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ORIGINAL ARTICLE

Effects of glutamine on the nuclear factor-kappaB signaling pathway of murine peritoneal macrophages

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Abstract The aim of this study was to evaluate the effect of glutamine on the expression of proteins involved in the nuclear factor-kappaB (NF-κB) signaling pathway of murine peritoneal macrophages. Since glutamine is essential for the normal functioning of macrophages, it was hypothesized that in vitro glutamine supplementation would increase NF-κB activation. Peritoneal macrophages were pretreated with glutamine (0, 0.6, 2 and 10 mM) before incubation with lipopolysaccharide (LPS), and the effects of glutamine on the production of tumor necrosis factor-alpha and on the expression and activity of proteins involved in the NF-κB signaling pathway were studied by an enzyme linked immuno-sorbent assay, Western blotting, and an electrophoretic mobility shift assay. Glutamine treatment (2 and 10 mM) increased the activation of NF-κB in LPS-stimulated peritoneal macrophages ($P < 0.05$). In non-stimulated cells, glutamine treatment (2 and 10 mM) significantly reduced IκB-α protein expression ($P < 0.05$). Glutamine modulates NF-κB signaling pathway by reducing the level of IκB-α, leading to an increase in NF-κB within the nucleus in peritoneal macrophages.

Keywords Macrophage · Glutamine · NF-κB · Inflammation · Tumor necrosis factor-α

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Introduction

Glutamine, traditionally considered a non-essential amino acid, currently appears to be a conditionally essential nutrient during stress, injury, or illness (Lacey and Wilmore 1990). These situations are associated with an increased susceptibility to infections and it has been suggested that this may be due in part to a diminished supply of glutamine to immunocompetent cells (Rogero et al. 2008a, b; Newsholme 2001). Glutamine is utilized at high rates by isolated cells of the immune system (Pithon-Curi et al. 2004; Curi et al. 1999; Newsholme et al. 1999), such as macrophages (Newsholme et al. 1996; Newsholme et al. 1986; Newsholme and Newsholme 1989), which are involved in innate immunity and whose abilities to phagocytose pathogens, kill fungi and produce interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), nitric oxide (NO) and reactive oxygen species are dependent on the extracellular concentration of glutamine (Rogero et al. 2008c; Curi et al. 2005; Murphy and Newsholme 1998, 1999; Bellows and Jaffe 1999; Yassad et al. 2000; Yassad et al. 1997; Spittler et al. 1997; Costa Rosa et al. 1995; Wallace and Keast 1992). Furthermore, several clinical (Déchelotte et al. 2006; Ziegler et al. 2005) and experimental (Singleton and Wischmeyer 2007; Singleton et al.

2005) studies have suggested that intravenous glutamine may prevent the occurrence of lung injury, tissue metabolic dysfunction, improving survival after sepsis.

The nuclear factor-kappaB (NF-κB) signaling pathway involves the enzymatic complex IκB kinase (IKK), which is composed of two catalytic subunits (IKKα and IKKβ), as well as a regulatory subunit (IKKγ) and induces the phosphorylation of inhibitor-κB (IκB). The phosphorylation of IκB—the protein that inactivates the p50/p65 (NF-κB) heterodimer—results in its polyubiquitination, which, in turn, leads to its degradation mediated by the 26S proteasome. This degradation of IκB allows NF-κB to translocate to the nucleus and to activate the transcription of several κB-dependent genes such as those encoding proinflammatory cytokines, like TNF-α (Vallabhapurapu and Karin 2009; Hayden and Ghosh 2008; Li and Verma 2002).

Considering that macrophages play an essential role in the inflammatory response (Hamilton and Tak 2009) and that glutamine is crucial for the function of these cells (Newsholme 2001), the aim of this study was to evaluate the effect of glutamine on the expression of proteins involved in the NF-κB signaling pathway of murine peritoneal macrophages. Furthermore, taking into account that glutamine is essential for the proper functioning of macrophages, we hypothesized that supplementation with glutamine in vitro would increase NF-κB activation.

