Anti-inflammatory effects of glutamine on LPS-stimulated human dental pulp cells correlate with activation of MKP-1 and attenuation of the MAPK and NF-kB pathways


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Running title: Anti-inflammatory effects of glutamine in pulp cells

Key Words: Glutamine; Human Dental Pulp Cells; LPS; Proinflammatory mediators; MKP-1; NF-kB; MAPK

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/iej.12303
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ABSTRACT

Aim The purpose of this study was to evaluate the anti-inflammatory effects of glutamine and the underlying signal pathway mechanisms in lipopolysaccharide (LPS)-stimulated human dental pulp cells (HDPCs).

Method HDPCs cells were exposed to 10μg/mL LPS and various concentrations of glutamine for 24 h. The production of PGE₂ and nitric oxide was determined by enzyme-linked immunosorbent assay (ELISA) and Griess reagent kit, respectively. Cytokines were examined by ELISA, reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time PCR. iNOS and COX protein expression as well as signal pathways were accessed by Western blot. The data were analysed by ANOVA with Bonferroni’s test (α =0.05).

Results Glutamine reduced LPS-induced iNOS and COX-2 protein expression as well as production of NO and PGE₂ in a dose-dependent fashion. Additionally, glutamine suppressed the production and mRNA expression of inflammatory cytokines including IL-1β, TNF-α, and IL-8. Furthermore, glutamine attenuated phosphorylation of extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal kinase (JNK) and IκB-α, and nuclear translocation of NF-kB p65, but enhanced mitogen-activated protein kinase phosphatase-1 (MKP-1) expression in LPS-treated HDPCs.

Conclusion Glutamine exerted an anti-inflammatory effect via activation of MKP-1 and inhibition of the NF-kB and MAPK pathways in LPS-treated HDPCs.

INTRODUCTION

Dental pulp tissues are frequently infected or inflamed due to bacterial ingress from dental caries (Stashenko et al. 1998). Both gram-negative and gram-positive bacteria have been detected in infected root canals and pulpitis (Hosoya & Matsushima 1997). Lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative bacteria, plays a key role in pulpal, periodontal,
and periapical disease through the production of pro-inflammatory mediators including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8) (Rupf et al. 2000, Coil et al. 2004).

Human dental pulp cells (HDPCs) also have the capacity to produce pro-inflammatory cytokines such as IL-6 and IL-8, and express adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in response to LPS (Tokuda et al. 2001, Li & Verma 2002, Yang et al. 2003, Nakanishi et al. 2010). Previously, it was reported that heat stress (42°C, 30 min) and LPS upregulate SIRT1 and immune and defense genes such as interleukin (IL)-8 and heme oxygenase-1 (HO-1) in HDPCs (Lee et al. 2011).

Glutamine is the most abundant amino acid and is produced in sufficient quantities in a healthy, physiologically stable body. However, under metabolic stresses, this amino acid becomes limited and the body becomes vulnerable to infections and immune responses (Parry-Billings et al. 1990, Newsholme 1996). The decrease in glutamine is associated with a strong positive correlation to the individual loss of CD4+ T cells (Hack et al. 1997). In addition, lymphocyte proliferation (Rohde et al. 1996), production of IL-2 and IFN—gamma (Horig et al. 1993, Rohde et al. 1996), and expression of surface activation markers such as CD25, CD45RO, and CD71 (Horig et al. 1993) were inhibited by low glutamine levels. In contrast, glutamine supplementation reduced pro-inflammatory cytokine production in human intestinal mucosa (Ko et al. 2009), protects against endotoxic shock in mice (coeffier et al. 2001), decreased inflammation in infant rat endotoxemia (Garrett-Cox et al. 2009), and attenuated LPS-induced intestinal inflammation in rats (Li et al. 2004). Recently, it was demonstrated that glutamine promotes growth, migration, and differentiation in human dental pulp cells (HDPCS) through the BMP-2 and mitogen-activated protein kinase (MAPK) pathways (Kim et al. 2014). However, the effect of glutamine on LPS-induced pulpal inflammation has not been reported.

