). After 24 h, the medium was replaced by fresh serum medium (2 ml) and cells were treated by IL-1β (1 nmol/l) alone or with glutamine (2.5 or 10 mmol/l). The culture medium was used as blanks. After incubated for 24 h, culture medium and hepatocytes were collected and frozen at −80 °C.

Alanine aminotransferase (ALT) measurement

The concentration of ALT in the culture medium, which indicated the severity of hepatocyte injury, was measured using commercially available assay kits from Sigma (St. Louis, USA) according to the manufacturer's instructions.

Nitrite determination

Accumulation of nitrite in the medium was used as a measure of NO formation. Nitrite was determined by the Griess method, adapted from Green et al. [11].

Determination of mitochondrial membrane potential ($\Delta\psi$m)

$\Delta\psi$m was measured by using the lipophilic cation JC-1, which potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (JC-1 at monomeric form, 527 nm) to red (JC-1 at aggregative form, 590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. For each sample, cells were suspended in 1 ml warm PBS at approximately 1 $\times$ 10^6 cells/ml and incubated in complete medium with JC-1 (10 μg/ml) for 10 min at 37 °C in the dark. Cells were then washed in PBS, resuspended in 1 ml incubation buffer without JC-1, and immediately analyzed on a flow cytometer (BD, Franklin Lakes, USA) with 488 nm excitation. A total of 10,000 cells were analyzed for green fluorescence with a 527-nm filter and for red fluorescence with a 590-nm filter. All data were analyzed with Elite software version 4.02. Mitochondria membrane damage, which goes along with a lower $\Delta\psi$m, was always associated with an increase in percent cells with depolarized mitochondria (%$\Delta\psi$m). The %$\Delta\psi$m determined by fluorescence ratio detection is used to make comparisons in this study [17].

Quantitative real-time RT-PCR

Total cellular RNA was extracted and purified from the hepatocytes using the protocol of the RNeasy Kit (Invitrogen,Carlsbad, USA). The purity and concentrations of RNA were determined by spectrophotometry. Samples with a ratio of OD_{260}/OD_{280} values greater than 1.8 were used in this experiment. cDNA was synthesized with a Reverse-it (ABgene, Epsom, UK) first-strand synthesis kit

using 1 μg total RNA in a 20 μl reaction volume. The RT reaction was carried out at 37 °C for 60 min, followed by 5 min at 95 °C. For real-time PCR, the cDNA was amplified using a Rotor-Gene 3000 Real-time PCR instrument (Corbett Research, Cambridgeshire, UK). Briefly, the double-strand DNA-specific dye SYBR Green I (Invitrogen, Carlsbad, USA) incorporated in a total 25-μl PCR reaction buffer containing 1 μl cDNA, 2.5 μl deoxynucleotide triphosphate mix (HyTest, Turku, Finland), 2.5 μl reaction buffer (Promega, Madison, USA), 1.5 μl $MgCl_2$, 1 unit Fast-Start Taq DNA Polymerase (Promega, Madison, USA), and 10 pmol iNOS- or GAPDH-specific primers to allow quantitative detection of the PCR product. The following primers were used: 5′-CATTCAGATCCCGAAACGCTA-3′ 5′-AGCC TCATGGTGAACACGTTCT-3′ for iNOS, and 5′-AAG AAGGTGGTGAAGCAGGC-3′ and 5′-TCCACCACCCT GTTGCTGTA-3′ for GAPDH (the internal housekeeping gene, 203 bp). The conditions for PCR were: 95 °C for 5 min, 40 cycles of 95 °C for 10 s (denaturing), 59 °C for 15 s (annealing), and 72 °C for 20 s (extension). After the PCR reactions were finished, dissociation curves of the PCR products were generated using the program of Rotor-Gene Real-Time Analysis Software 6.0. The differences in the threshold cycles for GAPDH and iNOS were used to calculate the expression levels of iNOS mRNA in hepatocytes.

