Bioorganic & Medicinal Chemistry 16 (2008) 8390–8396

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Suppression of cytokine production in lipopolysaccharide-stimulated mouse macrophages by novel cationic glucosamine derivative involves down-regulation of NF-κB and MAPK expressions

Eresha Mendis [a], Moon-Moo Kim [b], Niranjan Rajapakse [a], Se-Kwon Kim [c,d,*]

[a] Department of Food Science and Technology, Faculty of Agriculture, University of Peradeniya, Peradeniya, Sri Lanka
[b] Department of Chemistry, Dong-Eui University, Busan 614-714, South Korea
[c] Marine Bioprocess Research Center, Pukyong National University, Busan 608-737, South Korea
[d] Department of Chemistry, Pukyong National University, Busan 608-737, South Korea

ARTICLE INFO

Article history:
Received 26 June 2008
Revised 14 August 2008
Accepted 15 August 2008
Available online 22 August 2008

Keywords:
Glucosamine (Glc)
Quaternized amino glucosamine (QAGlc)
Cytokines
Nuclear factor κB (NF-κB)
Mitogen-activated protein kinases (MAPKs)

ABSTRACT

Exposure of macrophages to bacterial lipopolysaccharide (LPS) induces release of proinflammatory cytokines that play crucial roles in chronic inflammation. Glucosamine has reported to possess anti-inflammatory properties and currently is the oral supplement of choice for the management of inflammation related complications including osteoarthritis. In this study, quaternized amino glucosamine (QAGlc), a newly synthesized cationic glucosamine (Glc) derivative was found to inhibit LPS-stimulated production of IL-1β, IL-6, TNF-α, and PGE_2 in RAW264.7, mouse macrophages more potently than its starting material Glc. Since production of cytokines is regulated mainly via activation of NF-κB and regulation of mitogen-activated protein kinases (MAPKs), we examined if QAGlc could be responsible for the suppression of NF-κB pathway and MAPKs. We used reporter gene assay and Western blotting to examine the effects of QAGlc on activation and translocation of NF-κB. Further, QAGlc-mediated inhibition of NF-κB was accompanied with a suppression of its translocation. Apparently, QAGlc was shown to attenuate LPS-induced activation of p38 MAPK and JNK in RAW264.7 cells suggesting that inhibition of MAPK-mediated LPS signaling also contribute to suppression of cytokine production following stimulation of macrophages with LPS.

1. Introduction

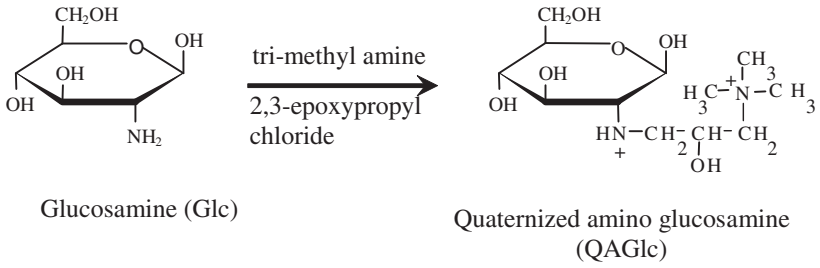
Activation of innate immune cells is a key step in mounting inflammatory responses against invading pathogens.[1] Bacterial lipopolysaccharides (LPS) is one of the principal agent that activates monocytes and macrophages leading the production of cytokines and other inflammatory mediators, including IL-1β, IL-6, TNF-α, and prostaglandin E_2 (PGE_2).[2] However, exuberant production of proinflammatory cytokines leads to severe immunopathologies such as endotoxic shock[3] and tissue damage.[4] Therefore, prolonged production of proinflammatory cytokines is thought to constitute a link between cellular injury and pathogenesis of diseases characterized by chronic inflammation.[5]

Proinflammatory cytokines such as TNF-α and IL-1β initiate cascade of destructive events in part by activating the transcription factor NF-κB, which in turn stimulates proinflammatory genes.[6] The ability of NF-κB to regulate proinflammatory gene expression is controlled by chemical modifications such as addition of phosphate groups, and by interactions with other proteins notably members of the IκB family (inhibitors of NF-κB). The binding of NF-κB to IκB helps to localize NF-κB in the cytoplasm. Upon activation of the NF-κB signaling pathway by LPS, IκB kinases (IKKs) target IκB for degradation.[7] This allows NF-κB to translocate into the nucleus, where it binds to promoters of proinflammatory genes activating their expressions. Many of the anti-inflammatory drugs suppress expression of these genes by inhibiting one or several targets of NF-κB activation pathway.[8] Therefore, from the upstream kinases such as IKK and NF-κB inducing kinase (NIK), to their downstream effector IκB, all represent attractive targets for novel drugs selectively regulating NF-κB function.

Mitogen-activated protein kinases (MAPKs) also regulate key proinflammatory pathways following stimulation with LPS.[9] The three major MAPKs proteins, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK are thought to play different roles in inflammatory diseases in different capac-

* Corresponding author. Tel.: +82 51 620 6753; fax: +82 51 621 0047.
E-mail address: sknkim@pknu.ac.kr (S.-K. Kim).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.08.037



Scheme 1. Synthesis of QAGlc from Glc.

ities.[10] Specifically, previous studies have indicated that p38 MAPK regulates processes that control cytokine expression.[11] Therefore, participation of MAPKs has been frequently implicated in occurrence of diseases and expression of immune or inflammatory responses.

