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Research Article

Glucosamine inhibits LPS-induced COX-2 and iNOS expression in mouse macrophage cells (RAW 264.7) by inhibition of p38-MAP kinase and transcription factor NF-κB

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Glucosamine supplements are very promising nonsteroidal anti-inflammatory agents widely used for the treatment of arthritis in animals and humans. In this study, we have proposed the molecular mechanism underlying the anti-inflammatory properties of glucosamine hydrochloride (GLN) using mouse macrophage cell line (RAW 264.7). Treatment with GLN inhibited LPS-stimulated nitric oxide (NO) production. Western blotting and RT-PCR analysis showed that GLN treatment decreased LPS-induced inducible nitric-oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein and mRNA expression in RAW 264.7 cells, respectively. To further elucidate the mechanism of inhibitory effect of GLN, we studied the LPS-induced phosphorylation of mitogen-activated protein kinases (pp44/42 and pp38). Our results clearly indicated that GLN treatment resulted in a reduction of pp38, whereas activation of p44/42 was not affected. In addition, LPS-induced activation of nuclear factor-κB (NF-κB) DNA binding suggests an inhibitory effect of GLN. These results indicate that GLN suppresses the LPS-induced production of NO, expression of iNOS and COX-2 by inhibiting NF-κB activation and phosphorylation of p38 MAP kinase.

Keywords: COX-2 / Glucosamine / iNOS / NF-κB / p38 MAPK

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1 Introduction

Glucosamine (GLN) supplements are potent anti-inflammatory agents widely used for the treatment of osteoarthritic and rheumatoid arthritic symptoms in animals and humans [1, 2]. The widely used and now commonly available GLN supplements in the market are in three different forms: GLN, glucosamine sulfate, and *N*-acetyl-glucosamine [3]. Glucosamine (2-amino-2-deoxy-D-glucose) is an amino monosaccharide, present in the cartilage and connective tissues that contributes to the strength, flexibility, and elasticity of the joint tissues [4]. A comprehensive review by Anderson and coworkers [3, 5] reports that GLN is an essential component of glycosaminoglycans present in mucous secretions, connective tissues, skin, tendon, ligaments, and cartilage. GLN is also recognized as a natural metabolite synthesized in the body from glutamate and fructose-6-phosphate.

Rheumatoid arthritis affects approximately 1% of Americans typically beginning in the age range of 30 – 50 years [6], while osteoarthritis is the most common form of arthritis with an estimated 12.1% of Americans 25 years and older and 80% of Americans 75 years and older with clinical signs and symptoms of osteoarthritis [7, 8].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed for arthritis. However, the common side effects of NSAIDs are associated with potential toxicity, gastrointestinal bleeding with approximately 16 500 deaths occurring annually among patients with rheumatoid arthritis or osteoarthritis in the US [9]. Therefore, individuals with arthritic pain have turned to alternative therapies such as omega-3 containing fish oils and several other nutraceuticals including green tea, herbal extracts, chon-

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Abbreviations: COX-2, cyclooxygenase-2; **EMSA,** Electrophoretic Mobility Shift Assay; **ERK,** extracellular signal-regulated kinases; **GLN,** glucosamine hydrochloride; **iNOS,** inducible nitric-oxide synthase; **MAPKs,** mitogen-activated protein kinases; **NF-κB,** nuclear factor-κB; **NO,** nitric oxide; **NSAIDs,** nonsteroidal anti-inflammatory drugs

droitin, and GLN to alleviate arthritic complaints [10]. Oral administration of GLN results in less significant side effects compared to the commonly prescribed NSAIDs [3]. Anderson and coworkers have evaluated GLN toxicity in several animal and human clinical studies. They reported that large oral doses of GLN (5000 – 15 000 mg/kg) can be administered without toxicity and that daily oral administration of GLN (159 – 2700 mg/kg/day) for one year shows no adverse effects in animals [3]. In humans, GLN (1500 mg/day) was well tolerated for periods up to 3 years [3]. Although several *in vitro* and *in vivo* studies have been conducted with GLN, the molecular mechanism of action of GLN in arthritic complaints is not yet known.