Western blot analysis

Cultured hepatocytes were placed on ice and washed twice with a cold phosphate buffer (PH 7.4) before being solubilized in a Tris buffer (50 mM, PH 7.4) that contained 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml trypsin inhibitor, 5 μg/ml aprotinin, and 5 μg/ml leupeptin. A crude cytosolic fraction was obtained as the supernatant after centrifugation at 12,000*g* for 15 min. Protein concentration was determined by the Bradford method [3] using bovine serum sample albumin as a standard. The total protein (50 μg) was subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride (Bio-Rad, Hercules, USA) membrane. After blocking in Tris buffer NaCl-Tween (TBST) containing 5% nonfat dried milk, the membrane was incubated overnight with a monoclonal antibody for the iNOS (Biomol, Plymouth, USA). After a TBST washing procedure, the bolts were incubated with horseradish-peroxidase labeled anti-rabbit antibody (the secondary antibody, KangCheng Biotech, Nanjing, China) for 1 h at room temperature. The immune reaction was detected by enhanced chemiluminescence and bands were quantified by scanning densitometry and expressed as arbitrary unites.

Nuclear protein extract and EMSA

Nuclear extracts of hepatocytes were isolated using a nuclear extract kit (Active Motif, Carlsbad, USA) and stored at −80 °C until use. Protein concentration was determined with a bicinchoninic acid assay kit (Jiancheng Biotech, Nanjing, China). EMSA was performed using a commercial kit (Gel Shift Assay System; Promega, Madison, USA). The NF-κB consensus oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was end-labeled with [γ-^{32}P]ATP (Yahui Biotech, Beijing, China) with T4-polynucleotide kinase. Nuclear protein (10 μg) was preincubated in 9 μl of a binding buffer, consisting of 10 mM Tris–Cl, pH 7.5, 1 mM MgCl2, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 4% glycerol, and 2 g of poly-(dexoyinosinic-deoxycytidylic acid) for 10 min on ice. After addition of the ^{32}P-labeled oligonucleotide probe, the incubation was continued for 20 min at room temperature. The reaction was stopped by adding 1 μl of gel loading buffer and the mixture was subjected to nondenaturing 4% polyacrylamide gel electrophoresis in 0.25 × TBE buffer (Tris borate–EDTA). The gel was vacuum-dried and autoradiographed at −70 °C overnight. Signals were analyzed densitometrically.

Statistical analysis

The results are presented as mean ± SEM. Statistic software of SPSS 11.5 was used for the data analysis. Significant differences between means were evaluated by ANOVA and Duncan's post hoc tests. A difference was considered significant at $p < 0.05$.

Results

ALT level in the culture medium

IL-1β markedly increased ALT enzyme release into the culture medium. This effect was significantly attenuated by simultaneous Gln administration in a dose-dependent way from 2 to 10 mmol/l. No significant increase of ALT level was observed when hepatocytes were treated with 10 mmol/l Gln alone (Fig. 1).

Nitrite release

Stimulation of hepatocytes with IL-1β caused a significant increase in nitrite release into the culture medium. This effect was inhibited by simultaneously addition of Gln in a dose-dependent way and the nitrite release was reverted to the control level by Gln at 10 mmol/l. 10 mmol/l Gln alone

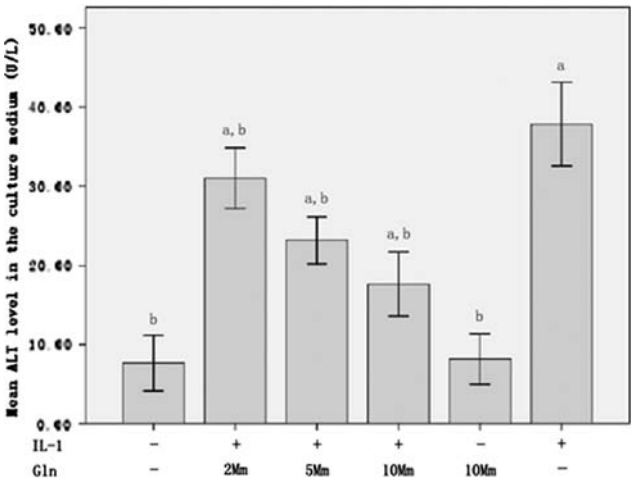

Fig. 1 ALT release into cultures of rat hepatocytes incubated with IL-1β (1 μmol/l) alone or with various concentrations of Gln for 24 h. Values are means ± SEM, $n = 6$. *a* Different from blank (hepatocytes stimulated by culture medium), $p < 0.05$; *b* different from positive control (hepatocytes stimulated by IL-1β alone), $p < 0.05$

