

Chitosan oligosaccharides block LPS-induced O-GlcNAcylation of NF- κ B and endothelial inflammatory response

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ABSTRACT

It is known that chitosan oligosaccharides (COS) suppress LPS-induced vascular endothelial inflammatory response by mechanism involving NF- κ B blockade. It remains unknown how COS inhibit NF- κ B. We provided evidence both in cultured endothelial cells and mouse model supporting a new mechanism. Regardless of the endothelial cell types, the LPS-induced NF- κ B-dependent inflammatory gene expression was suppressed by COS, which was associated with reduced NF- κ B nucleus translocation. LPS enhanced O-GlcNAc modification of NF- κ B/p65 and activated NF- κ B pathway, which could be prevented either by siRNA knockdown of O-GlcNAc transferase (OGT) or pretreatment with COS. Inhibition of either mitogen-activated protein kinase or superoxide generation abolishes LPS-induced NF- κ B O-GlcNAcylation. Consistently, aortic tissues from LPS-treated mice presented enhanced NF- κ B/p65 O-GlcNAcylation in association with upregulated gene expression of inflammatory cytokines in vascular tissues; however, pre-administration of COS prevented these responses. In conclusion, COS decreased OGT-dependent O-GlcNAcylation of NF- κ B and thereby attenuated LPS-induced vascular endothelial inflammatory response.

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1. Introduction

Vascular endothelial cells are key regulator of inflammatory response which provide an anti-inflammatory, anticoagulatory surface in the steady state and a controlled inflammatory response in injury (infection) state (Kadl & Leitinger, 2005). A properly regulated inflammatory response in these blood vessel-lining cells is crucial because it allows maintaining vascular homeostasis which usually becomes impaired over the course of inflammatory diseases including atherosclerotic cardiovascular diseases and diabetes. Bacterial Lipopolysaccharides (LPS) have a well-established role in the induction of inflammatory response through promoting the production of pro-inflammatory cytokines in many cell types (Raetz & Whitfield, 2002). It is also well recognized that LPS impair vascular endothelial function through aberrant inflammatory reactions (Bierhaus, Chen, Liliensiek, & Nawroth, 2000). Emerging evidence highlighted that LPS-induced vascular endothelial inflammatory response can be efficiently blocked by administration of

chitosan oligosaccharides (COS) (Liu, Li, et al., 2010; Liu et al., 2011).

COS are depolymerized products, as oligomers of D-glucosamine (Arvanitoyannis, Nakayama, & Aiba, 1998), from the naturally-occurring compounds chitin and chitosan through chemical and enzymatic hydrolysis. Biological activities of COS have been extensively studied due to their high solubility (Da Silva, Pochard, Lee, & Elias, 2010; Kim & Rajapakse, 2005), absorption (Eijsink et al., 2010) and biocompatibility (Du, Wang, Yuan, Wei, & Hu, 2009). Increasingly emerging evidence indicate that COS exhibit anti-inflammatory activities in experimental models in vitro (Liu, Li, et al., 2010; Liu et al., 2011) and in vivo (Qiao, Bai, & Du, 2011), in addition to those of antitumor (Fernandes et al., 2012), antifungal (Hussain, Singh, & Chittenden, 2012), antimicrobial (Malcata et al., 2010; Tavaría et al., 2012), and free radical scavenging (Kim, Ahn, Kong, & Kim, 2012) activities. As such, COS have in recent years been recommended as healthy food supplements in Asian countries due to these properties (Nam, Kim, & Shon, 2007; Nishimura et al., 1984). Although it remains largely unknown exactly how COS exert these potential beneficial effects, studies with animals have observed an anti-inflammatory feature shared among various models treated with COS. These have included a rabbit model of breast capsular contracture (Marques et al., 2011), a mouse model of sepsis (Qiao et al., 2011), chemical-induced paw edema (Fernandes et al., 2010), asthma (Chung, Park, & Il Park, 2012),

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and diet-induced obesity (Choi, Yang, & Chun, 2012). Further in-depth studies with cultured cell models support the notion that the anti-inflammatory feature of COS may attribute to the suppression of NF- κ B-dependent inflammatory gene expression. Indeed, pre-treatments with COS significantly abolish the otherwise increased inflammatory cytokines by LPS, such as IL-1 β (Pangestuti, Bak, & Kim, 2011; Qiao et al., 2011), IL-6 (Liu, Li, et al., 2010; Ma et al., 2011; Pangestuti et al., 2011; Yoon, Moon, Park, Im, & Kim, 2007), IL-8 (Liu et al., 2011) and TNF- α (Ma et al., 2011; Pangestuti et al., 2011; Qiao et al., 2011; Yoon et al., 2007) in endothelial cells, as well as in macrophage and microglia. However, it has yet to be established how COS suppress NF- κ B activation induced by LPS.

NF- κ B is present as a dimer consisting of p65 (RelA) and p50 subunits in most cell types. This dimer is localized to the cytoplasm and binds the inhibitor I κ B. Treatment with LPS or other activating agents stimulate I κ B kinase, which phosphorylates I κ B and thereby induces its degradation. The degradation of I κ B leads to dissociation and translocation of NF- κ B into the nucleus and activation of target genes. In this study, we sought to provide evidence to test a potentially novel hypothesis involving NF- κ B modulation through which COS exerted their anti-inflammatory effects induced by LPS. The mechanism will be tested both in cultured cell and mouse models, which may represent COS as the anti-inflammatory agent in vascular endothelial cells.