Materials and methods

Materials

Lipopolysaccharide (LPS) (Escherichia coli strain 055:B5) was purchased from Sigma (St. Louis, MO, USA). T4 polynucleotide kinase and [γ-^{32}P] ATP were purchased from Amersham Biosciences (Pittsburgh, PA, USA). Rabbit anti-mouse antibodies against NF-κB-p65 and IκB-α were purchased from Calbiochem (Darmstadt, Germany). The antibody against IKK-β was purchased from Cell Signaling Technology (Beverly, MA, USA). The F4/80 FITC antibody (Clone A3–1) was purchased from Serotec (Oxford, UK). Polyvinylidenedifluoride membranes were purchased from Millipore (Billerica, MA, USA). Cell culture medium (RPMI 1640), fetal bovine serum, and penicillin and streptomycin were obtained from Cultilab (Campinas, SP, Brazil). All other chemicals were purchased from Sigma, unless otherwise specified.

Animals

Male BALB/c mice were obtained at 12 weeks of age from the Animal Laboratory of the Faculty of Pharmaceutical Sciences at São Paulo University. The mice were kept in a room at an ambient temperature of 22 ± 2°C and a relative humidity of 55 ± 10% under a 12-h light/12-h dark cycle (lights on at 7:00 a.m.). All of the animals were killed in the morning between 08:00 a.m. and 12:00 p.m. All procedures performed on the animals were approved by the Ethics Committee on Animal Experimentation of the Faculty of Pharmaceutical Sciences, São Paulo University, according to the guidelines of the Brazilian College on Animal Experimentation.

Cell culture

Peritoneal macrophages were obtained by washing the peritoneal cavity with 5 mL of sterile, pyrogen- and glutamine-free RPMI 1640 medium, pH 7.4. Macrophages were spun down (1,500 rpm, 10 min, 4°C) and re-suspended in glutamine-free RPMI 1640 culture medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL). Cell viability was determined by Trypan Blue exclusion. Cultures rich in macrophages were obtained by incubating 3×10^6 cells per dish in six well polystyrene culture plates for 2 h at 37°C in a 5% CO_2 humidified air environment. Non-adherent cells were removed by vigorous washing three times with glutamine-free RPMI 1640. Flow cytometric analyses were carried out in order to determine the fraction of F4/80$^+$ cells using the F4/80 fluorescein isothiocyanate antibody (Clone A3–1, Serotec) and a FACSCalibur flow cytometer (Becton & Dickinson, San Jose, CA, USA). Peritoneal cells constituted more than 70% of the F4/80$^+$ macrophages and cells adhered to the wells constituted more than 90% of the F4/80$^+$ macrophages (data not shown).

Following the period of cell adhesion, macrophages were treated with glutamine (0, 0.6, 2 or 10 mmol/L) for 24 h in sterile, pyrogen-free RPMI 1640 medium with fetal bovine serum (10%). The reasons for incubating cells with 0.6, 2 and 10 mmol/L of glutamine are the following: 0.6 mmol/L of glutamine corresponds to the concentration normally found in the plasma of healthy rodents and humans; 2 mmol/L is the standard concentration used in cell cultures and it provides better results when incubating cells for a prolonged period (24–72 h) (Eagle 1955; Eagle et al. 1955); 10 mmol/L was used since, in some studies using other cell types, such as enterocytes and peripheral blood mononuclear cells, glutamine concentration varying from 2 to 10 mmol/L had opposing effects on the expression of genes coding for cytokines (Hubert-Buron et al. 2006; Wischmeyer et al. 2003). After glutamine treatment, macrophages were incubated with LPS (15 μg/mL) for 30 min for the evaluation of IκB-α, IKK-β, and NF-κB-p65 protein expression and of the NF-κB DNA-binding capacity. Macrophages were incubated with LPS (15 μg/mL) for 24 h to determine the TNF-α concentration in the culture supernatant.