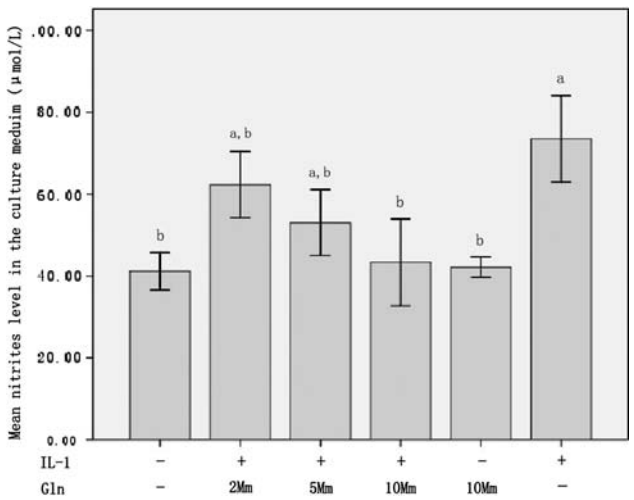

Fig. 2 Nitrite concentration in cultures of rat hepatocytes incubated with IL-1β alone or with various concentrations of Gln for 24 h. Values are means ± SEM, $n = 6$. *a* Different from blank, $p < 0.05$; *b* different from positive control, $p < 0.05$

had no influence on the nitrite release in rat hepatocytes (Fig. 2).

Mitochondria membrane potential ($\Delta\psi$m)

An elevated percentage of hepatocytes with depolarized mitochondria (%$\Delta\psi$m) was observed after stimulation with IL-1β. This effect was significantly attenuated by administration of Gln in a dose-dependent way from 2 to 10 mmol/l. 10 mmol/l Gln alone had no effect on $\Delta\psi$m. (Figs. 3, 4).

iNOS mRNA expression

Incubation of hepatocytes with IL-1β caused a marked induction of iNOS mRNA expression. This effect was significantly attenuated by simultaneously administration of Gln in a dose-dependent way. 10 mmol/l Gln alone had no effect on iNOS mRNA expression (Fig. 5).

iNOS protein level

After IL-1β stimulation, the iNOS protein level in hepatocytes was increased markedly. This effect was significantly attenuated by simultaneous addition of Gln at 5 and 10 mmol/l. 10 mmol/l Gln alone had no influence on iNOS protein expression (Fig. 6).

NF-κB activation

Incubation of hepatocytes with IL-1β caused a marked activation of NF-κB. This effect was significantly augmented by the simultaneous treatment of Gln at concentration of 10 mmol/l; a significant increase in NF-κB activity was also observed when hepatocytes were treated with 10 mmol/l Gln alone (Fig. 7).

Discussion

In this study, we investigated the ability of Gln to regulate iNOS gene expression and mitochondria function in the isolated rat hepatocytes stimulated by IL-1β. Only one concentration of IL-1β was used and only one time point was studied here because we had previously found that IL-1β at 1 nmol/l markedly enhanced NO production and mitochondria depolarization in primary cultured hepatocytes. Both effects were time dependent and achieved the peak at the incubation time of 24 h. The results demonstrate that Gln inhibits IL-1β-induced NO production and iNOS gene expression as shown by decreasing of both mRNA and protein level in rat hepatocytes. This inhibition of iNOS was consistent with the prevention of mitochondria depolarization. To our knowledge, this is the first time a down-regulation of iNOS expression and mitochondria protection induced by Gln in rat hepatocytes have been demonstrated. An in vivo study carried out by Yang et al. [24] offers partial support for these findings, reporting an inhibition of hemorrhagic shock induced iNOS gene expression in rat liver after administration of alanine-glutamine dipeptide.