Arthritis is associated with inflammation and more specifically with the generation of nitric oxide (NO) [2]. The possible mechanism of action of GLN is by the modulation of inflammatory responses and reduction in NO production [2]. The bacterial endotoxin (LPS) is able to induce an inflammatory response by activation of macrophages through the binding of the LPS-binding protein to CD14, a membrane glycophosphatidylinositol anchored protein. Pro-inflammatory genes are activated *via* signal transduction pathways which lead to NO production [11]. NO is produced from L-arginine through the action of NO synthase (NOS) enzyme. In the biological system, NO has many functions such as vasodilation, neurotransmission, and inflammatory response [12]. However, excess production of NO can lead to the formation of mutagenic peroxynitrites ($ONOO^-$) leading to septic shock and autoimmune disorders. Production of peroxynitrite results in modifications to DNA and other forms of cellular injury [13]. Therefore, regulating the production of NO using various nutraceuticals can be considered an important therapeutic approach for the potential treatment and prevention of inflammatory diseases.

In this study, we have made an attempt to understand the molecular mechanism of action of GLN in inflammation using an *in vitro* model system of mouse macrophage cells (RAW 264.7) induced with LPS. We have shown that GLN can potentially suppress NO production, inducible nitric-oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) levels at the mRNA and protein levels by inhibiting the phosphorylation of p38 and inhibiting the activation of transcription factor nuclear factor-κB (NF-κB).

2 Materials and methods

2.1 Reagents and cells

Mouse macrophage cell lines (RAW 264.7) were obtained from the American Type Culture Collection (ATCC) and grown in DMEM supplemented with 15% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). DMEM, LPS, Tri-Reagent, Griess reagent, monoclonal anti-β-actin, and glucosamine hydrochloride were purchased from Sigma – Aldrich (St. Louis, MO). The relative RT-PCR kit for mouse iNOS and COX-2 were obtained from Ambion (Austin, TX), mouse monoclonal anti-iNOS and COX-2 were purchased from BD Biosciences Pharmingen (San Diego, CA), and antimouse and antirabbit IgG conjugated with horseradish peroxidase were purchased from DAKO Corporation (Carpinteria, CA). Mouse mAb against phosphorylated p44/42, rabbit polyclonal antibody for p44/42, phosphorylated p38 and p38 were obtained from Cell Signaling Technology (Beverly, MA).

2.2 NO measurement

The mouse macrophage cells (RAW 264.7) were cultured in DMEM supplemented with 15% FBS, an antibiotic and antimycotic mixture. The cell suspension of 50 000 cells/well was cultured in flat bottom microtitre plate in quadruplicate for 12 h. Thereafter, 100 μL of media was replaced with fresh medium containing either LPS (0.5 μg/mL) alone or LPS with various concentrations of GLN and further cultured for 24 h. The culture supernatant was collected at the end of culture for nitrite assay, which was used as a measure of NO production. The culture supernatant (100 μL) was mixed with equal volume of Griess reagent and the absorbance was measured at 570 nm. The concentration of nitrite (μM) was calculated from a standard curve drawn with known concentration of sodium nitrite dissolved in DMEM. The results are presented as mean ± SD of four replicates of one representative experiment and this experiment has been repeated five times.

2.3 Cell viability

To determine if GLN treatment was cytotoxic, RAW 264.7 mouse macrophage cells were treated with various concentrations of GLN (1.25 – 10 mM) for 18 h, and a cell viability assay was performed using MTT dye [14]. It was observed that GLN treatment displayed a dose-dependent response in cell viability and GLN at 10 mM was observed to be the highest nontoxic dose (data not shown).