Glucosamine (Glc), an amino sugar has received a considerable attention due to its therapeutic effectiveness in the treatment of arthritis and well tolerance in patients.[12] Moreover, recent studies have demonstrated that anti-inflammatory actions of Glc compounds can be related to the suppression of neutrophil functions[13] and proinflammatory cytokines.[14] Moreover, structural modifications to Glc by introducing new functional groups can be expected to improve its therapeutic effects.[15] In our laboratory, a series of Glc derivatives were synthesized to obtain molecules having different functional groups and different charged properties. They exerted different therapeutic potentials based on their functional groups in different cellular models.[16,17] Among them, Glc derivative having quaternized amino functionality (QAGlc) highly suppressed cytokine production in LPS-stimulated mouse macrophages (RAW264.7) than that of Glc. The present study was conducted to find out a possible signaling pathway of inhibiting cytokine production by QAGlc in RAW264.7 cells.

2. Results

2.1. Synthesis of QAGlc from Glc

In order to synthesize this Glc derivative having quaternized amino functionality, Glc was reacted with trimethylamine and 2,3-epoxypropyl chloride at same molar ratio as shown in Scheme 1. Reaction of trimethylamine with epoxide compounds involves nucleophilic substitution and cleavage of the epoxide ring. The reaction of trimethylamine and 2,3-epoxypropyl chloride gives (2,3-epoxypropyl) trimethylammonium chloride which subsequently reacts with Glc. However, an amino group of the Glc is the most favorable nucleophilic center under alkaline conditions

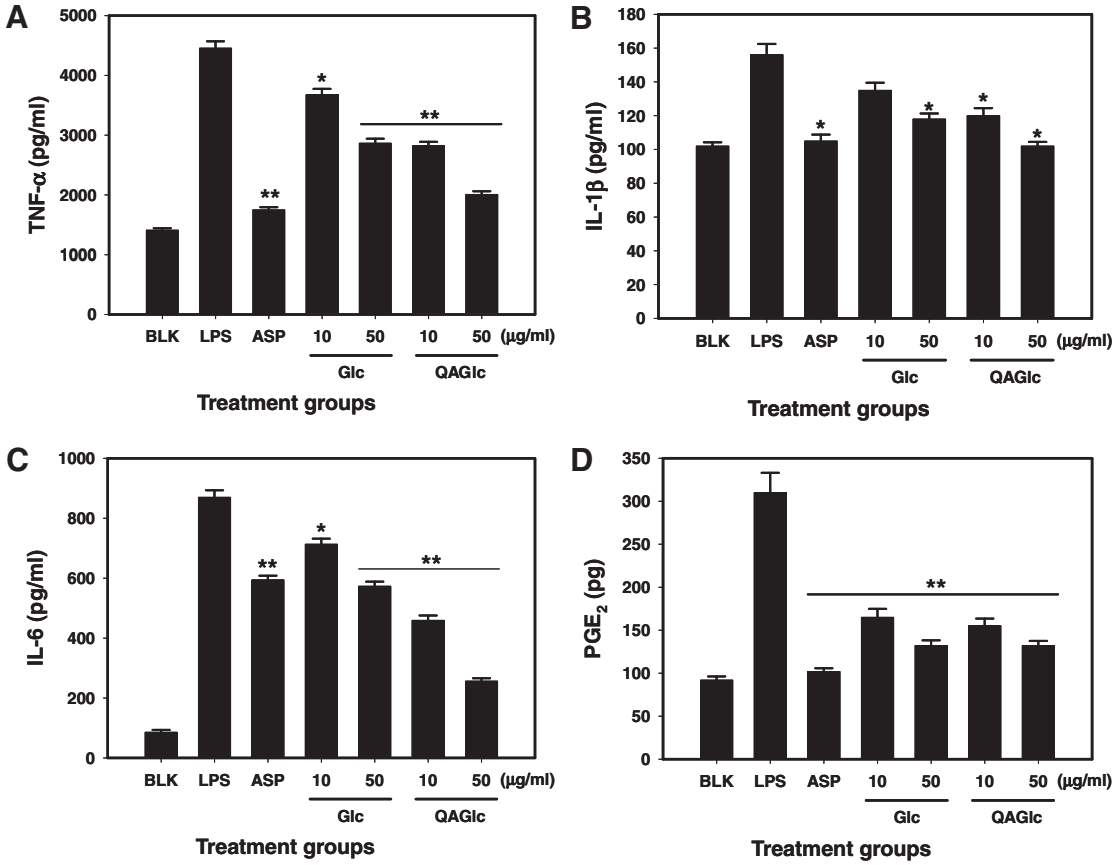


Figure 1. Effect of QAGlc and Glc on production of TNF-α, IL-1β, IL-6, and PGE_2 in LPS-stimulated RAW264.7 cells. Cells were treated with different concentrations of test materials or 10 nM aspirin (ASP) as a positive control for 1 h, and stimulated with LPS (1 μg/ml) for another 4 h. Following incubation, amounts of above proteins released were determined by their specific antibody-coated ELISA kits as described in Section 4.

(pH 9.00) to react with this intermediate. Subsequently, the reaction between (2,3-epoxypropyl) trimethylammonium chloride and amino group of the Glc forms QAGlc. Alkaline pH as well as the addition of trimethylamine, 2,3-epoxypropyl chloride and Glc at same mole ratio is critical for this reaction. After extraction of the product using series of solvents, QAGlc was obtained as a dark brown fluffy solid. Removal of remaining 2,3-epoxypropyl chloride and trimethylamine was possible by using 100 Da dialysis membrane because both these materials have smaller molecular weights compared to Glc and QAGlc. Following dialysis, lyophilized product which contained un-reacted Glc and newly synthesized QAGlc, was separated using a cationic resin at pH 4.0. At this pH, both Glc and QAGlc are adsorbed to cationic resin and they can be eluted at different salt concentrations due to difference in their cationic properties. Finally the purified end product was obtained as a dark brown fluffy solid. This amount represented 50% yield (w/w) of the weight of initial material Glc.

