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Glucosamine Inhibits Lipopolysaccharide-Induced Microglial Activation via a Down-Regulation of iNOS Gene

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Abstract

This study investigated the effects of glucosamine on lipopolysaccharide (LPS)-induced cellular activation, inducible nitric oxide synthase (iNOS) expression in microglia, and the molecular and signaling mechanisms involved. LPS with 100 ng/mL was used for activation of BV2 microglial cells. LPS-induced mRNA and protein expressions for iNOS and other signaling factors were analyzed by reverse-transcription polymerase chain reaction and Western blot analysis, respectively. Glucosamine attenuated LPS-induced iNOS protein and mRNA expression in dose- and time-dependent manners, but did not affect iNOS protein stability. However, the inhibitory actions of glucosamine on LPS-induced iNOS expressions were independent of nuclear factor-κB signals and mitogen-activated protein kinases activations. Glucosamine inhibited LPS-induced phosphorylation of ribosomal p85 S6, translational regulatory protein, which may down-regulate translation in iNOS mRNA. These results suggest that inhibitory action mechanisms of glucosamine on LPS-induced microglial activation include down-regulation of microglial activator gene iNOS herein.

Key Words : Glucosamine, Inducible nitric oxide synthase, Lipopolysaccharide, Microglia

Introduction

Microglia, a resident macrophage-like population of brain cells, have been proposed to play a role in host defense and tissue repair in the central nervous system (CNS) [1]. Microglial activation occurs rapidly after brain injuries such as inflammatory, infectious, ischemic, and neurodegenerative pathologies of the CNS [2]. Presence of activated

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microglia has been demonstrated in pathological lesions in several neurological diseases including Alzheimer's disease, Parkinson's disease, multiple sclerosis, and acquired immune deficiency syndrome dementia complex [1]. In addition to these pathologic conditions, microglia become activated by inflammatory mediators such as lipopolysaccharide (LPS) and various interleukins [2,3].

LPS is a major component of the outer membrane constituent of Gram-negative bacteria and a potent activator of microglia [4]. LPS binds to a CD14-like receptor in microglia and activates the Toll-like receptor 4 in the brain [5]. The LPS signal induces activations of a variety of proteins including mitogen-activated protein kinases (MAPKs) [6], a transcriptional factor nuclear factor-κB (NF-κB) [7], or inducible nitric oxide synthase (iNOS) [8].

Various functions of microglia have been correlated with the state of activation, ranging from the ramified resting microglia to the ameboid reactive microglia [9]. The process of microglial activation is accompanied by marked morphological changes, phagocytosis, induction of various pro-inflammatory cytokines, and superoxide radicals [1, 4]. One of the central mediators of the microglial activated response is nitric oxide (NO). NO is synthesized from L-arginine and molecular oxygen by nitric oxide synthase (NOS). So far three isoforms of NOS have been identified and molecularly cloned. Of the three NOS isoforms, iNOS is expressed in microglial cells as a response to bacterial endotoxins like LPS or pro-inflammatory cytokines such as interleukins and TNF-α [7]. It has been suggested that NO from iNOS may be a key mediator of glial-induced neuronal death in various neurodegenerative disorders [10].

NF-κB activation in response to LPS stimulation is well correlated with transcriptional activation of iNOS in macrophages [11]. Interestingly, glucosamine is known to inhibit interleukin-1β-induced expression of inflammatory mediators, COX-2 and iNOS, in chondrocytes through inhibition of NF-κB activation [12]. In addition to NF-κB, activation of signaling proteins like MAPKs is well associated with LPS-induced iNOS expression in macrophages [13]. In general, MAPKs consist of three subfamily, including extracellular signal-regulated kinases (ERKs), c-*jun* N-terminal kinases (JNKs), and p38 MAPK. These MAPKs are known to be phosphorylated and activated in response to extracellular stimuli including LPS. S6Ks are known to participate in translation of mRNAs encoding ribosomal proteins or elongation factors [14] and consists of two isoforms, including p70 and p85 S6K. Of particular interest, some data have shown that p70 S6K involves in translation of iNOS in response to LPS in macrophages [15].