Western blotting

Cells (3×10^6) were washed three times with PBS and lysed with RIPA buffer (0.1% SDS, 1% Igepal CA-630, 1% sodium deoxycholate, 10 mM Tris–HCL, pH 7.5, 150 mM NaCl, 2 μg/mL aprotinin, 1 μg/mL leupeptin, 100 μg/mL PMSF, 0.5 mM EDTA). The protein content of the cell homogenates was determined using a BCA Protein Assay kit (Pierce Biotechnology, IL, USA). Equal amounts of protein were separated on 7.5% SDS-polyacrylamide minigels and transferred to Immobilon polyvinylidene difluoride membranes. After incubation with the appropriate primary antibodies, membranes were incubated for 1 h at room temperature with a secondary antibody conjugated to horseradish peroxidase. Following three washes in TBST, immunoreactive bands were visualized using the ECL detection system (Amersham Biosciences, Pittsburgh, PA, USA). To standardize and quantify the immunoblots, a digital detection system (IMAGE QUANT™ 400 version 1.0.0, Amersham Biosciences, Pittsburgh, PA, USA) was used. Results were expressed in relation to the intensity of β-actin and as a percentage of the control value.

Electrophoretic mobility shift assay

Nuclear factor-kappaB activation was evaluated in peritoneal cells either with or without LPS treatment. Nuclear extracts from peritoneal adherent cells were obtained as previously described (Rong and Baudry 1996). Oligonucleotides containing an NF-κB consensus-binding site (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were end-labeled using T4 polynucleotide kinase and [γ-^{32}P] ATP. Binding reactions of the probe (30,000 cpm) were performed for 20 min at room temperature with 10 μg of protein from nuclear extracts in 12 μL of the binding buffer, which consisted of 20 mM HEPES, pH 7.6, 50 mM KCl, 10% glycerol, 0.2 mM EDTA, 1 mM DTT and 2 μg polydeoxyinosinic–deoxycytidylic acid (poly[dI–dC]). Competitive-binding assays were conducted under the same conditions with the addition of 2 pmol (100-fold molar in excess) of unlabeled competitor oligonucleotides. The DNA–protein complexes were electrophoresed on 4% non-denaturing polyacrylamide gels at 4°C in 45 mM Tris, 45 mM borate and 1 mM EDTA buffer. The gels were dried and subjected to autoradiography. The blots were analyzed by scanner densitometry (Image Master 1D®, Amersham Biosciences, Pittsburgh, PA, USA), and results related to NF-κB activation were obtained through densitometric analysis. Results are expressed relative to the control condition (PBS).

Enzyme linked immuno-sorbent assay

The levels of TNF-α in the culture supernatant were measured using enzyme linked immuno-sorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). The ELISA results were quantitated using an ELISA plate reader at a wavelength of 450 nm and corrected for the absorbance at 540 nm, according to the manufacturer's instructions.

Statistics

Results were tested for variance homogeneity (Cochran *C* tests, Hartley, Bartlett) and then submitted to variance analysis (ANOVA) followed by a Tukey *t* test. Calculations were performed using the statistics computer software GraphPad Prism, version 4.0. The significance level (alpha) was set at 0.05.

Results

TNF-α synthesis

Lipopolysaccharide-stimulated macrophages displayed significantly higher TNF-α synthesis compared to non-stimulated cells, however, glutamine supplementation had no influence on TNF-α synthesis (Fig. 1).