2.4 Western blotting

At the indicated time points, cells were rapidly washed with ice-cold PBS and solubilized in cold lysis buffer containing 10 mM Tris-base, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 5 mM PMSF, 2 mM sodium orthovanadate, 10 μg/mL leupeptin, 25 μg/mL aprotinin, 1 mM sodium pyrophosphate, and 20% glycerol. After incubation for 30 min on ice, lysates were centrifuged (12 500 rpm, 15 min) and supernatants were collected and protein concentration in samples was estimated by BioRad protein assay reagent (BioRad laboratories, Hercules, CA) following manufacturer's instruction.

An equal amount of protein (40 μg) from each sample was loaded on SDS-polyacrylamide electrophoresis gel

(10% separating gels) and resolved for 1.5 h at 120 V in buffer containing 95 mM Tris-HCl, 960 mM glycine, and 0.5% SDS. After electrophoresis, the proteins were transferred to a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ) at 200 mA for 1 h in a buffer containing 25 mM Tris-base, 192 mM glycine, and 20% methanol. After transfer, the membrane was blocked in PBST (20 mM sodium phosphate buffer, pH 7.6, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk for 1 h at room temperature and incubated with primary antibody in the blocking solution at 4°C overnight. Thereafter, the membrane was washed four times with PBST, incubated with secondary antibody in the blocking solution for 1 h at room temperature and washed four times with PBST for 5 min each time. Specific bands were detected by an enhanced chemiluminescence detection system (Amersham Biosciences) and the membrane was exposed to X-ray film.

2.5 Determination of iNOS and COX-2 mRNA expression

The RAW 264.7 cells were cultured (10^6/well) in a 96-well plate for 24 h and the medium was then replaced by fresh medium with different concentrations of GLN either alone or in combination with LPS and further cultured for 12 h. Total RNA was isolated using Tri-reagent (Sigma – Aldrich) and 5 μg of this total RNA was reverse transcribed to make cDNA using random hexamer and superscript reverse transcriptase (Invitrogen, Grand Island, NY), following manufacturer's instruction. The linear range of amplification of iNOS and COX-2 cDNA (2 μL for COX-2 and 1 μL for iNOS of RT reaction mixture) was determined using gene specific primers from Ambion, following the manufacturer's instruction. The optimum amount of 18S primer and competitor for iNOS and COX-2 gene were also determined as per manufacturer's instruction. The PCR for iNOS (2 μL cDNA, 30 cycles) and COX-2 (1 μL cDNA, 25 cycles) was performed in a final volume of 50 μL containing dNTPs (each at 2.5 mM), 1 × PCR buffer, 5 U of *Taq* DNA polymerase, 0.4 μM of gene specific primer, and optimum ratio of 18S primer and competitor (3:7). After an initial denaturation for 30 s at 94°C, aforementioned cycles of amplification (95°C for 30 s, 55°C for 45 s, and 72°C for 45 s) were performed followed by a 10 min final extension at 72°C. Finally, PCR products from each sample (10 μL) were resolved in 2% agarose gel, stained with ethidium bromide and an image of the gels were captured at appropriate exposure times and magnification.

2.6 Electrophoretic mobility shift assay (EMSA)

RAW 264.7 cells were treated with either LPS (0.5 μg/mL) alone or with various concentration of GLN for 2 h. Thereafter, nuclear extracts were prepared using a modified method [11]. Briefly, cells were washed once with PBS (pH 7.2) and were suspended in hypotonic buffer A (10 mM HEPES (pH 7.6), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) for 10 min on ice, and vortexed for 10 s. Nuclei were pelleted by centrifugation at $12\,000 \times g$ for 5 min. Then the pellets were suspended in buffer B (20 mM HEPES (pH 7.6), 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at $12\,000 \times g$ for 20 min and stored at −80°C. For EMSAs, 6 μg of each nuclear extract was mixed with the ^{32}P-labeled double stranded NF-κB binding consensus oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (Promega, Madison, WI) and incubated at room temperature for 20 min. The incubation mixture contains 1 μg of poly(dI-dC) in a binding buffer (25 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 5% glycerol, and 50 mM NaCl). The DNA/protein complex was electrophoresed on 5% nondenaturing polyacrylamide gel in Tris/acetate/EDTA buffer (0.04 M Tris, 0.04 M acetate, 0.002 M EDTA). The specificity of binding was also examined by competition with the unlabeled oligonucleotides. Mobility shift of DNA due to binding of NF-κB complex was detected by Phosphor Imager-445 SI (Molecular Dynamics, Amarsham-Pharmacia, NJ, USA).