The alteration of bioenergetic status in both vital (liver) and nonvital (skeletal muscle) organs caused by mitochondria dysfunction during sepsis has been well recognized in the past three decades and is implicated as an

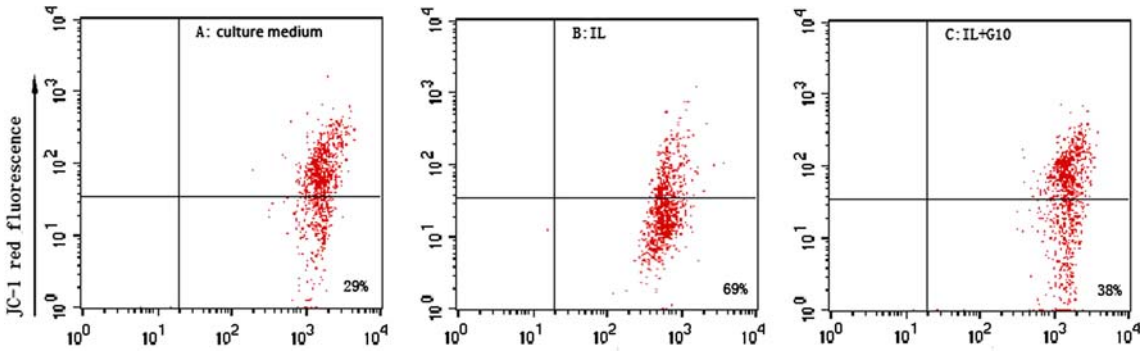

Fig. 3 Cytofluorometric analysis of mitochondrial membrane potential ($\Delta\psi$m). One representative analysis of $\Delta\psi$m in hepatocytes stimulated by culture medium (**a**), 1 nmol/l IL-1β (**b**) and IL-1 + 10 mmol/l Gln (**c**). Respective percentage of cells with depolarized mitochondria (%$\Delta\psi$m) are indicated in the lower right corner of each group (29, 69 and 38%, respectively). Thus, a decrease in $\Delta\psi$m corresponds to an increase in percent of hepatocytes with depolarized mitochondria (%$\Delta\psi$m)

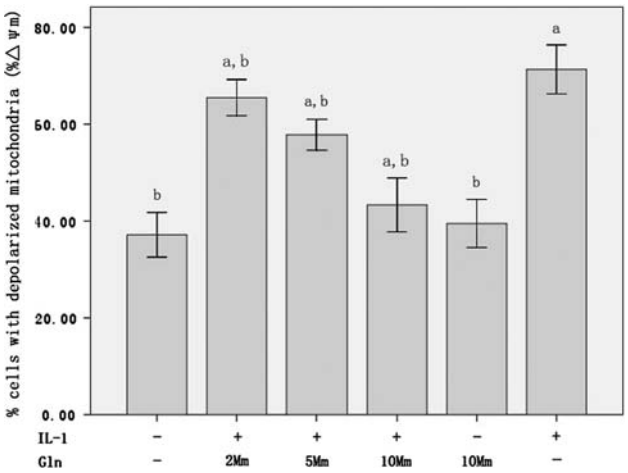

Fig. 4 Mitochondria membrane potential ($\Delta\psi$m) of rat hepatocytes incubated with IL-1β alone or with various concentrations of Gln for 24 h. Values are means ± SEM, $n = 6$. A decrease in $\Delta\psi$m corresponds to an increase in percent of hepatocytes with depolarized mitochondria (%$\Delta\psi$m). *a* Different from blank, $p < 0.05$; *b* different from positive control, $p < 0.05$