Glucosamine is a dietary supplement for rheumatoid arthritis or osteoarthritis and as a pain reliever. Many studies have demonstrated that glucosamine modulates interleukin-1β-induced NF-κB activation in chondrocytes [12,16] and inhibits LPS-induced NO production in RAW 264.7 macrophages [17]. Glucosamine can be distributed to brain at relevant quantity after 8 h of oral administration [18], thus suggesting this agent as part of a new inhibitory strategy in targeting activated microglial cells in the CNS. However, at present, it is largely unknown about the inhibitory action mechanisms of glucosamine

against LPS-induced activation and iNOS expression of microglial cells.

Therefore, this study investigated the effects of glucosamine on LPS-induced activation of microglia, iNOS expression in LPS-activated microglia, and the molecular and signaling mechanisms involved.

Materials & Methods

Materials

RPMI 1640, Dulbeccos Modified Eagle's Medium (DMEM), penicillin and streptomycin were obtained from GIBCO-BRL (Grand Island, NY, USA). Fetal bovine serum was from Hyclone (Logan, UT, USA). D-glucosamine and LPS were from Sigma (St. Louis, MO, USA). Cycloheximide and rapamycin were from Biomol (Plymouth, PA, USA). Bradford reagent was from Bio-Rad (Hercules, CA, USA) . iNOS polyclonal antibody was from Upstate Biotechnology (Lake Placid, NY, USA). Antibodies against phospho-ERKs (p-ERKs), phospho-JNKs (p-JNKs), JNKs, phospho-p38s (p-p38s), phospho-S6K (p-S6K), S6K, phospho-S6 (p-S6), and S6 were from Cell Signaling Tech (Beverly, MA, USA). Antibodies against NF-κB and IκB-α were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other laboratory chemicals were purchased from Sigma.

Cell culture and preparation of whole cell lysates

BV2 murine microglia (BV2 cells) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 ?g/mL streptomycin. Whole cell lysates for detecting phospho-proteins as well as iNOS were prepared as previously reported [19].

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with the RNAzol-B (Tel-Test, Friendswood, TX, USA). Three micrograms RNA were reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Single stranded cDNA was amplified by PCR with primers: iNOS sense, 5'-GACAAGCTGCA TGTGACATC-3'; iNOS anti-sense, 5'-GCTGGTAGGTTCCTGTTGTT-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5-GGTGAAGGTCGGT GTGAACG-3; GAPDH anti-sense, 5-GGTAGGAACACGGAAGGCCA-3. The following PCR conditions were applied: iNOS, 25 cycles of denaturation at 94˚C for 30 s, annealing at 52˚C for 30 s, and extension at 72˚C for 30 s; GAPDH, 18 cycles of denaturation at 94˚C for 30 s, annealing at 57˚C for 30 s, and extension at 72˚C for 30 s. GAPDH was used as an internal control to evaluate relative expression of iNOS.

Western blot analysis

Western blot was carried out as previously reported [19]. After electrophoresis and transferring, the membrane (Millipore, Billerica, MA, USA) was washed with Tris-buffered saline (TBS, 10 mM Tris, 150 mM NaCl) containing 0.05% (vol/vol) Tween 20 (TBST) and blocked in TBST containing 5%

(wt/vol) non-fat dried milk. The membrane was incubated with p-JNKs (1:1,000), JNKs (1:1,000), p-ERKs (1:2,000), p-p38s (1:2,000), p-S6K (1:1,000), S6K (1:1,000), IκB-α (1:2,000), NF-κB (1:2,000), iNOS (1:2,000), or actin (1:10,000) antibodies. The membrane was then treated with secondary antibodies coupled to horseradish peroxidase, and developed in the ECL Western detection reagents.