3 Results

3.1 Inhibition of LPS-induced NO production from RAW 264.7 cells by GLN

In order to study the inflammatory response, which is evident in arthritis and related illnesses, LPS was used to stimulate the release of NO from the macrophage cells. Nitrite was used as an indicator of NO production, due to the short half-life of NO. The nitrite concentration (micromolar) was determined in the supernatant after treatment with LPS (0.5 μg/mL) alone or cotreated with GLN (2.5, 5.0, and 10 mM) for 12 h using Griess reagent. As shown in Fig. 1, GLN suppressed the LPS-induced NO formation significantly (2.5 mM: $p < 0.05$; 5 mM: $p < 0.01$; 10 mM: $p < 0.001$), in dose-dependent fashion. The GLN had no effect on the basal level of NO formation in RAW 264.7 cells.

3.2 Inhibition of LPS-induced iNOS and COX-2 protein expression by GLN

To confirm whether the inhibition of NO production is due to less enzymatic activity or decreased protein expression of iNOS, we further studied the effect of GLN on iNOS protein expression by Western blotting. In addition to iNOS, we have also studied the effect of GLN on the expression of COX-2 protein, known to be activated in LPS-stimulated macrophages. Equal amount of proteins (40 μg) were resolved to detect the expression of iNOS and COX-2 pro-

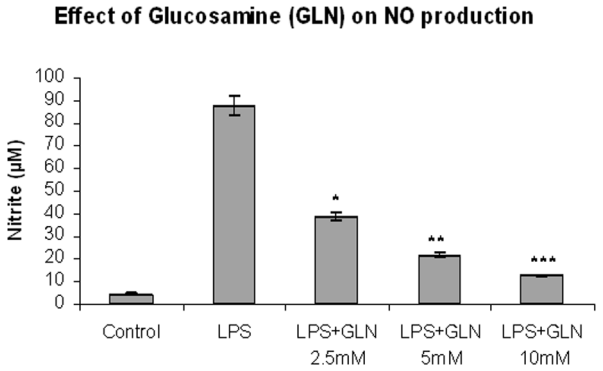

Figure 1. Effect of glucosamine on NO production. RAW 264.7 macrophage cells were treated with LPS (0.5 μg/mL) and/or various concentrations of GLN for 12 h, and the amount of nitrite in the supernatant from each treatment group was measured using Griess reagent. Each bar represents mean± SD of five replicates of a representative experiment of a total of four experiments. *Statistical significance of the difference between LPS and LPS + GLN treatment groups: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

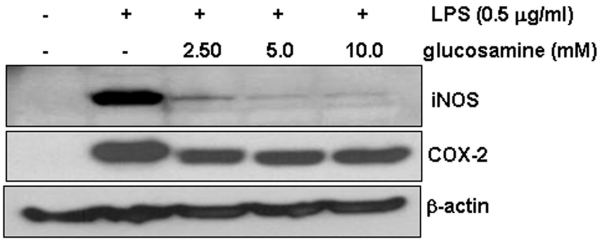

Figure 2. Inhibition of LPS-induced iNOS and COX-2 protein expression. RAW 264.7 macrophage cells were treated with LPS (0.5 μg/mL) and/or various concentrations of GLN for 18 h. Western blot analysis was performed using 40 μg of the total protein lysate as discussed in Materials and methods. This experiment was repeated three times with similar observation. The detection of B-actin was done in the same blot as a loading control.

teins by Western blot. Our results suggest that GLN significantly inhibits iNOS, in dose-dependent fashion. Furthermore, LPS-induced COX-2 was also inhibited by 2.5, 5.0, and 10 mM of GLN. However, COX-2 inhibition by GLN did not exhibit dose response (Fig. 2). The detection of β-actin was also performed in the same blot as loading control.