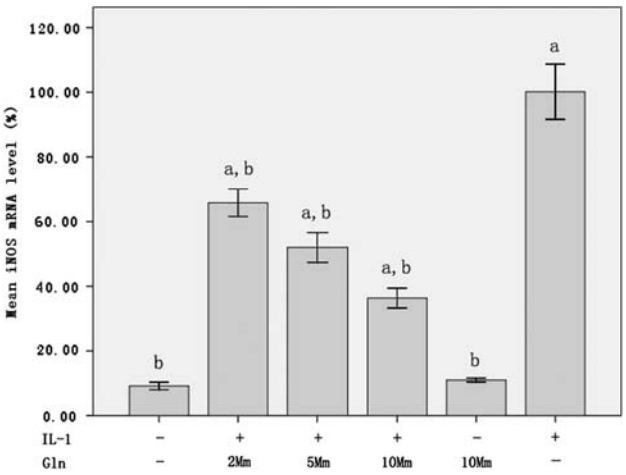

Fig. 5 iNOS/GAPDH mRNA level (real-time quantitative RT-PCR) in cultures of rat hepatocytes incubated with IL-1β alone or with various concentrations of Gln for 24 h. Values are means ± SEM expressed as a percentage of IL-1β values, $n = 6$. *a* Different from blank, $p < 0.05$; *b* different from positive control, $p < 0.05$. GAPDH was used to normalize differences in RNA isolation, RNA degradation, and/or efficiency of the RT reaction

important pathophysiological mechanism for the development of multiple organ failure [4, 5, 7, 9, 18]. The development of new pharmacologic strategies to minimize mitochondria injury may have the potential to greatly improve the clinical treatment of sepsis and other inflammatory diseases. $\Delta\psi$m alteration and subsequent cell death had been observed ex vivo in monocytes from septic patients and alterations in $\Delta\psi$m were more pronounced in non-survivor than in survivors, suggesting $\Delta\psi$m to be a marker for disease outcome [1]. Our data show that IL-1β stimulation causes significant cellular injury as attested by enhanced ALT level and alteration of mitochondrial function as attested by $\Delta\psi$m decrease in cultured hepatocytes. These effects can be attenuated by administration of Gln. It is the first time the direct protective effect of Gln on mitochondria under inflammatory stress has been delineated.

Overexpression of the inducible form of nitric oxide synthase (iNOS), which occurs in nearly all vital organs in models of sepsis, results in dramatic increases in NO production [6]. NO is beneficial in host defense in lower concentration ranges, but excessive release of NO and its metabolites, such as peroxynitrite, will initiate a wide range of toxic oxidative reactions [13, 20]. Studies both in a human septic shock [4] and in vivo models of sepsis [5] have demonstrated a relationship between mitochondrial dysfunction and NO overproduction. In an attempt to determine the potential mechanism of Gln's protective effects on mitochondria, we investigated the influence of Gln on the iNOS gene expression. Our current data show that Gln supplementation down-regulates iNOS gene expression attested by preventing both mRNA and protein induction in IL-1β stimulated hepatocytes, which was

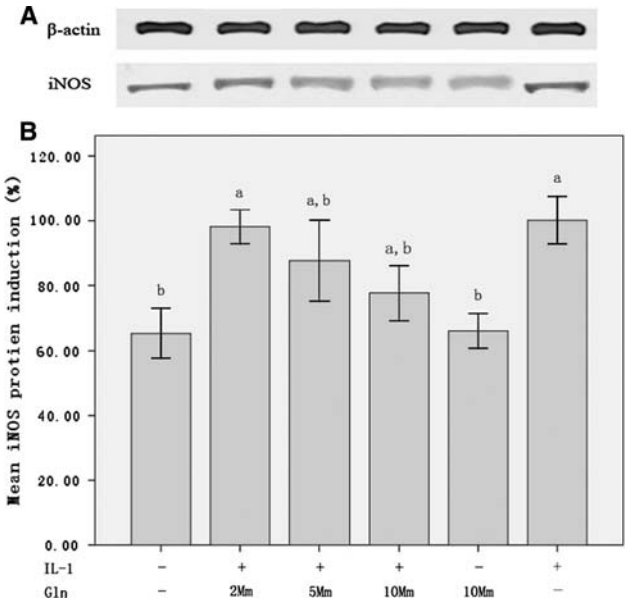

Fig. 6 Western blot analysis of iNOS protein in cultures of rat hepatocytes incubated with IL-1β alone or with various concentrations of Gln for 24 h. **a** Representative Western blot photographs. **b** Values are means ± SEM expressed as a percentage of IL-1β values, normalized to β-actin protein, $n = 6$. *a* Different from blank, $p < 0.05$; *b* different from positive control, $p < 0.05$