Results

LPS increases iNOS protein expression in BV2 microglial cells

The effects of different concentrations of LPS, a well-known iNOS inducer, on iNOS protein expression in BV2 microglial cells were initially determined by Western blot experiment. As shown in Fig. 1A, LPS dose-dependently induced iNOS protein expression in BV2 cells. The 100 ng/mL concentration of LPS was sufficient to highly induce iNOS protein expression in BV2 cells. Data of cytotoxic tests, including MTS cell viability test and trypan blue dye-based cell counting assays, demonstrated no cytotoxicity in BV2 cells by the 100 ng/mL concentration of LPS treatment (data not shown). The kinetic of iNOS expression by LPS with 100 ng/mL was next determined. As shown in Fig. 1B, iNOS protein was weakly induced at 4 h LPS treatment, followed drastic increase of iNOS protein thereafter in a time-dependent manner. Control actin protein was not changed by treatment with LPS in different doses and times (Fig. 1A & B).

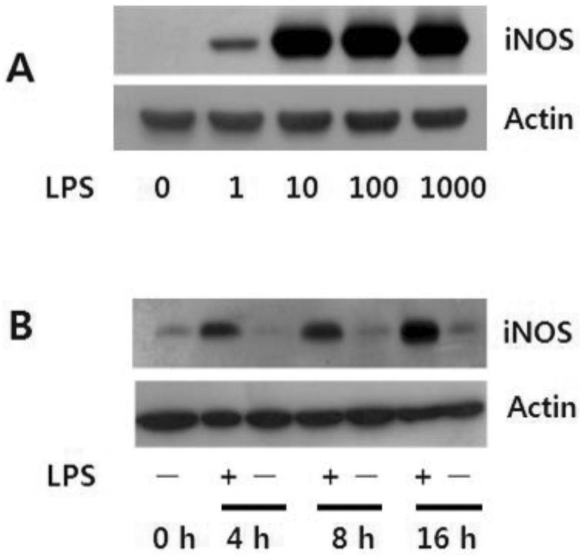

Fig. 1. Effects of LPS on inducible nitric oxide synthase (iNOS) protein expression in BV2 microglial cells. Microglial cells were treated with different concentrations (ng/mL) of LPS for 16 h (A). Microglial cells were treated with 100 ng/mL LPS for the indicated times (B). Whole cell lysates and total RNA were prepared and analyzed for iNOS or actin Western blot. LPS induces iNOS protein expression in dose- and time-dependent manners. Actin was used as a reaction standard.

Glucosamine attenuates LPS-induced iNOS protein and mRNA expression with no change in iNOS protein stability in BV2 microglial cells

The effects of different concentrations of glucosamine on LPS- induced iNOS protein and mRNA expression in BV2 cells were next evaluated by Western blot and RT-PCR methods, respectively. Glucosamine inhibited LPS-induced expression of iNOS protein in a dose-dependent manner in BV2 cells (Fig. 2A). Similar to inhibition of iNOS protein expression, there was a dose-dependent inhibition of LPS-induced iNOS mRNA

expression by glucosamine (Fig. 2B).

Notably, 5 mM of glucosamine was sufficient to completely inhibit the expression of iNOS mRNA, induced by LPS, in BV2 cells. Kinetic results interestingly demonstrated that there was strong suppression of LPS-induced expression of both iNOS protein and mRNA after 16 h treatment with glucosamine (Fig. 2C & D), suggesting that long time treatment of glucosamine may be effective in inhibiting expression of iNOS protein and mRNA in response to LPS. Data of the down-regulation of LPS-induced expression of iNOS mRNA and protein by glucosamine suggested that the inhibition might be due to reduction of transcription and translation or