The aim of this study was to investigate the anti-inflammatory activity of glutamine in pulpal inflammation by focusing on pro-inflammatory cytokines in LPS-stimulated HDPCs and comparing the effects of glutamine to treatment with the selective COX-2 inhibitor NS398. The hypothesis was that exogenous glutamine in HDPCs might be a potential strategy by which to treat LPS-mediated
MATERIALS AND METHODS

Cell culture

Immortalized HDPCs, transfected with human telomerase catalytic component (hTERT), were kindly provided by Professor Takashi Takata (Hiroshima University, Japan) (Kitagawa et al. 2007). Cells were cultured in α-modified Eagle medium supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Bio. Whittaker, Walkersville, MD, USA) in a humidified atmosphere of 5% CO2 at 37°C.

Secretion of PGE2 and cytokines by ELISA

Cells were cultured in 12-well plates with 1 × 10^4 cells/well. The cultures were serum-starved for 24 h and treated with experimental agents. After incubation of the cultures in experimental conditions, the medium of each sample was collected and assayed for prostaglandin E2 (PGE2), TNF-α, IL-1β and IL-8 synthesis according to the manufacturer’s protocol using a commercial enzyme-linked immunosorbent assay (ELISA) (R&D Systems, MN) and calibrated spectrophotometrically with a standard curve. The experiments were performed in triplicate.

Determination of NO production

In brief, 100μL of cell culture medium was mixed with 100μL of Griess reagent [1% sulfanilamide and 0.1% naphthylethlenediamine dihydrochloride in 2.5% phosphoric acid], the mixture was incubated at room temperature for 10 min, and the absorbance at 540nm was measured in a microplate reader. Fresh culture medium was employed as a blank in every experiment. The quantity of nitrite was determined from a sodium nitrite standard curve.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

mRNA expression of cytokines and beta-actin was monitored by semiquantitative RT-PCR. Briefly, total RNA was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD, USA)

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according to the manufacturer’s instructions. Briefly, the cells were lysed directly in the plate wells using 0.5 ml Trizol reagent per well. After chloroform extraction, the total RNA was recovered from the aqueous phase and precipitated with an equal volume of isopropanol and diethylpyrocarbonate (DEPC)-treated water after a brief wash with 75% ethanol. Reverse transcription of RNA was performed using AccuPower RT PreMix (Bioneer, Daejeon, The Republic of Korea). Thereafter, the RT-generated DNA (2–5 μl) was amplified using AccuPower PCR PreMix (Bioneer, Daejeon, The Republic of Korea). The PCR cycle number was optimized for each experimental condition and primer set. Representative samples were run for a varying number of cycles (22-38 cycles), and the optimal cycle number was selected in the region of linearity between the cycle number and PCR product intensity. To confirm a linear relationship between the template and PCR product intensity at the optimal cycle number, PCR was run at different concentrations of cDNA obtained from representative samples. All reactions included a negative control in which cDNA was omitted from the PCR. To prove sample equality, amplification of the beta-actin was used as an internal control. The PCR products were subjected to electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

**Real-Time RT-PCR**

Total RNA was reverse transcribed using AccuPower RT Premix (Bioneer) according to the instructions of the manufacturer. The resulting cDNA (equivalent to 40 ng total RNA) was used in quantitative real-time PCR using FastStart DNA MasterPLUS SYBR Green I (Roche Applied Science, Indianapolis, IN, USA) and LightCycler according to the instructions of the manufacturer. PCR was performed using the following specific primers: TNF-α: sense primer, 5’- CAT CTT CTC AAA ATT CGA GTG ACA A-3’ and antisense primer, 5’- TGG GAG TAG ACA AGG TAC ACA ACT GCT-3’; IL-1β; sense primer, 5’-CAA CCA ACA AGT GAT ATT CTC CAT G-3’ and antisense primer, 5’-CAC GAT TTC CCA GAG AAC ATG TG-3’; IL-8: sense primer, 5’-ATG ACT TCC AAG CTG GCC GTG GCT-3’ and antisense primer, 5’-TCT CAG CCC TCT TCA AAA ACT TCT C-3’; GAPDH: sense primer, 5’-ACC ACA GTC CAT GCC ATC AC-3’ and antisense primer, 5’-TCC ACC ACC
CTG TTG CTG TA-3’. All samples were normalized to GAPDH. A comparison was done for each sample using a relative cycle threshold (Ct) comparison. An average Ct was calculated for the triplicate reactions and normalized to GAPDH (DCT = CtSample – CtGAPDH). The Ct values were then compared between different treatments and time points (DDCt) with normalization to the untreated samples. Finally, a fold change was calculated from the DDCt (fold change = 2DDct).