3.3 Effect of GLN on LPS-induced iNOS and COX-2 mRNA expression

Since iNOS and COX-2 expressions were decreased at the protein level, we investigated whether the decrease in NO production was due to a down-regulation of iNOS and COX-2 gene expression at the mRNA level using RT-PCR. Using gene specific primers, 2 μL of cDNA was amplified

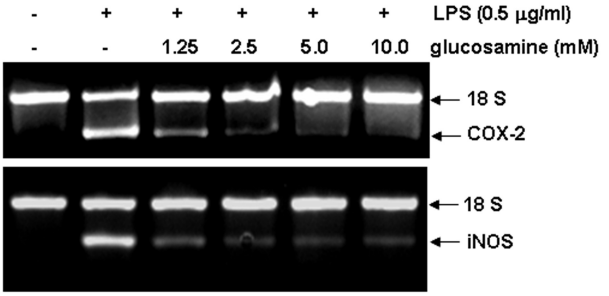

Figure 3. Inhibition of LPS-induced iNOS and COX-2 gene expressions. RAW 264.7 macrophage cells were treated with LPS (0.5 μg/mL) and/or various concentrations of GLN for 12 h and total RNA was isolated and 2 μg of the total RNA was reverse transcribed to make cDNA and amplified using gene specific primers. Equal volume of each PCR reaction was resolved in 2% agarose gel. 18S ribosomal RNA was amplified in the same reaction tube to serve as internal controls.

for 349 bp of iNOS, 297 bp of COX-2, and 495 bp of 18S ribosomal RNA by PCR as described in Materials and methods. We have observed that various concentrations of GLN inhibited the LPS (0.5 μg/mL) stimulated mRNA expression of iNOS after 12 h of treatment with RAW 264.7 cells. This inhibition of mRNA correlates to the inhibition of protein expression by GLN. As an internal control 18S rRNA was used which can be seen as a constant band size (Fig. 3). Treatment with GLN resulted in reduced expression for COX-2 and iNOS. Reductions in COX-2 and iNOS expressions were also noticeable at the lowest treatment levels. A dose-dependent experiment was carried out to determine the effect of the lowest concentration of GLN at the mRNA level and revealed that doses between 1.25 and 10 mM exhibited a dose-dependent inhibition (Fig. 3).

3.4 Inhibition of LPS-induced activation of p38-MAP kinase

Since activation of mitogen-activated protein kinases (MAPKs) have been shown to be involved in iNOS induction mediated by LPS in mouse macrophages [11, 15], we investigated the effects of GLN on the activation of p44/42- and p38-MAP kinase in LPS-stimulated RAW 264.7 macrophage cells. The phosphorylation of threonine and tyrosine residues is required for the activation of MAP kinases [16]. Maximum reduction in NO production as well as iNOS and COX-2 protein and mRNA expressions were observed after 10 mM treatment with GLN. When the cells were cotreated with GLN (10 mM) and LPS (0.5 μg/mL) for 15 and 30 min, the LPS-induced phosphorylation of p38-MAP kinase (pp38) was inhibited by GLN (10 mM) at the 15-min time point by 22% compared to LPS (Fig. 4), while activation of p44/42 MAP kinase (extracellular signal-regulated kinases (ERK) 1/2) was not inhibited (Fig. 4).