IKK-β, IκB-α and NF-κB-p65 protein expression

We investigated the effect of LPS and glutamine on IKK-β, IκB-α and NF-κB-p65 protein expression (Fig. 2a). IKK-β expression was significantly lower in LPS-stimulated peritoneal macrophages than in non-stimulated cells; the presence of varying glutamine concentrations in the culture media did not affect IKK-β expression (Fig. 2b). Incubation with LPS significantly decreased IκB-α protein expression. In cells not stimulated with LPS, treatment with 2 and 10 mM of glutamine lowered IκB-α protein expression when compared to 0 and 0.6 mM of glutamine

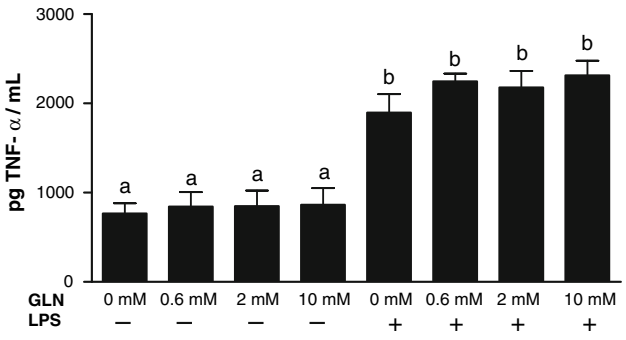

Fig. 1 TNF-α synthesis (mean pg/ml ± SEM) in mouse peritoneal macrophage culture ($n = 5$). Cells were treated in the growth medium (see "Materials and methods") with different glutamine (*GLN*) concentrations (0, 0.6, 2 and 10 mM) for 24 h and, subsequently, incubated in the growth medium with 15 μg/mL of lipopolysaccharide (*LPS*) for 24 h. *Different letters* above *bars* denote statistical significance ($P < 0.05$; ANOVA plus Tukey *t* test)