2.2. Effect of QAGlc on activities of inflammatory cytokines

Non-toxic concentrations of both Glc and QAGlc were selected following cytotoxicity testing to study further the signaling pathways of their activities. RAW264.7 cells treated with QAGlc or Glc were stimulated with LPS to assess the effect of QAGlc on the production of proinflammatory mediators mainly involved in chronic inflammation and protein levels of TNF-α, IL-1β, IL-6 and PGE_2 in culture medium were measured by ELISA. Treatment of LPS stimulated production of TNF-α (Fig. 1A), IL-1β (Fig. 1B), IL-6 (Fig. 1C), and PGE_2 (Fig. 1D) in 3-fold, 2-fold, 20-fold, and 3-fold, respectively. Treatment of QAGlc suppressed the production of TNF-α (Fig. 1A), IL-1β (Fig. 1B), IL-6 (Fig. 1C), and PGE_2 (Fig. 1D) to different extents in a concentration dependant manner. In particular, about 45% inhibition of LPS-stimulated TNF-α activity was observed when QAGlc was treated with 50 μg/ml concentration. Treatment with QAGlc at 50 μg/ml concentration suppressed the IL-1β concentration from 160 pg/ml to 100 pg/ml. Much higher reduction was observed in IL-6 level when the cells were treated with QAGlc at the same concentration. It was about 3-fold decrement from the level of LPS group. Apparently, nearly 50% inhibition of PGE_2 activity was observed when QAGlc treated to RAW264.7 cells at 50 μg/ml concentration. Glc exerted suppressive effects on TNF-α, IL-1β, IL-6, and PGE_2 in a concentration-dependent manner but at all instances these effects were lower compared to those of QAGlc. A prominent difference between activities of QAGlc and Glc were observed with regard to production of TNF-α and IL-6 compared to that of IL-1β and PGE_2. Further, protein levels of these inflammatory cytokines in the presence of QAGlc were assessed using Western blot analysis with phosphorylated forms of antibodies following stimulation with LPS. In line with the results obtained from immunoassays, clear decrements in protein expression levels of TNF-α, IL-1β, and IL-6 were observed and it followed a concentration-dependent pattern (Fig. 2) when cells were treated with QAGlc compared to LPS treatment group alone. At the highest concentration of QAGlc examined (60 μg/ml) these protein expressions were almost completely inhibited.

2.3. QAGlc inhibits NF-κB-promoter activity

To investigate whether QAGlc could modulate the NF-κB-promoter activity, RAW264.7, mouse macrophage cells were co-transfected with a NF-κB-promoter containing luciferase reporter construct and β-galactosidase expression vector. β-Galactosidase staining confirmed nearly 85% transfection efficiency in RAW264.7 cells. Transfected cells were then treated with nontoxic concentrations of QAGlc. To see the effect of QAGlc on NF-κB-promoter activity, transfected cells were stimulated using bacterial endotoxin, LPS. LPS resulted about 4-fold induction in NF-κB gene promoter activity in transfected cells compared to that of group performed in the absence of LPS. A dose-dependent decrease in luciferase activity that represents the suppression of NF-κB-promoter activity was observed following treatment of QAGlc in LPS-stimulated cells (Fig. 3A). Concurrent reductions in NF-κB-promoter activities in Glc-treated groups were observed, but these activities were much lower than that of QAGlc.

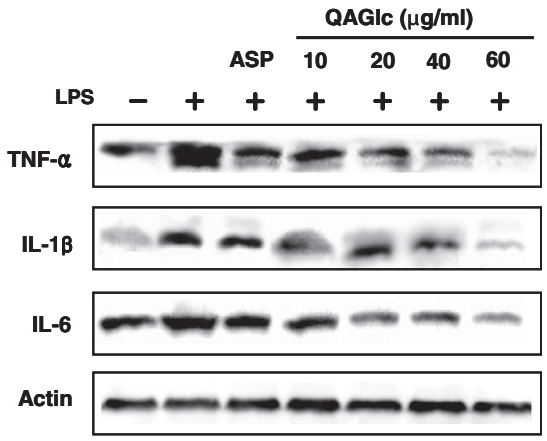


Figure 2. Western blot analysis of TNF-α, IL-1β, and IL-6 protein expressions in RAW264.7 cells treated with QAGlc. QAGlc-treated cell extracts were obtained and equal amounts of protein were resolved by SDS–PAGE. Separated protein bands were transferred onto nitrocellulose membranes and incubated with specific primary and secondary antibodies, respectively. Western blots were developed with enhanced chemiluminescence reagent. β-Actin was used normalize total proteins. LPS, bacterial lipopolysaccharide; ASP, aspirin.

2.4. QAGlc reduces nuclear translocation of NF-κB

To delineate the role of QAGlc on NF-κB translocation, plasma and nuclear protein extracts were separated from QAGlc-treated LPS-stimulated cells. NF-κB p65 subunit protein expression levels were then tested using Western blot analysis employing p65 antibody. Lower NF-κB levels were observed in plasma protein analysis of QAGlc-treated cells compared to LPS treatment group (Fig. 3B). This reduction was much prominent following the treatments with 40 and 60 μg/ml concentrations of QAGlc. Moreover, this was much higher than that of aspirin at 10 μM concentration. Interestingly, a clear reduction in translocation of NF-κB to nucleus in the presence of QAGlc was observed when nuclear extracts from LPS-stimulated cells were analyzed. Therefore, these results suggested the involvement of QAGlc to mediate both inhibition of NF-κB activation and translocation.