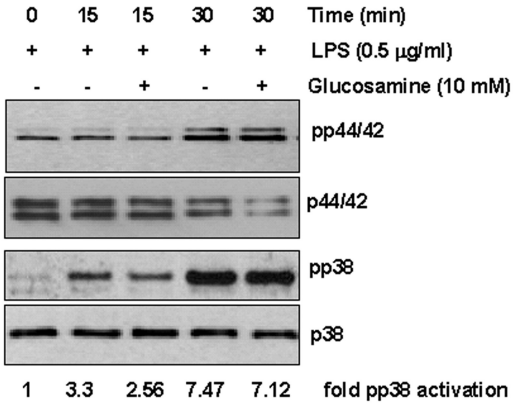

Figure 4. Effect of GLN on LPS-induced MAPK activation. Total protein lysates of RAW 264.7 cells were prepared after treatment with LPS and/or GLN (10 mM) for 15 and 30 min. A total 40 μg of protein lysates were resolved and probed for phosphorylated (pp42/44, pp38) and basal p42/44 and p38 using specific monoclonal antibodies as described in Materials and methods. This experiment was repeated three times with similar observation, and total fold change for pp38 has been calculated by measuring the integrated density of bands.

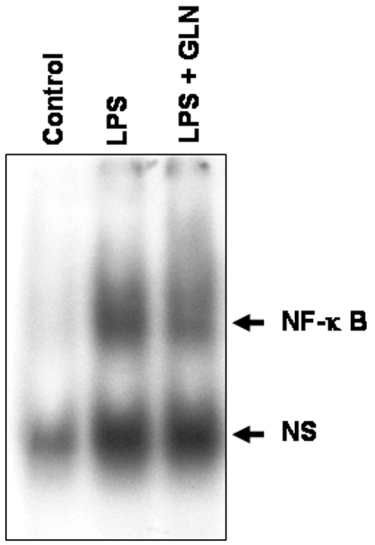

Figure 5. Effect of GLN on LPS-induced NK-κB activation: Nuclear protein lysates of RAW 264.7 cells were prepared after 2 h of treatment with LPS (0.5 μg/mL) either alone or with GLN (10 mM). Six micrograms of nuclear protein was used in the DNA binding assay, and the total binding reaction was resolved in 5% nondenaturing polyacrylamide gel. NS indicates nonspecific binding.

3.5 Effect of GLN on LPS-induced NF-κB activation

The involvement of transcription factor NF-κB in the expression of iNOS stimulated by proinflamatory cytokines and LPS is well known. Therefore, we investigated the possibility of inhibition of iNOS expression by GLN mediated through modulation of NF-κB activation. As shown in Fig. 5, the induction of specific NF-κB DNA binding activity by LPS was inhibited by GLN. The effect of GLN alone did not show any activation of NF-κB binding (data not shown). The specificity of binding was examined by competition with the addition of unlabeled/cold oligonucleotides, in excess (data not shown). The intensity of NF-κB binding was analyzed using BioRad Quantity One® software (Molecular Imager ChemiDoc XRS System, BioRad, Richmond. CA). Densitometry analysis showed that GLN at 10 mM inhibited NF-κB binding activity by 16%.

4 Discussion

GLN, a naturally occurring monoaminosaccharide present in connective and cartilage tissues, has been widely used for relieving symptoms from arthritis [2, 4]. Macrophages play an important role in response to inflammation by producing proinflammatory mediators, namely NO, superoxide anions, and cytokines [17]. The anti-inflammatory potential of GLN was investigated by Meininger and coworkers using *in vivo* and *in vitro* studies. A well-established rat model of LPS-induced inflammation intravenously administered with D-glucosamine before and after LPS, indicated that GLN decreased urinary nitrate excretion compared to nitrate level prior to D-glucosamine administration. An *in vitro* study also showed a decrease in NO production after LPS-induced mouse macrophage cells were treated with various concentrations of D-gluosamine [2]. Hua and coworkers evaluated the effect of GLN on rat adjuvant arthritis, a model of rheumatoid arthritis. Oral administration of GLN inhibited swelling in both hind paws of the mice where Friend's complete adjuvant (FCA) was injected. Plasma concentrations of NO and prostaglandin E_2 were also reduced after treatment of GLN, suggesting its anti-inflammatory potential in rheumatoid arthritis [18]. Although GLN is commonly taken to alleviate symptoms associated with arthritis, the mechanisms of action are yet to be definitively determined [19]. To further understand the molecular mechanism of GLN activity on elicited macrophages, we investigated the effect of GLN on the production of NO, expression of COX-2 and iNOS, activation of MAPKs (p38, p42/44) and transcription factor NF-κB.