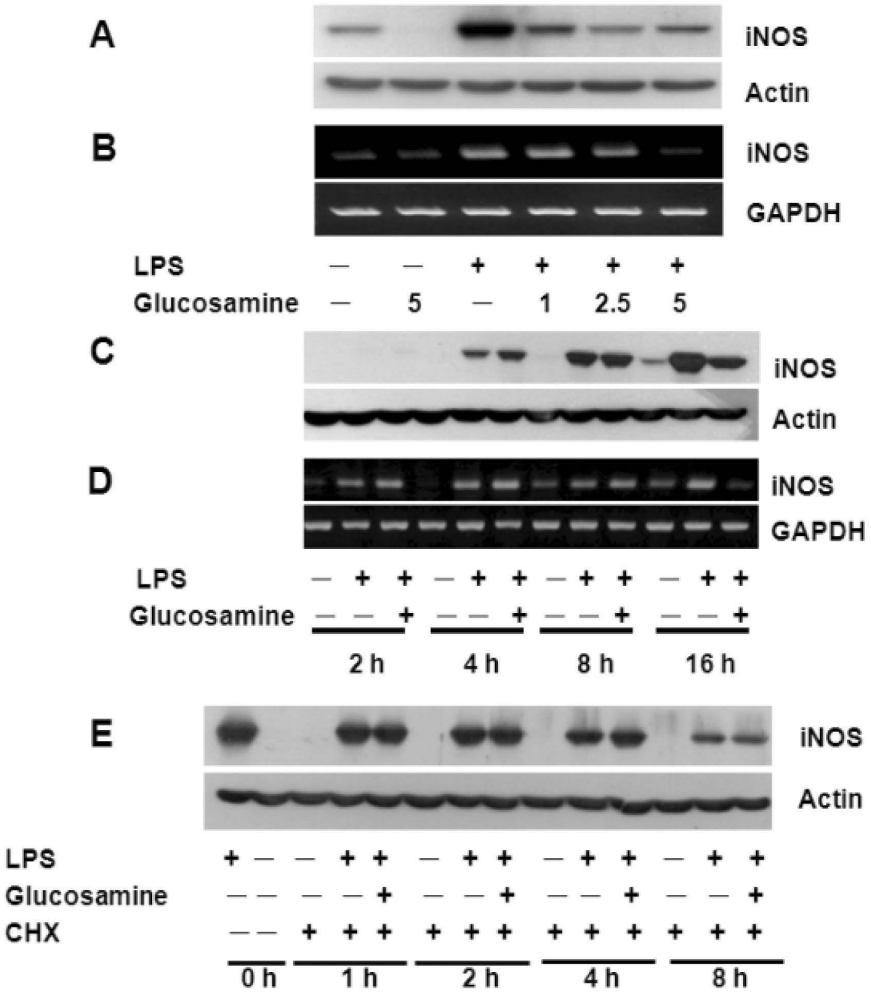

Fig. 2. Effects of glucosamine on LPS-induced iNOS expressions and protein stability in BV2 microglial cells. Microglial cells were treated with LPS (100 ng/mL) and/or different concnetrations (mM) of glucosamine for 16 h (A & B). Microglial cells were treated with 5 mM glucosamine and/or LPS for the indicated times (C & D). Whole cell lysates and total RNA were prepared and analyzed for iNOS or actin Western, and iNOS or GAPDH RT-PCR, respectively. Glucosamine does not affect LPS-induced iNOS protein stability in the presence or absence of protein synthesis inhibitor, cycloheximide (CHX, 2 μg/mL) as E.