2.5. QAGlc suppresses NF-κB via inhibition of IKK

Activation of NF-κB is preceded via induction of cascade of molecules. Therefore it was hypothesized that QAGlc could affect targets of the NF-κB signaling cascade. Therefore, the level of NF-κB inducing kinase (NIK), IKK, upstream effecters, and IκB, a downstream effecter levels in QAGlc-treated RAW264.7 cells were studied using Western blot analysis. Treatment with LPS resulted in clear increments in protein levels of IKK and NIK and clearly reduction in amount of IκB (Fig. 4). The results revealed that QAGlc treatment did not exert any effect on protein expression level of NIK. However, treatment of QAGlc blocked the protein expression levels of IKK in a concentration-dependent manner. Moreover, increments in IκB levels were observed in the presence of QAGlc compared to that of group treated with LPS alone.

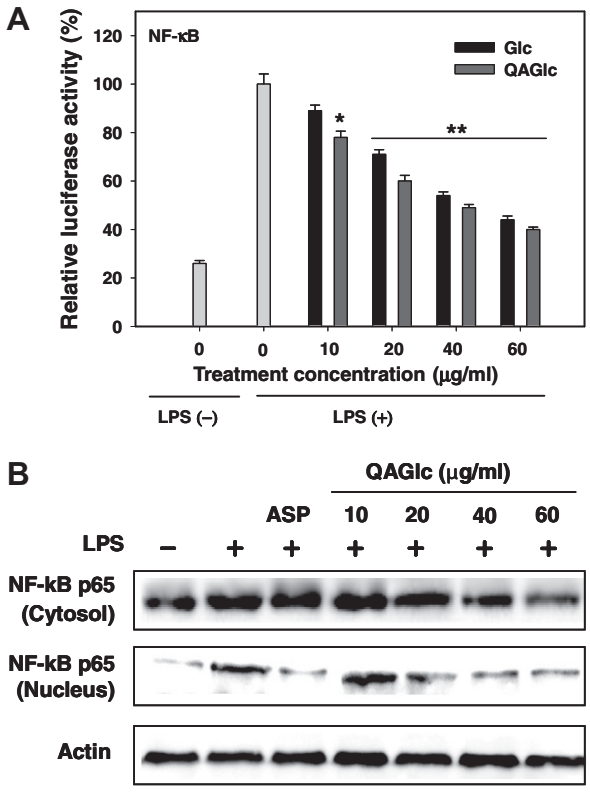


Figure 3. Effect of QAGlc on NF-κB expression in LPS-stimulated RAW264.7 cells. (A) Cells were co-transfected with NF-κB-luciferase reporter vector and β-galactosidase expression vector by Lipofectamine™ 2000 reagent (Invitrogen, USA). Transfected cells were treated with different concentrations of QAGlc followed by stimulation with LPS and luciferase enzyme activity that represents NF-κB-promoter activity was determined. The level of reporter gene expression was plotted as a relative luciferase activity compared with non-treated cells. Statistical comparisons, $^{*}P < 0.05$ and $^{**}P < 0.01$. (B) Western blot analysis of NF-κB p65 protein expressions in RAW264.7 cells treated with QAGlc. Total proteins in nucleus and cytosol were separately extracted and resolved by SDS–PAGE. Proteins transferred onto nitrocellulose membranes were incubated with anti-p65 NF-κB antibody and p65 protein bands were developed. β-Actin was used normalize total proteins. LPS, bacterial lipopolysaccharide; ASP, aspirin.

2.6. Effect of QAGlc on protein expressions of ERK, JNK, and p38 MAPK

To verify whether QAGlc exert any effect on MAPKs, their protein levels following treatment with QAGlc were assessed using Western blot analysis. Treatment of LPS increased the protein levels of JNK, p38 MAPK and ERK assessed with phosphorylated forms of antibodies in Western blots. The results of this study exhibited that treatment of QAGlc inhibited mainly JNK and p38 MAPK protein levels in a dose-dependent manner (Fig 5). However, a clear reduction of ERK could not be observed in RAW264.7 cells treated with different concentration of QAGlc compared to its level in cells treated with LPS alone.

3. Discussion

The results of this study present a novel Glc derivative having suppressive effects on production of proinflammatory cytokines, crucial agents involved in immune responses. Although, Glc had significant effects, was not able to achieve suppression levels comparable to those of QAGlc. Particularly, these effects were observed in macrophage, cells that thought to play major roles in chronic inflammatory diseases. Activated monocytes/macrophages liberate cytokines at the site of inflammation and involved in the progression of disease states resulted from chronic inflammation.[3] Further, this study presents a facile way of modifying Glc structure to have anti-inflammatory effects. The characteristic of Glc that is most important in the field of chemistry is this monomer has nucleophilic primary amino group. The reactivity of this amino group is convenient as they allow Glc derivatives to be generated with a range of facile chemistries or biochemistries. Moreover, the results of this study confirm the substitution of quaternized amino functionality to Glc increases the potential of Glc to act against LPS-stimulated production of cytokines.