In this study, we evaluated the nontoxic doses of GLN's effect on LPS-induced NO production in RAW 264.7 cells. In agreement with a previous study [2], our results also confirm the dose-dependent inhibition of NO production by GLN in LPS-induced RAW 264.7 cells (Fig. 1). Furthermore, we demonstrated that inhibition of NO production is concomitant with the suppression of iNOS expression at mRNA and protein levels, as shown by RT-PCR and Western blots (Figs. 2 and 3). In addition, we studied another important mediator of inflammation, COX-2, which acts on

arachidonic acid to release prostaglandins that further orchestrate the process of inflammation [20]. Similar to iNOS inhibition, GLN also inhibits the COX-2 protein and mRNA expression (Figs. 2 and 3).

Numerous studies have suggested that LPS regulates iNOS and COX-2 expression by the MAPK signaling pathway [21, 14, 22, 23]. However, signaling from MAPKs to transcription factors mediating iNOS and COX-2 expression are not fully understood. Stimulation of monocyte/macrophage by LPS activates several intracellular signaling pathways that include the NF-κB pathway and three MAP kinase pathways: ERK (p42/44) 1 and 2, c-Jun N-terminal kinase (JNK) and p38 [24]. ERK1/2 belongs to the Ser/Thr MAPK family and is activated in a concentration-dependent manner by LPS [11]. Previously, it has been demonstrated that GLN inhibits the fMLP (formyl-Met-Leu-Phe) induced activation of p38 MAPK in human peripheral blood neutrophils [4]. However, another study could not demonstrate any inhibitory effect of GLN on IL-1β induced MAPKs in human chondrocytes [19]. In our study, we have shown that p38 and p44/42 were phosphorylated by stimulation with LPS. Furthermore, phosphorylation of p38 was inhibited (22%) by GLN at 15 min of LPS stimulation, whereas there was no effect of GLN on pp44/42 (Fig. 4). However, the inhibitory effect of GLN on pp38 was not observed after 30 min of LPS stimulation. Therefore, for the first time we are able to demonstrate that GLN selectively inhibits LPS-induced pp38, while no effect was observed on pp44/42 MAPK in RAW 264.7 cells.

The transcription factor NF-κB is important in inflammatory response specifically through the regulation of COX-2 and iNOS [13]. NF-κB is composed of members of the Rel family including p65 (RelA), p50, p52, *etc.* NF-κB is normally a heterodimer of p50 and p65. It is a *cis* acting element located at the 5′ flanking region of COX-2 and iNOS [25, 26]. The promoter for iNOS has two binding sites for NF-κB at 55 and 971 bp upstream from the TATA box [27, 28]. In its inactive form, in the cytoplasm, NF-κB is complexed with IκB. When NF-κB is stimulated it can then enter the nucleus and activate genes. Upon stimulation, for example with LPS, NF-κB-inducing kinase (NIK) and MAPK/ERK kinase kinase-1 (MEKK1) are stimulated and phosphorylate IKK1 and IKK2, components of the IκB complex [13].

The protective effect of GLN has been suggested by inhibiting the production of reactive oxygen species (ROS), expression of proinflammatory genes (iNOS, COX-2), and activation of transcription factors such as NF-κB [29]. Previous studies have shown that GLN-inhibited IL-1 beta induced NF-κB activation in human chondrocytes [30]. In the present study, using EMSA we demonstrated that GLN inhibits LPS-induced NF-κB in RAW 264.7 cells. These data together suggest that GLN regulates the expression of iNOS and COX-2 by inhibiting pp38 and NF-κB. Therefore, the results of this study propose the underlying anti-inflammatory molecular mechanism of action of GLN in mouse macrophage cell lines.

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5 References

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