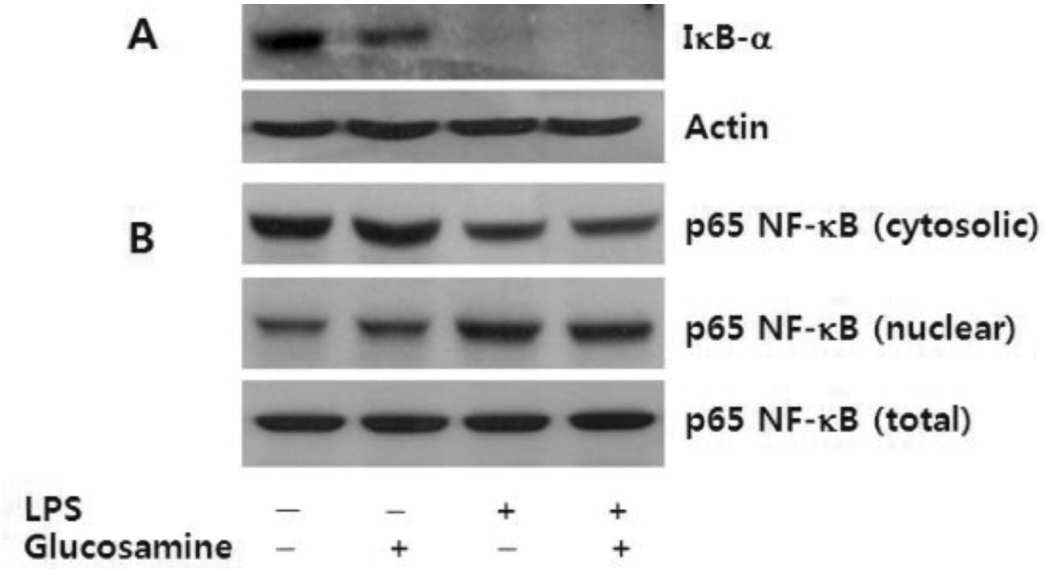

Fig. 3. Effects of glucosamine on LPS-induced inhibitory κB (IκB)-α degradation and nuclear factor (NF)-κB nuclear localization in BV2 microglial cells. Microglial cells were treated with LPS (100 ng/mL) and/or glucosamine (10 mM) for 16 h. Whole cell lysates were prepared and analyzed for IκB-α or actin Western blot (A). LPS-induced cellular fractioned or total NF-κB expressions were analyzed in the absence or presence of glucosamine (B). Glucosamine does not affect LPS-induced NF-κB signals in BV2 microglial cells.

protein stability of iNOS, respectively. Whether glucosamine affects iNOS protein stability at post-translational level was next evaluated. For the purpose, the protein synthesis inhibitor cycloheximide (2 μg/mL) was used to assess any change of LPS-induced iNOS protein stability in the absence or presnece of glucosamine.

As shown in Fig. 2E, there was natural iNOS protein degradation in the absence of glucosamine, but there was no significant decrease or change in iNOS protein levels in the presence of glucosamine, suggesting no effect of glucosamine on iNOS proteins stability. Thus, these results suggest that inhibition of LPS-induced iNOS expression by glucosamine is not through inhibition of iNOS protein stability but via reduction of steady-state iNOS mRNA levels or transcription.

Glucosamine attenuates LPS-induced iNOS protein expression independent of nuclear factor (NF)-κB signals in BV2 microglial cells

The effects of glucosamine on LPS-induced NF-κB activation in BV2 cells were next examined. As expected, LPS induced the activation of NF-κB, which was determined by increased proteolytic degradation of IκB-α, a NF-κB inhibitory protein (lane 3, Fig. 3A). Activation of NF-κB by LPS was further shown in which there was decreased level of cytoplasmic p65 NF-κB but increased level of nuclear p65 NF-κB in no change of total protein level of p65 NF-κB (lane 3, Fig. 3B). However, such NF-κB activation elicited by LPS was not affected by addition of glucosamine, thus, suggesting that suppressive effect of glucosamine on iNOS