Preparation of nuclear and cytosol extracts

The cytosolic and nuclear protein fractions from LPS and glutamine-treated cells were separated. Briefly, cells were washed with ice-cold PBS, and pelleted. The cell pellet was resuspended in hypotonic buffer (mM): HEPES 10, pH 7.9, KCl 10, DTT 0.5, aprotinin 10, leupeptin 10 and PMSF 20 for 15 min on ice, and vortexed for 10 s. Nuclei were pelleted by centrifugation at 15,000×g for 1 min. The supernatants containing cytosolic proteins were collected. A pellet containing nuclei was resuspended in hypertonic buffer (mM) HEPES 20, pH 7.6, 25% glycerol, MgCl₂ 1.5, EDTA 4, DTT 0.05, PMSF 20, aprotinin 10 and leupeptin 10, for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 15,000×g for 2 min and stored at −70°C. In study of NF-κB activation, nuclear and cytosolic extracts were used in p65 NF-κB and phospho-IκB α, respectively.

Western blot analysis

Cells (1 × 10⁶) from each set of experiments were harvested and washed twice in cold tris-buffered saline. Cells were solubilized in ice-cold 1% Triton X-100 lysis buffer. After 30 min on ice, the lysates were clarified by centrifugation. Proteins (20 µg) were resolved by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose membranes. After blocking nonspecific sites with 1% bovine serum albumin (BSA), the membrane was incubated overnight with specific primary antibody for phospho-ERK (1:1500, Cell Signaling, Beverly, MA, USA), ERK (1:1500, Cell Signaling), phospho-p38 (1:1500, Cell Signaling), p-p38 (1:1500, Cell Signaling), phospho-JNK (1:1500, Cell Signaling), JNK (1:1500, Cell Signaling), MKP-1 (1:1000, Sigma-Aldrich Chemical Co, St. Louis, Missouri, USA), respectively.
Mo), p65 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phospho-IκB p65 (1:1000, Santa Cruz Biotechnology) at 4 °C. The membrane was then incubated for an additional 60 min with a peroxidase-conjugated secondary antibody (1:5000, Vector Laboratories, Burlingame, CA, USA) at room temperature. The immunoactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit. Desitometric analysis of each blot was performed with a computerized image processing system (Quantity One; Bio-Rad, Hercules, CA, USA).

Statistical analysis
The results were expressed as the mean ± SD, and an analysis of variance (ANOVA) with Bonferroni’s test was used for multiple comparisons.

RESULTS
Effects of glutamine on LPS-induced pro-inflammatory cytokines and mediators
To explore the potential anti-inflammatory properties of glutamine in HDPCs, the cells were treated with glutamine and LPS (10 μg/mL) for 24 h. Glutamine significantly attenuated the LPS-elicited increase in NO and PGE2 levels (P < 0.05) as well as iNOS and COX-2 protein expression, in a concentration-dependent manner (Fig. 1 A, B, C). The effect of 20 mM glutamine on NO and PGE2 levels as well as iNOS and COX-2 protein expression was similar to that of 10 μM NS-398. Since TNF-α, IL-1β, and IL-8 are the major inflammatory cytokines in pulpal and periapical disease (Coil et al. 2004), the inhibitory effect of glutamine on their mRNA expression and secretion into the medium were measured by RT-PCR, real-time PCR and ELISA kits. Glutamine significantly decreased LPS-induced TNF-α, IL-1β, and IL-8 concentrations in a dose-dependent manner (P < 0.05) (Fig. 2A, B, C).

In addition, the effect of glutamine on LPS-induced TNF-α, IL-1β, and IL-8 protein secretion into the supernatant corresponded with the effect on mRNA expression (P < 0.05) (Fig. 2 D). The effect of 20 mM glutamine on expression and secretion of cytokines was similar to that of 10 μM NS-398.

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Effects of glutamine on LPS-activated signal transduction pathways

To identify the molecular mechanisms involved in the response to glutamine, the effects of glutamine on LPS-induced activation of MAPK and the downstream MKP-1 and NF-κB pathways were examined. As shown in Fig. 3A, glutamine (10 mM) treatment inhibited the LPS-induced phosphorylation of ERK1/2, JNK, and p38 MAPKs in HDPCs (P < 0.05). In addition, glutamine enhanced the LPS-induced increase in both the levels of total mitogen-activated protein kinase phosphatase 1 (MKP-1) protein and its phosphorylation (Fig. 3) (P < 0.05). Treatment with glutamine abolished LPS-induced nuclear NF-κB p65 expression as well as phosphorylation of IkBα (Fig. 4) (P < 0.05).