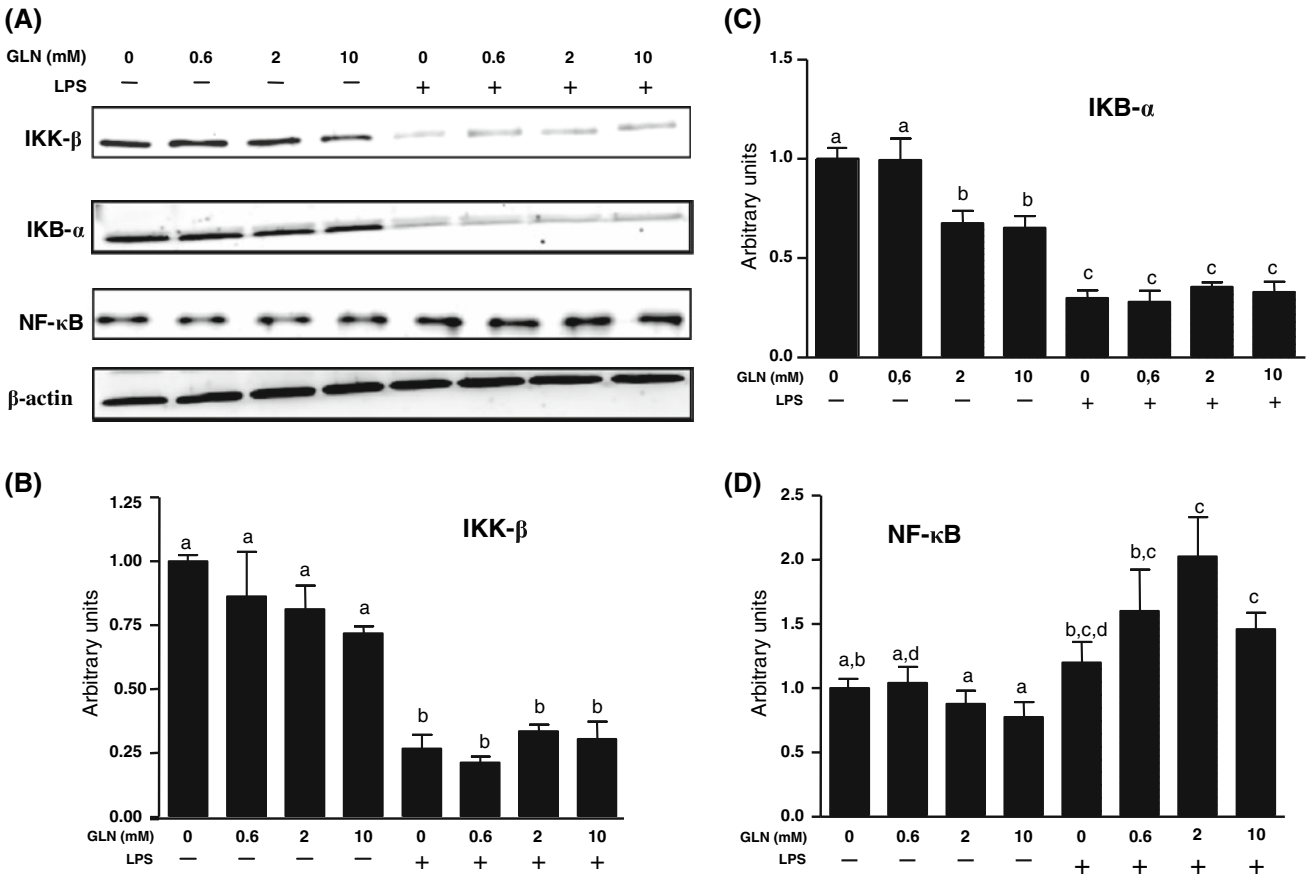

Fig. 2 Effect of LPS and glutamine on IKK-β, IκB-α and NF-κB protein expression (mean arbitrary units ± SEM; **b–d**, respectively) in mouse peritoneal macrophages ($n = 5$). The levels of IKK-β, IκB-α and p65 subunit of NF-κB were determined by Western blot analysis (**a**). The protein expression of IKK-β (**b**), IκB-α (**c**) and the p65 subunit of NF-κB (**d**). After densitometric analysis, results related to the protein expression of IKK-β, IκB-α and the p65 subunit of NF-κB were normalized to β-actin values. Cells were treated with different glutamine (*GLN*) concentrations (0, 0.6, 2 and 10 mM) for 24 h and then incubated in the growth medium (see "Materials and methods") with 15 μg/mL of lipopolysaccharide (*LPS*) for 30 min. *Different letters* above *bars* denote statistical significance ($P < 0.05$; ANOVA plus Tukey *t* test)

(Fig. 2c). Macrophages stimulated with LPS and supplemented with 0.6, 2 and 10 mM of glutamine showed increased NF-κB-p65 protein expression compared to their respective non-stimulated controls (Fig. 2d).

NF-κB activation

We investigated the effect of LPS and glutamine on DNA-binding activity of NF-κB using an electrophoretic mobility shift assay (EMSA). NF-κB activation was increased in cells incubated with LPS and treated with 2 and 10 mM of glutamine in comparison to their non-stimulated controls and to LPS-simulated macrophages treated with 0 and 0.6 mM of glutamine (Fig. 3).

Discussion

Due to the fact that glutamine is the most abundant amino acid in human plasma (Rowbottom et al. 1996) and skeletal muscle (Rogero et al. 2004, 2006), a number of studies have recently explored its mode of action on gene expression, revealing a large variety of target genes involved in major functions in the organism (Curi et al. 2005, 2007). We investigated the effect of glutamine on NF-κB signal transduction pathways. NF-κB is a member of transcriptional activator proteins, and the NF-κB/Rel transcription factor family consists of p50, p52, p65 (Rel A), c-Rel and Rel B, which form homo- or heterodimers. The important role of NF-kB in coordinately controlling inflammatory gene activation is firmly established (Ghosh and Hayden 2008; Vanden Berghe et al. 2003). With the exception of mature B cells where NF-κB is constitutively nuclear, in all other cell types including macrophages, NF-κB is retained in the cytoplasm through association with the IκBs, which mask their nuclear localization motif (Vallabhapurapu and Karin 2009). When cells are activated by proinflammatory cytokines (Osborn et al. 1989), oxidants (Cooke and Davidge 2002), or LPS (Sen and