Beneficial effects of Glc in arthritis have been suggested to perform partly via anti-inflammatory effects including suppression of cytokines.[13] However in stimulated macrophages, QAGlc exerted much higher suppressive effect against production of TNF-α and IL-6 among others than that exerted by Glc. TNF-α is an inducible cytokine with a broad range of proinflammatory and immunostimulatory activities.[18] Selective removal of TNF-α in patients with RA using monoclonal antibodies had remarkable ameliorative effects

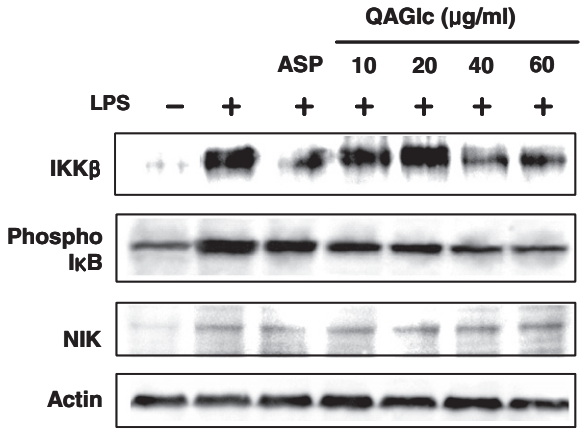


Figure 4. Western blot analysis of IKKβ, IκB, and NIK. RAW264.7 cells were treated with different concentrations of QAGlc and total proteins in whole cell extracts were transferred onto nitrocellulose membranes followed by resolving on SDS–PAGE gel. Membranes were separately labeled with primary antibodies of IKKβ, IκB, and NIK. Protein bands were detected with secondary antibody and chemiluminescence reagents. Detection of β-actin was used as control for equal loading of protein. LPS, bacterial lipopolysaccharide; ASP, aspirin.

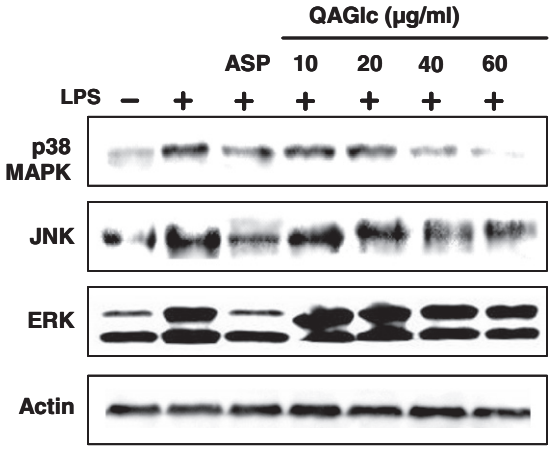


Figure 5. Western blot analysis of p38, JNK, and ERK. RAW264.7 cells were treated with different concentrations of QAGlc and total proteins in whole cell extracts were transferred onto nitrocellulose membranes followed by resolving on SDS–PAGE gel. Membranes were separately labeled with primary antibodies of p38, JNK, and ERK. Protein bands were detected with secondary antibody and chemiluminescence reagents. Detection of β-actin was used as control for equal loading of protein. LPS, bacterial lipopolisaccharide; ASP, aspirin.

on disease activity, in support for a pivotal role of TNF-α in RA.[19] Further, suppression of IL-6 has also shown to be similarly effective in reducing inflammatory markers in patients with arthritis.[20] However, according to the results obtained in this study the levels of PGE_2 and IL-1β in the presence of QAGlc were lower than in those the presence of Glc. However, these differences were not significantly different. The inhibitory effects observed with this novel Glc derivative in this study were specific and could not be attributed to cell death as this material was well tolerated by cells.

The results of the present study suggest the inhibition of NF-κB activation in the presence of QAGlc contributes partly to its potential to suppress production of NF-κB-dependent cytokines including TNF-α, IL-1β, IL-6, and PGE_2 following stimulation with LPS. Cytokines that are stimulated by NF-κB such as TNF-α and IL-1β can also directly activate the NF-κB pathway, thus establishing a positive autoregulatory loop that can amplify the inflammatory response and increase the duration of chronic inflammation. Western blot analysis performed using protein extracts from nucleus explained that, substantial inhibition of NF-κB translocation was accompanied its suppressive effect. Further, results obtained from Western blotting performed with antibodies of other associate genes revealed that the primary target of QAGlc was IKK. This would be a logic step of QAGlc to target NF-κB inhibition because IKK is the critical kinase involved in activating the NF-κB pathway.

QAGlc was shown to block LPS-induced activation of p38 MAPK and JNK in RAW264.7 cells suggesting that inhibition of ERK-mediated LPS signaling also contribute to suppression of cytokine production in these cells in the presence of QAGlc. The results of this study does not give any clear effects on ERK activity with the treatment of QAGlc and these observations are in agreement with the results obtained in many other studies that ERK involved in transducing growth factor-dependant proliferative signals but not in inflammation-related pathways. However, some studies have come up with findings that inhibition of ERK and p38 MAPK in combination causes a markedly greater suppression in LPS-stimulated TNF-α production.[21]

Our data raise the possibility that modification of the structural features of Glc could introduce better molecules having improved pharmacological effects. Widespread availability of this compound further potentiates emergence of similar studies to confer long term therapeutic effects of structurally modified molecules. Further, understanding the inhibitory mode of these materials in the production of proinflammatory cytokines from immune cells will beneficial for the development of therapeutic potentials of Glc in treating certain chronic inflammatory disease states including RA.

4. Materials and methods

4.1. Materials

Chitosan was kindly donated by Kitto Life Co. (Seoul, South Korea). Chemicals required for synthesis, including 2,3-epoxypropyl chloride and trimethylamine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Mouse macrophage tumor cell line, RAW264.7 was obtained from American Type of Culture Collection (Manassas, VA, USA). Materials required for culturing of cells including culture media were purchased from Gibco BRL, Life Technologies (USA). RC-DCTM protein assay kit was obtained from Bio-Rad (CA, USA). Dowex-50WX2 cation exchange resin was purchased from Dow Chemical Company (Michigan, USA. Dialysis membrane having 100 Da pore size was purchased from Spectrum Laboratories Inc. (RanchonDominguez, CA). MTT reagent was also purchased from Sigma Chemical Co. (St. Louis, MO, USA). NF-κB gene promoter reporter vectors were purchased from Clontech (CA, USA).