DISCUSSION

Glutamine supplementation shows benefits in infectious complications (Griffiths et al. 1997), endotoxin shock and inflammatory disease (Coeffier et al. 2001, Li et al. 2004, Garrett-Cox et al. 2009), and improves the metabolic and clinical condition of catabolic patients (Ziegler et al. 1996). However, the role of glutamine in pulpitis has not been clarified. The results of the present study, have for the first time demonstrated that glutamine has an anti-inflammatory effect on LPS-treated HDPCs.

LPS has been shown to have pro-inflammatory effects and to strongly stimulate bone resorption by acting on the production and release of cytokines (Rupf et al. 2000, Coil et al. 2004). However, there is no single microbial species responsible for the pathogenesis of periradicular diseases. Porphyromonas gingivalis (P. gingivalis) and P. endodontalis have been closely associated with acute symptoms of endodontic infections (Sundqvist et al. 1989). To determine whether HDPCs respond to bacterial infection, HDPCs were treated with LPS extracted from P. gingivalis, which has previously been found to induce IL-8 expression and activate NF-κB in dental pulp stem cells (Chang et al. 2005). This study utilized LPS at a concentration of 10 μg/mL, since this concentration has been shown to induce inflammatory reactions such as upregulation of MMP-2, MMP-9, ICAM-1 and VCAM-1 in HDPCs (Lee et al. 2008, Yu et al. 2009). In addition, 10 μM NS-398 (a selective COX-inhibitor) was used as positive control (Kojima et al. 2006).

In inflamed pulp, iNOS is primarily expressed in neutrophils and macrophages, and a NOS
inhibitor effectively suppressed the expression of pro-inflammatory cytokines and COX-2 mRNA (Kawashima et al. 2005), implicating NO synthesis in the production of various mediators of pulpal inflammation. Inflammatory mediators such as PGE$_2$, TNF-$\alpha$, IL-1$\beta$, and IL-8 are elevated in pulpal inflammation (Coil et al. 2004). This study in an LPS-activated HDPC culture system demonstrated that glutamine inhibits NO and PGE$_2$ production via the suppression of iNOS and COX-2 expression. Furthermore, glutamine suppressed the production and mRNA expression of IL-1$\beta$, TNF-$\alpha$, and IL-8 in LPS-induced HDPCs. These results are in agreement with several previous reports indicating that glutamine reduced LPS-induced TNF-$\alpha$ release in human peripheral blood mononuclear cells (Wischmeyer et al. 2003), and improved outcomes in a LPS-induced rat model of endotoxemia (Wischmeyer et al. 2001). In addition, these anti-inflammatory effects of 20 mM glutamine were similar to those observed with 10 $\mu$M NS-398, suggesting that glutamine may be useful for the treatment of pulpal and periapical disease.

LPS exerts its inflammatory effects via the activation of both the MAPK signaling pathway and the classical NF-$$\kappa$$B pathway (Lee et al. 2011). Thus, the inhibition of these signaling pathways may explain the potent activity of glutamine as a suppressor of inflammatory mediators and cytokines. In this study, it was shown that LPS treatment induces phosphorylation of p38, ERK, and JNK in HDPCs, results which are consistent with those of a previous study in mouse macrophages (Choi et al., 2012). In addition, the experiments demonstrated that LPS-induced activation of MAPKs was inhibited by glutamine. Similarly, glutamine blocked LPS-induced p38 and JNK phosphorylation in murine alveolar macrophages and LPS-injected mouse lung (Coeffier et al. 2001). MKP-1 is the major enzyme responsible for the dephosphorylation and thus inactivation of all three MAPKs. Moreover, upregulation of MKP-1 and other anti-inflammatory proteins is increasingly being recognized as an important molecular mechanism responsible for the anti-inflammatory activity of some treatments (Issa et al. 2007). The present study also showed that glutamine increased the level of total MKP-1 protein and its phosphorylation, which may in turn result in attenuation the p38, JNK and ERK activity and ultimately result in the down-regulation of the expression of various inflammation-related genes in LPS-stimulated HDPCs.