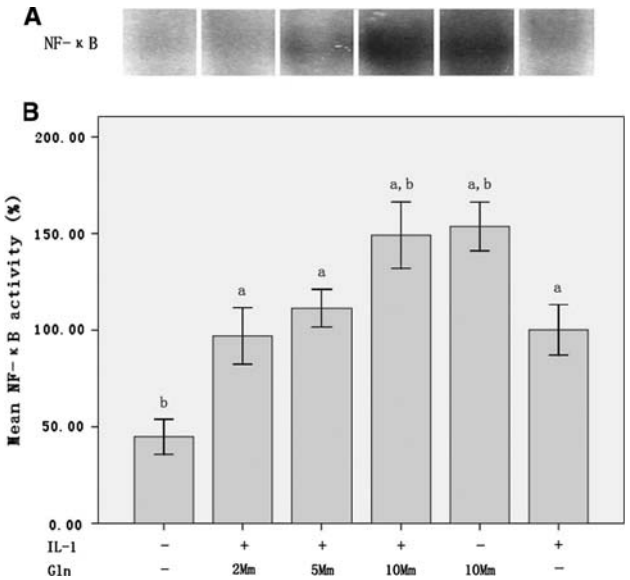

Fig. 7 NF-κB activity of rat hepatocytes incubated with IL-1β alone or with various concentrations of Gln for 24 h. **a** Representative autoradiograph of EMSA. **b** Values are means ± SEM expressed as a percentage of IL-1β values, $n = 6$. *a* Different from blank, $p < 0.05$; *b* different from positive control, $p < 0.05$

associated with its ability to maintain mitochondria membrane potential. These data may be clinically important because a recent study using an iNOS inhibitor did not show benefit in sepsis [14]. On the other hand, many clinical trials have demonstrated that Gln does have a beneficial effect on reducing complication and mortality in critical illness [8, 15], which may be partially due to its attenuation, but not blockade of iNOS overexpression under inflammatory stress.

It must be realized that the supplemental dose of Gln in this study was above the normal concentrations seen in human plasma (0.5–1.0 mmol/l) and the mitochondria protective effect of Gln was observed only when its concentration was more than 5 mmol/l. An in vivo study has revealed that this concentration can be achieved in rat models of intravenous Gln administration without adverse consequences to the organism [23]. As the alanine-glutamine peptide is commercially available, it is possible to achieve Gln concentrations needed to influence iNOS gene expression in the clinical setting.

At present, the mechanistic step by which Gln downregulates iNOS expression is unknown. As the promoters of genes encoding iNOS contain a consensus sequence for the binding of nuclear factor (NF)-κB, which is necessary to confer inducibility in rat and human hepatocytes by IL-1β stimulation [10, 16]. And a recent study in a rat model has demonstrated that Gln can prevent NF-κB activation and iNOS expression in lung tissue following septic shock which is associated with attenuation of ARDS and mortality [19]. In the current study, we tested NF-κB activity and found the results in contrast to what we had expected. Gln at 2 and 5 mmol/l did not inhibit NF-κB activation induced by IL-1β and Gln at 10 mmol/l significantly augmented the inducibility of IL-1β on NF-κB activation. It is unclear whether some downstream products, which may be relevant to Gln's beneficial role, are produced after NF-κB activation in liver. However, we can demonstrate that the regulatory potential of Gln on iNOS expression is independence on NF-κB pathway.

In summary, our data indicate that Gln can prevent IL-1β induced mitochondria injury in rat hepatocytes and this protective activity seem to be due to it's inhibitory effect on iNOS gene expression. It may be of clinical importance because Gln, which traditionally was considered a non-essential amino acid, now appears to be a conditionally-essential nutrient during serious illness and injury in which iNOS play an important role.

Conflict of interest statement The authors have no commercial associations or sources of support that might pose a conflict of interest.

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