4.2. Instrumental analysis

Infrared spectra were recorded using KBr plates in Spectrum 2000® FT-IR spectrophotometer (Perkin Elmer, USA). ^1H NMR and ^{13}C NMR spectra were recorded in a D_2O environment using a JNM-ECP-400® (400 MHz) spectrometer (JEOL, Japan). Elemental analysis (C, N, and H) was performed using a Vario-EL Elementar Analysesysteme® (USA) and optical density was measured using GENios® microplate reader (Tecan Austria GmbH, Austria). To quantify protein expression levels, Western blot bands were visualized using LAS3000® Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan).

4.3. Preparation of Glc from chitosan

For the preparation of Glc, chitosan (average deacetylation degree of 90–97%) was mixed with 6 N HCl and stirred at 100 °C while refluxing. After 3 h, distilled water and activated carbon powder were added and stirring was continued for additional 30 min at 60 °C. Reaction mixture was then filtered using a glass filter and addition of activated carbon powder to the filtrate was repeated to obtain a clear solution. This solution was then vacuum evaporated and the resulting solid was thoroughly washed with ethanol and diethyl ether to give Glc.

Glc. Fine white solid; IR (KBr) ν_{max} 3412 (OH), 2864 (CH), 1578 (NH), 1309 (CN), 1108, 1065, 1036 (pyranose) cm^{-1}; ^1H NMR (D_2O, 400 MHz) δ 5.4, 4.9 (1H, H-1_α, H-1_β), 3.2, 2.9 (1H, H-2_α, H-2_β), 3.4–3.9 (1H, H-3, 1H, H-4, 1H, H-5 and 2H, H_2-6), 4.7 (D_2O); ^{13}C NMR (D_2O, 400 MHz) δ 89.0, 92.1 (C-1_α, C-1_β), 54.3, 56.2 (C-2_α, C-2_β), 69.0 (C-3), 76.3 (C-4), 71.2, 72.4 (C-5_α, C-5_β), 61.1 (C-6); elemental analysis C% (33.48), N% (6.49), H% (6.61).

4.4. Synthesis and purification of quaternized amino glucosamine (QAGlc)

Glc was quaternized by reacting with 2,3-epoxypropyl chloride and trimethylamine according to the method of Ronghua et al.[22] Same molar ratio of 2,3-epoxypropyl chloride was added to trimethylamine solution following adjustment of the pH of trimethylamine solution to 9. Glc was then added at same molar ratio to the reaction mixture containing trimethylamine and 2,3-epoxypropyl chloride. Then the quaternization reaction was preceded at room temperature while stirring for 24 h. Reaction product was extracted using ethanol, methanol and trihydrofluoride and finally QAGlc was obtained. Then QAGlc was subjected to dialysis using 100 Da molecular weight cut-off membrane to remove un-reacted 2,3-epoxypropyl chloride and trimethylamine. After series of dialysis attempts, lyophilized QAGlc was passed through a Dowex-50WX2 cation exchange resin and amount of carbohydrate in fractions eluted with 1 N NaCl was measured using phenol–sulfuric acid method by measuring the absorbance at 420 nm. After plotting the graph, fractions were pooled and QAGlc was separated as a solution from un-reacted Glc. QAGlc solution was further subjected to dialysis using a 100 Da molecular weight cut-off dialysis membrane and lyophilized to give QAGlc.

QAGlc. Dark brown fluffy solid; IR (KBr) ν_{max} 3411 (OH), 2929, 2805 (CH), 1639 (CO), 1480 (Me), 1309 (CN), 1115, 1092, 1033 (pyranose) cm^{-1}; ^1H NMR (D_2O, 400 MHz) δ 5.4, 4.9 (1H, H-1_α, H-1_β), 3.2, 2.9 (1H, H-2_α, H-2_β), 3.4–3.9 (1H, H-3, 1H, H-4, 1H, H-5 and 2H, H_2-6), 4.7 (D_2O), 2.8 (2H, H_2-7 and 2H, H_2-9), 3.1 (9H, H_9-10); ^{13}C NMR (D_2O, 400 MHz) δ 89.2, 92.4 (C-1_α, C-1_β), 54.5, 56.1 (C-2_α, C-2_β), 69.3 (C-3), 76.1 (C-4), 71.4, 72.3 (C-5_α, C-5_β), 61.3 (C-6), 64.4 (C-7), 57.2 (C-8), 65.1 (C-9), 54.2 (3C,10-NMe); elemental analysis C% (43.61), N% (8.52), H% (8.19).

4.5. Cell culture

RAW264.7 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 μg/ml penicillin–streptomycin. For experiments, cells were detached with trypsin–EDTA and plated onto 24- or 96-well plates at a plating density of 7×10^5 and 1.5×10^5 per well, separately.

4.6. Cytotoxicity determination of RAW264.7 cells following treatment with QAGlc

To assess cell cytotoxicity levels of Glc and QAGlc on RAW264.7 cells, MTT assay was performed with different concentrations and incubation time intervals using MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) method as described elsewhere. For this purpose, cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were washed with fresh medium and were treated with different concentrations of Glc and QAGlc. After 48 h of incubation, cells were rewashed and 20 μl of MTT (5 mg/ml) was added and incubated for 4 h. Finally, DMSO (150 μl) was added to solubilize the formazan salt formed and amount of formazan salt was determined by measuring the OD at 540 nm using an GENios® microplate reader (Tecan Austria GmbH, Austria). Relative cell viability was determined by the amount of MTT converted into formazan salt. Viability of cells was quantified as a percentage compared to the control (OD of treated cells − OD of blank/OD of control − OD of blank × 100) and dose response curves were developed. The data were expressed as mean from at least three independent experiments and $P < 0.05$ was considered significant.