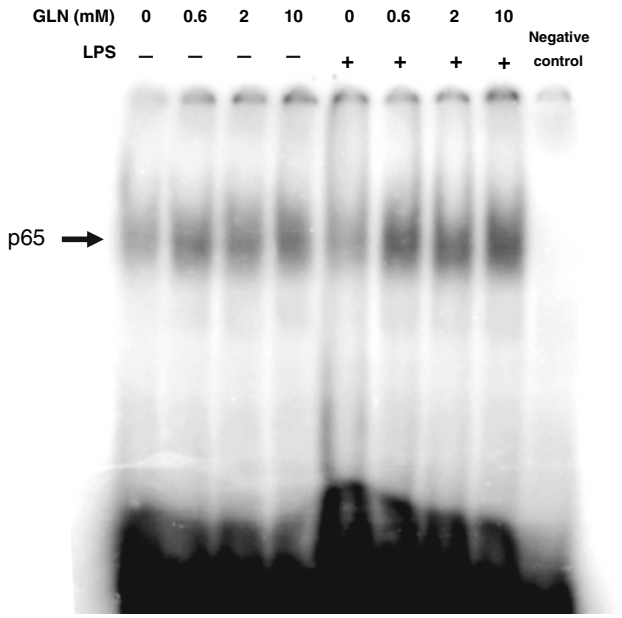

Fig. 3 Effect of LPS and glutamine on NF-κB activation in mouse peritoneal macrophages ($n = 5$). Nuclear extracts were prepared and analyzed for DNA-binding activity of NF-κB using an electrophoretic mobility shift assay. Results related to NF-κB activation were obtained through densitometric analysis. Cells were treated with different glutamine (*GLN*) concentrations (0, 0.6, 2 and 10 mM) for 24 h, then incubated in vitro with 15 μg/mL of lipopolysaccharide (*LPS*) for 30 min

Baltimore 1986), the IκBs are rapidly phosphorylated at two serines within their amino-terminal regulatory domains (Perkins 2007). Phosphorylation of these two sites triggers polyubiquitination of IκBs and their subsequent degradation. Paralleling the loss of IκB in the cytoplasm is the appearance of NF-κB in the nucleus. Activated NF-κB then binds to the cognate DNA-binding sites and induces gene transcription. The ability of different dimers to recognize slightly different DNA targets probably increases the ability of NF-κB to differentially regulate gene expression. In macrophages, the most important components to form dimers are the p50 and p65 subunits (Perkins 2007; Sharif et al. 2007; Gilmore 2006).

Corroborating these facts, the present study verified that incubation with LPS reduced IKK-β and IκB-α protein expression while increasing NF-κB expression and activation, which consequently increased TNF-α production. It is important to emphasize that treatment with glutamine augmented NF-κB expression and activation in LPS-stimulated macrophages, which suggests an important role for glutamine in increasing the inflammatory response in macrophages during infectious processes. Furthermore, cells not stimulated with LPS showed lower IκB-α protein expression after treatment with glutamine (2 and 10 mM). This fact indicates that the presence of glutamine leads to a preactivated state in macrophages in the absence of stimuli that induces an inflammatory response. Thus, these results demonstrate the importance of glutamine in modulating innate immunity during infectious conditions, since activated macrophages play an important role in the regulation of immune and inflammatory responses by releasing several mediators including lipids, reactive oxygen and nitrogen species, chemokines and cytokines (Gordon 1986).

Several studies related to the inflammatory response have highlighted the physiological importance of glutamine, which, by counteracting activation of the NF-κB pathway, contributes to the attenuation of local inflammation in the gut (Hubert-Buron et al. 2006; Erbil et al. 2005; Deger et al. 2006; Fillmann et al. 2007) and lung (Kim et al. 2006; Zhang et al. 2008). The pathway by which glutamine attenuates NF-κB activation is not yet clear, although it may involve either enhanced intracellular glutathione (Exner et al. 2000), which in turn inhibits NF-κB activation, or an increase in the *O*-linked *N*-acetylglucosamine protein levels (Chatham et al. 2008). On the other hand, the present study demonstrated that glutamine-treated macrophages showed an increase in NF-κB activation, indicating that glutamine supplementation may exert anti- or pro-inflammatory effects depending on the tissue or cell type studied.