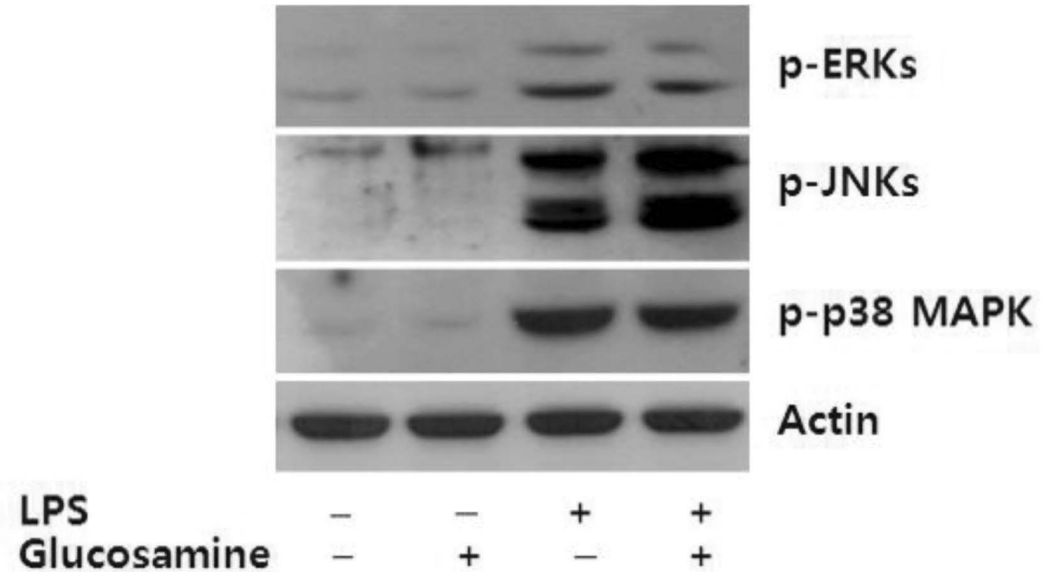

Fig. 4. Effects of glucosamine on LPS-induced mitogen-activated protein kinases (MAPKs) in BV2 microglial cells. Microglial cells were treated with LPS (100 ng/mL) and/or glucosamine (10 mM) for 16 h. Whole cell lysates were prepared and analyzed for p-ERKs, p-JNKs, p-p38 MAPK or actin Western blot with respective antibodies. Glucosamine does not affect LPS-induced MAPKs activation in BV2 microglial cells.

expression in BV2 cells is not through inhibition of NF-κB pathway.

Glucosamine attenuates LPS-induced iNOS protein expression independent of mitogen-activated protei kinases (MAPKs) activation in BV2 microglial cells

There was increased phosphorylation of ERKs, JNKs and p38 MAPK in BV2 cells after 30 min LPS treatment (lane 3, Fig. 4). However, pretreatment with glucosamine had no effect on LPS-induced phosphorylation of these MAPKs (lane 4, Fig. 4), addressing the ability of glucosamine to inhibit LPS-induced iNOS expression in BV2 cells independent of MAPKs.

Glucosamine inhibits LPS-induced phosphorylation of ribosomal p85 S6 kinase (p85 S6K) in BV2 microglial cells

The effects of LPS on phosphorylation of S6Ks in BV2 cells were next examined. Clearly, LPS induced the phosphorylation of p70 and p85 S6Ks in BV2 cells (lane 4, Fig. 5). The effects of glucosamine on LPS-induced phosphorylation of these S6Ks in BV2 cells were next determined. Notably, glucosamine inhibited LPS-induced phosphorylation of p85 S6K, but not p70 S6K, in BV2 cells (lane 2 & 4, Fig. 5). The specific inhibition of LPS-induced phosphorylation of p85 S6K by glucosamine was confirmed by rapamycin, a well-known pharmacological inhibitor of mTOR/p70 S6K that specifically blocked basal or LPS-induced phosphorylation of p70 S6K in BV2 cells (lane 3 & 6 , Fig. 5). Total protein levels of p70 S6K and actin were not significantly changed by glucosamine or