4.7. Determination IL-1β, IL-6, and TNF-α activity following treatment with QAGlc

Production of IL-1β, IL-6 and TNF-α in RAW264.7 cells was assayed using Biotrak™ ELISA kits (Amersham Pharmacia Biosciences, NJ, USA) following the instructions. Cells were treated with different concentrations of test material or 10 μM aspirin for 1 h and production of IL-1β and TNF-α was stimulated by 1 μg/ml final concentration of LPS followed by incubation for another 24 h. Following incubation, conditioned medium was used for the experiment. For that, 50 μl of IL-1β and TNF-α standards (prepared for calibration) or same volume of test samples were added to wells of IL-1β or TNF-α antibody-coated 96-well plates in duplicate. Biotinylated antibody reagent (50 μl) was added and incubated for 3 h at room temperature. Reaction mixture was aspirated and washed using a microplate washer (Tecan Austria GmbH, Austria). Streptavidin–HRP conjugate (100 μl) was added and incubate for 30 min at room temperature. After complete washing, 100 μl of TMB substrate solution was added and incubated for 30 min at room temperature and reaction was stopped by adding 100 μl of stop solution. Optical density was determined at 450 nm using GENios® microplate reader (Tecan Austria GmbH, Austria).

4.8. Determination PGE_2 activity following treatment with QAGlc

RAW 264.7 cells were cultured in 24-well plates with serum free media and pre-treated with different concentrations of QAGlc for 1 h. Production of PGE_2 was stimulated by adding 1 μg/ml final concentration of LPS and incubated for 24 h. The conditioned medium was used for PGE_2 determination by Biotrak™ Prostaglandin E_2 direct assay kit (Amersham Pharmacia Biosciences, NJ, USA) according to manufacture's instructions. For this purpose 50 μl supernatant (includes PGE_2 released from cells) from each treatment group was added into anti-mouse antibody-coated wells and mixed with 50 μl of diluted mouse-anti-PGE_2. Plate was incubated at room temperature (20–25 °C) for 1 h on a microplate shaker following addition of 50 μl of diluted conjugate into all wells except the blank. The reaction mixture was then aspirated and wells were washed with wash buffer. Immediately 150 μl of room temperature equilibrated TMB enzyme substrate was pipetted into all wells. Cover the plate with the lid provided and mix on a microplate shaker for exactly 30 min at room temperature (15–30 °C). The reaction was halted by the addition of 100 μl of 1 M sulfuric acid to all wells. The optical density was read at 450 nm after 30 min using GENios® microplate reader (Tecan Austria GmbH, Austria).

4.9. NF-κB reporter gene assay

RAW264.7 cells cultured in 10 cm^3 culture dishes were transiently transfected with NF-κB-promoter containing luciferase reporter vector. β-Galactosidase expression vector was co-transfected with the reporter vector to serve as an internal control of transfection efficiency. Transfected cells sub-cultured in 24-well plates were treated with different concentrations of QAGlc and Glc. Following stimulation with LPS (1 μg) cells were washed once with cold PBS and lysed with 200 μl/well lysis buffer (25 mM Tris–HCl, pH 8.0, containing 2 mM DDT and 1% Triton-X 100). Aliquots of cell lysate and luciferase substrate (Promega) were mixed in equal amounts in a 96-well plate and luminescence intensity was measured with a luminescence microplate reader (Tecan Austria GmbH, Austria). β-Galactosidase activity was measured with ONPG buffer. The level of reporter gene expression was determined as a ratio, compared with cells stimulated by PMA (10 ng/ml) alone and represented as relative luciferase activity.

Transfection efficiency was determined by X-Gal staining method. Briefly, transfected cells were fixed with 0.5% glutaraldehyde and stained with X-Gal solution containing 20 mM $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, and 1 mM $MgCl_2$. After 24 h of incubation at 37 °C, transfected cells were visualized with blue color under a light microscope.

4.10. Extraction of nuclear and plasma protein

For separate extraction of nuclear and cytoplasmic proteins, CelLytic™ NuCLEAR™ Extraction kit (S26-36-23, Sigma–Aldrich Co., MO, USA) was used following manufacturer's instructions. Briefly, cells treated with QAGlc were collected and lysed with 0.5 ml of lysis buffer (500 μl, hypotonic lysis buffer, 5 μl, 0.1 M DTT, and 5 μl protease inhibitor cocktail) and incubated for 15 min on ice. Igepal CA-630 solution (36 μl) was added and vortex for 20 seconds. Nuclei were separated by centrifugation at 13,000g and supernatant (cytoplasmic protein) was collected. Precipitated nuclei were lysed with 70 μl of extraction buffer mix (98 μl, extraction buffer, 1 μl of 0.1 M DTT and 1 μl protease inhibitor cocktail) for 30 min and nuclei protein were collected by centrifugation at 13,000*g*.