NF-$$\kappa$$B is a transcription factor that plays a critical role in the regulation of many genes.
particularly in the inflammatory response (Tak & Firestein, 2001). Activation of NF-κB proceeds following the phosphorylation and degradation of the inhibitory subunit IκB-α (Li & Verma 2002). The experiments showed that glutamine inhibits the phosphorylation of IκB-α and the nuclear translocation of p65 proteins, resulting in lower levels of NF-κB transactivation. This result implies that the inhibitory effects of glutamine on the production of inflammatory mediators and cytokines can mediated by blocking MAPKs pathways with activation MKP-1, and subsequent inhibition of NF-κB translocation in HDPCs. The results suggest that glutamine treatment has an anti-inflammatory effect and may contribute to the inhibition of inflammatory reaction in the pulp, root canal systems, and periapex, thus prompting more favorable healing in endodontic disease.

CONCLUSION

Glutamine may exert its anti-inflammatory effects through MKP-1, MAPK, and NF-kB signaling pathways in HDPCs. These results suggest that exogenous glutamine supplementation may be useful in the treatment of pulpal and periapical disease.

ACKNOWLEDGEMENTS

The authors deny any conflicts of interest. This work was supported by a grant from the National Research Foundation of Korea (NRF) funded by the Korean government (MSIP) (2012R1A5A2051384).

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**FIGURE LEGENDS**

**Figure 1** Effect of glutamine on LPS-induced COX-2 and iNOS expression (A,B), and PGE2 (C) and NO production (D). Cells were treated with LPS (10 μg/mL) and the indicated dose of glutamine for 24 h. 10 μM NS-398 was used as a positive control. Protein expression was determined by Western blot. (B) The histogram shows the quantification of protein expression by densitometry, and is presented as fold increases compared to control cells. Concentrations of NO and PGE2 (C, D) were assessed by Griess reaction and ELISA, respectively. The data shown are representative of three independent experiments. Values represent the mean ± SD from three experiments. *, *p*<0.05 compared to the control; #, *p*<0.05 compared to cells treated with LPS alone.

**Figure 2** Effects of glutamine on LPS-induced production (A-C) and mRNA expression (D-G) of TNF-α, IL-1β, and IL-8. Cells were treated with LPS (10μg/mL) and the indicated dose of glutamine for 24 h. 10 μM NS-398 was used as a positive control. The amounts of TNF-α, IL-1β, and IL-8 in culture supernatants were determined by ELISA (A-C). mRNA expression was determined by RT-PCR analysis (D) and real-time polymerase chain reaction (E-G). The data shown are representative of three independent experiments. *, *p*<0.05 compared to the control; #, *p*<0.05 compared to cells
treated with LPS alone.

Figure 3 Effects of glutamine on the LPS-induced activation of MAPK and MKP-1 pathways in HDPCs. Cells were treated with LPS (10 μg/mL) and 10 mM glutamine for 120 min. (B) The histogram shows the quantification of protein expression by densitometry, and is presented as fold increases compared to control cells. Similar data were obtained from three independent experiments. #, p<0.05 compared to cells treated with LPS alone.

Figure 4 Effects of glutamine on the LPS-induced activation of NF-κB pathways in HDPCs. Cells were treated with LPS (10 μg/mL) and 10 mM glutamine for 120 min. (B) The histogram shows the quantification of protein expression by densitometry, and is presented as fold increases compared to control cells. Similar data were obtained from three independent experiments. The data shown are representative of three independent experiments. #, p < 0.05 compared to cells treated with LPS alone.

Table 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) primers and conditions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’-3’)</th>
<th>Size (bp)</th>
<th>Tm (°C)</th>
<th>Cycle</th>
</tr>
</thead>
</table>
| TNF-α | Forward: CTCTGGCCCAGGCAGTCAGA
Reverse: GGCCTTGGGAAGGTTGGAT | 519       | 60      | 30    |
| IL-1β | Forward: GGATATGGAGCAACAAGTGG
Reverse: ATGTACCAGTTGGGAACCTG | 288       | 60      | 30    |
| IL-6  | Forward: GGCCTTTCGATCCAGTTG
Reverse: AACCACATCGCAAGCTACC | 216       | 60      | 30    |
| β-actin | Forward: CATGGATGATGATATCGCCGCG
Reverse: ACATGATCTGGTACATTTTCG | 371       | 55      | 25    |
Figure 1.
Figure 2.
Figure 3.
Figure 4.

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