Macrophages are cells with a large capacity for cytokine production, in particular TNF-α, which is one of the most important products released from these cells (Murphy and Newsholme 1999). Despite the fact that glutamine augmented NF-κB activation in this study, it did not affect TNF-α production in LPS-stimulated peritoneal macrophages in culture. This result may be related to the fact that the LPS incubation in the assays evaluating TNF-α production may have favored the activation of other signaling pathways involved in the response to LPS stimulation. The mitogen-activated protein kinases (MAPK) signaling pathway acts on upstream regulators of NF-κB and AP-1 signaling pathways (Brown and Sacks 2008; Malemud and Miller 2008). Therefore, it is possible that LPS activation of the MAPK pathway—and the consequent activation of the transcription factor AP-1—affected TNF-α synthesis and accounted for the lack of significant differences in TNF-α concentrations in the presence of different glutamine concentrations despite the fact that extracellular glutamine influenced NF-κB activation.

Glutamine is important as a precursor for peptide and protein synthesis, amino sugar synthesis, purine and pyrimidine and thus nucleic acid and nucleotide synthesis, and it also provides a source of carbons for oxidation in some cells. However, the present results indicate that glutamine has also a regulatory role (rather than nutrient) in the cytokine production, since glutamine modulates NF-κB signaling pathway, which is crucial in triggering the inflammatory response (Roth 2008). Concerning glutamine modulation of cytokine production, it has been observed

that this amino acid influences the production of interferon-γ and tumor necrosis factor-α (Roth et al. 2002). Expression and production of TNF-α by cultured mononuclear cells stimulated with LPS can be suppressed by glutamine (Wischmeyer et al. 2003), while opposite effects were found for the synthesis and secretion of IL-1 and IL-6 in LPS-stimulated rat peritoneal macrophages, since the addition of glutamine increased the synthesis of both cytokines (Yassad et al. 2000).

Considering glutamine-induced effects on cellular metabolism and on gene expression regulation, it should be emphasized that this effects may be in part related to glutamate, since the immediate product of glutamine metabolism in most cells is glutamate, which is produced by the action of glutaminase, an enzyme found at high concentrations and associated with the mitochondria in cells which readily utilize glutamine. Glutamate is the most abundant intracellular amino acid (reported concentrations vary between 2 and 20 mM) and glutamine is the most abundant extracellular amino acid in vivo (0.7 mM compared to an approximate glutamate concentration of 20 μM) (Rogero et al. 2004, 2006; Curi et al. 2005). Glutamate is involved in a number of key functions, in addition to amino acid transamination, in lymphocytes, macrophages and neutrophils. Glutamate metabolism in these cells, via the action of NADP+-dependent malic enzyme, provides NADPH, which is required for biosynthetic reactions such as fatty acid synthesis or for the production of free radicals such as O_2^- or NO by NADPH oxidase and inducible NO synthase, respectively (Newsholme 2001). NADPH is also required by glutathione reductase and as such plays an important role in increasing reduced glutathione concentration (O'Neill et al. 2000). Glutamate may also serve as a precursor for glutathione synthesis and as such may play a direct role in antioxidant defenses (Newsholme 2001). In addition, glutamate might be involved in the transcription of genes of proinflammatory cytokines, since glutamate can act in glutamate-responsive elements, which were recently identified as a functional AP-1 site in the 5′-flanking sequence of some genes in mammalian neurons and glial cells (Brasse-Lagnel et al. 2009).

Conclusion

Our study is the first to report that glutamine decreased the level of IκB-α leading to an increase in NF-κB within the nucleus in peritoneal macrophages.

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Conflict of interest statement The authors declare no conflict of interest.

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