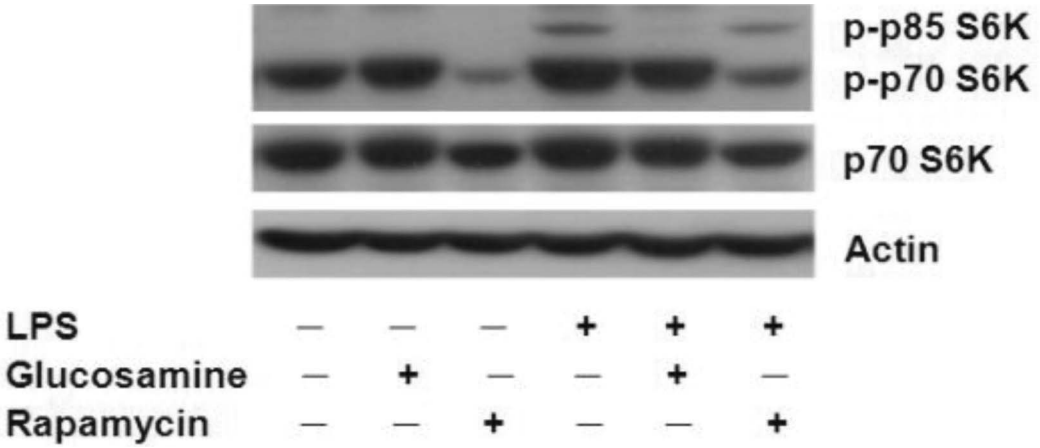

Fig. 5. Effects of glucosamine on LPS-induced phosphorylation of ribosomal p70 and p85 S6 kinases (S6Ks) in BV2 microglial cells. BV2 cells pretreated for 1 h with 6 nM rapamycin, an inhibitor of mTOR/p70 S6K, and then with LPS (100 ng/mL) and/or glucosamine (10 mM) for 16 h. Whole cell lysates were prepared and analyzed for p-p85 S6K, p-p70 S6K, p70 S6K, or actin Western blot with respective antibodies. Glucosamine inhibits LPS-induced phosphorylation of p85 S6K, but not of p70 S6K in BV2 microglial cells.

rapamyin. These results suggest that the ability of glucosamine to inhibit LPS-induced iNOS expression in BV2 cells may be in part associated with suppression of phosphorylation of p85 S6K translational regulatory protein, which may down-regulate translation in iNOS mRNA.

Discussion

Activation of microglia results in production of NO. Excess NO released by microglia cells as a consequence of iNOS induction may contribute to immunomodulation and neuronal damage in neurodegenerative disorders. iNOS is expressed in microglia as a response to bacterial endotoxins or pro-inflammatory cytokines [7,13]. Several studies have shown that LPS induces iNOS expression in microglia through the activation of NF-κB and/or phosphorylation of MAPKs [7,8,11,13]. Consistent with these previous results, the present experiment also demonstrated that LPS rapidly and strongly induced activation of NF-κB and MAPKs in BV2 microglial cells. This experiment also clearly demonstrated that LPS induced iNOS mRNA and protein expressions in BV2 cells.

Glucosamine has been used in treatment of osteoarthritis and reliever of joint pain for long period. Glucosamine increases the formation of new cartilage by stimulating chondrocyte synthesis of collagen, proteoglycans and hyaluronic acid, and by inhibiting proteolytic and lysosomal enzymes responsible for damage of joint cartilage [20]. Cartilage damage often seen in osteoarthritis is known to be largely mediated by interleukin-1β, a pro-inflammaory cytokine that initiates a number of events leading to cartilage destruction [16]. It has been reported that glucosamine inhibits interleukin-1β -induced activation of

chondrocytes by inhibition of NF-κB activation [12,16]. Though its action mechanism was not clearly determined, glucosamine was shown to inhibit NO production and iNOS protein expression in LPS-treated macrophages [17]. Consistent with this, this experiment also disclosed that glucosamine could inhibit the LPS-induced iNOS protein expression in BV2 cells. An interesting finding of the present study was that glucosamine inhibited LPS-induced phosphorylation of p85 S6K translational regulatory protein in BV2 microglial cells, while the drug had no effect on LPS-induced IκB-α degradation, NF-κB nuclear localization, and MAPKs activation. These results suggest that an inhibitory mechanism of glucosamine on LPS-induced iNOS expression may be, at least in part, associated with inhibition of p85 S6K. These results may, therefore, further suggest p85 S6K as a novel target of glucosamine in down-regulating iNOS expression induced by LPS signaling pathway.

Summary

This study investigated the effects of glucosamine on LPS-induced cellular activation, iNOS expression in microglia, and the molecular and signaling mechanisms involved.

LPS was used for activation of BV2 microglial cells. LPS-induced mRNA and protein expressions for iNOS and other signaling factors were analyzed by RT-PCR and Western blot analysis, respectively.

Glucosamine attenuated LPS-induced iNOS protein and mRNA expression in dose- and time-dependent manners, but did not affect iNOS protein stability. However, the inhibitory actions of glucosamine on LPS-induced iNOS expressions were independent of NF-κB signals and MAPKs activations. Glucosamine inhibited LPS-induced phos-phorylation of p85 S6K which may down-regulate translation in iNOS mRNA.

These results suggest that inhibitory action mechanisms of glucosamine on LPS-induced microglial activation include down-regulation of microglial activator genes iNOS herein.

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