4.11. Western blot analysis

After treatment of QAGlc for different time intervals, whole cell extracts were obtained and resolved by denaturing SDS–PAGE. Briefly, harvested cells were washed twice with ice-cold PBS; resuspended in cell lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EGTA, 1% Nonidet P-40, and 0.25% sodium deoxycholate) containing antipain (1 μg/ml), aprotinin (1 μg/ml), chymostatin (1 μg/ml), leupeptin (0.1 μg/ml), pepstatin (1 μg/ml), and 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride; and incubated on ice for 20 min. Supernatants were recovered by a 10-min centrifugation (12,000*g*) at 4 °C, and protein concentration was determined with

the Bio-Rad protein assay using bovine serum albumin as a standard. Proteins (20–40 μg) were diluted in 5× sample buffer (10% SDS and 100 mM each dithiothreitol, glycerol, bromphenol blue, and Tris–HCl) and resolved in 4–20% Novex gradient gel (Invitrogen, USA), electrotransferred onto a nitrocellulose membrane. Then proteins were transferred onto nitrocellulose membranes, and the blots were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline and 0.1% Tween 20 for 1 h at room temperature. Membranes were incubated for 1 h at room temperature with the appropriate dilution of primary antibodies (1:500) related to apoptosis and cell cycle progression. After three 5-min washes with Tris-buffered saline and 0.1% Tween 20, the blots were incubated with the corresponding peroxidase-conjugated secondary antibody (1:5000 dilutions) for 1 h at room temperature. They were then washed again three times with Tris-buffered saline and 0.1% Tween 20, rinsed briefly with PBS, and developed with enhanced chemiluminescence reagents (ECL, Amersham Biosciences, UK). Western blot bands were visualized using LAS3000® Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan). Detection of β-actin (1:5000 antibody dilutions) was used as control for equal loading of protein.

4.12. Statistical analysis

One-way analysis of variance was used for all statistical analyses using independent experiments and data are represented as means ± SEM. Individual values were compared by Dunnett's test and $P < 0.05$ considered as significant unless otherwise stated.

Acknowledgments

The authors acknowledge Marine Bioprocess Research Center of Marine Bio 21 Project, funded by the Ministry of Maritime Affairs and Fisheries Republic of Korea, for the support provided through the research Grant P-2004-01.

References and notes

1. Takeda, K.; Kaisho, T.; Akira, S. *Annu. Rev. Immunol.* **2003**, *21*, 335.
2. Beutler, B.; Rietschel, E. T. *Nat. Rev. Immunol.* **2003**, *3*, 169.
3. Aderem, A. *Crit. Care Med.* **2001**, *29*, 16.
4. Ritchlin, C. T.; Haas-Smith, S. A.; Li, P.; Hicks, D. G.; Schwarz, E. M. *J. Clin. Invest.* **2003**, *111*, 821.
5. Cerami, A.; Beutler, B. *Immunol. Today* **1988**, *9*, 28.
6. Baeuerle, P. A. *Curr. Biol.* **1998**, *8*, R19.
7. Hoffmann, A.; Levchenko, A.; Scott, M. L.; Baltimore, D. *Science* **2002**, *8*, 1241.
8. Gilroy, D. W.; Lawrence, T.; Perretti, M.; Rossi, A. G. *Nat. Rev. Drug Discov.* **2004**, *3*, 401.
9. Zhao, Q.; Shepherd, E. G.; Manson, M. E.; Nelin, L. D.; Sorokin, A.; Liu, Y. *J. Biol. Chem.* **2005**, *280*, 8101.
10. Morel, J.; Barenbaum, F. *Joint Bone Spine* **2004**, *71*, 503.
11. Prichett, W.; Hand, A.; Sheilds, J.; Dunnington, D. *J. Inflamm.* **1995**, *45*, 97.
12. Reginster, J. Y.; Deroisy, R.; Rovati, L. C.; Lee, R. L.; Lejeune, E.; Bruyere, O.; Giacovelli, G.; Henrotin, Y.; Dacre, J. E.; Gossett, C. *Lancet* **2001**, *357*, 251.
13. Ma, L.; Rudert, W. A.; Harnaha, J.; Wright, M.; Machen, J.; Lacomy, R.; Qian, S.; Lu, L.; Robbins, P. D. M.; Trucco, M.; Giannoukakis, N. *J. Biol. Chem.* **2002**, *277*, 39343.
14. Largo, R.; Alvarez-Soria, M. A.; Diez-Ortego, I.; Calvo, E.; Sanchez-Pernaute, O.; Egido, J.; Herrero-Beaumont, G. *Osteoarthr. Cartilage* **2003**, *11*, 290.
15. Hoffer, L. J.; Kaplan, L. N.; Hamadeh, M. J.; Grigoriu, A. C.; Baron, M. *Metabolism* **2001**, *50*, 767.
16. Kim, M. M.; Mendis, E.; Rajapakse, N.; Kim, S. K. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1938.
17. Mendis, E.; Kim, M. M.; Rajapakse, N.; Kim, S. K. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3105.
18. Vilcek, J.; Lee, T. H. *J. Biol. Chem.* **1991**, *266*, 7313.
19. Elliot, M. J.; Maini, R. N.; Feldmann, M.; Kalden, J. R.; Antoni, C.; Smolen, J. S.; Leeb, B.; Breedveld, F. C.; Macfarlane, J. D.; Bijl, H.; Woody, J. N. *Lancet* **1994**, *344*, 1105.
20. Choy, E. H.; Isenberg, D. A.; Garrood, T.; Farrow, S.; Ioannou, Y.; Bird, H.; Cheung, N.; Williams, B.; Hazleman, B.; Price, R.; Yoshizaki, K.; Nishimoto, N.; Kishimoto, T.; Panayi, G. S. *Arthritis Rheum.* **2002**, *46*, 3143.
21. Rutault, K.; Hazzalin, C. A.; Mahadevan, L. C. *J. Biol. Chem.* **2001**, *276*, 6666.
22. Ronghua, H.; Mendis, E.; Rajapakse, N.; Kim, S. K. *Life Sci.* **2006**, *78*, 2399.