2. Materials and methods

2.1. Chemicals and reagents

COS were prepared as previously described with degree of deacetylation over 95%, average molecular weight: ≤ 1000 Da, and endotoxin free (limulus amoebocyte lysate test) (Zhang, Du, Yu, Mitsutomi, & Aiba, 1999). The weight percentages of COS were 3.7%, 16.1%, 28.8%, 37.2% and 14.2%, respectively, with DP (degree of polymerization) 2–6 in oligomixture. Antibodies against mitogen-activated protein kinase (MAPK), MAPK kinase (MEK), phosphorylated MAPK, O-GlcNAc transferase (OGT), and β -actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against NF- κ B/p65, Histone H3, and all peroxidase conjugated secondary antibodies were from Cell Signaling (Danvers, MA, USA); a goat anti-rabbit IgG conjugated to a fluorescent green dye Alexa Fluor 488 was from Invitrogen (Carlsbad, CA, USA); LPS (from *Escherichia coli* 055:B5) was from Sigma–Aldrich (St. Louis, MO, USA); mito-TEMPO-H (mTempol) was from Enzo Life Sciences (Farmingdale, NY, USA); ST045849 was bought from TimTec LLC (Newark, DE, USA). Human control and OGT siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). NE-PER[®] Nuclear and Cytoplasmic Extraction Reagents were purchased from Thermo (Thermo, USA). Inhibitors SB203580 and PD98059 were bought from Fisher Scientific (Waltham, MA, USA). Ponceau S was obtained from Sigma (St. Louis, MO, USA). The NF- κ B p50/p65 Transcription Factor Assay Kit was from Abcam (Cambridge, MA, USA).

2.2. Treatment of mice

Male C57BL/6J mice, 10 weeks of age, 20–30 g, were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed under controlled temperature (21 °C) and lighting, with 12 h of light and 12 h of dark, and had free access to water and a standard mouse chow diet. Mice were handled in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center (Oklahoma City, OK). The mice were randomly assigned to two major groups: the COS and non-COS treatment groups

(COS-group: 1 mg/ml in drinking water, 2 weeks; non-COS group: normal drinking water). Each major group was further divided into two subgroups treated either with LPS or vehicle (LPS from *E. coli*: 3 mg/kg, i.p.; vehicle: PBS). Body weight and food/water uptake were recorded at the start and before the endpoints of experiments. All mice were euthanized 24 h after the acute injection of either LPS or vehicle and the aortic tissues were prepared as described previously and stored at -80°C .

2.3. Endothelial cells and the treatment with siRNA duplex

The endothelial cells: bovine aortic endothelial cells (BAEC) and human vascular endothelial cells (EA.hy926) were from ATCC (Manassas, VA). BAEC were grown in endothelial cells basal medium (EBM; Lonza, Walkersville, MD) containing 5% FBS, penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and growth factor; EA.hy926 cells were cultured with Dulbecco's modified Eagle medium (DMEM; ATCC, Manassas, VA) containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). All cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂. In all experiments, cells used were between passages the third and eighth grown to 70–80% confluence as previously reported (Liu, Yu, Xu, & Xu, 2012; Liu, Yu, Zhang, & Xu, 2012). Transfection of control or OGT siRNA was performed based on protocols provided by Santa Cruz Biotechnology (Santa Cruz, CA) as described previously (Liu, Yu, Xu, et al., 2012; Liu, Yu, Zhang, et al., 2012). All cells were incubated in a humidified atmosphere of 5% CO₂ + 95% O₂ at 37 °C.

2.4. Immunofluorescent staining of endothelial cells for NF- κ B protein

The cultured endothelial cells were subjected to immunofluorescent staining with a commercial immune-staining kit including ProLong[®] Gold and SlowFade[®] Gold Antifade obtained from Life Technologies (Carlsbad, CA) as described previously (Liu, Yu, Xu, et al., 2012). Briefly, cells cultured on glass cover-slips were treated with experimental reagents and fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.3% Triton X-100 for 25 min at RT. After blocking with 1% goat serum for 10 min, cells were incubated with a polyclonal antibody against NF- κ B p65 overnight at 4 °C, a goat anti-rabbit IgG conjugated with a fluorescent green dye Alexa Fluor 488 was used as the secondary antibody and cells were permanently mounted with DAPI. Fluorescent signals were captured and analyzed with fluorescence microscopy (Olympus, Japan).

2.5. Western blot analysis and immunoprecipitation

Protein samples were detected by Western blot. Western blotting and band densitometry were performed as previously reported (Liu, Yu, Xu, et al., 2012; Liu, Yu, Zhang, et al., 2012). Briefly, the treated vascular endothelial cells were washed with ice-cold PBS and lysed in RIPA lysis buffer (50 mM Tris with pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate and 0.05 mM EDTA) for 15 min on ice, and cell lysate was centrifuged at 12,000 $\times g$ for 10 min at 4 °C. The supernatant was collected and stored at -80°C . Protein content was determined with a BCA (bicinchoninic acid) assay kit from Pierce (Pierce, USA). Levels of studied proteins were determined by Western blot analysis with their respective antibodies. Briefly, total cell lysate was boiled in 5 $\times$ loading buffer (125 mM Tris-HCl, pH 6.8, 10% SDS, 8% dithiothreitol, 50% glycerol and 0.5% bromochlorophenol blue) for 10 min. Equal amount of proteins (50 μg) was subjected to 8–12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes

were blocked with 5% skim milk in PBS with 0.1% Tween 20 (PBST) for 1 h, and incubated with primary antibodies overnight at 4 °C. Antibodies were detected by means of HRP-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized with enhanced chemiluminescence reagents (ECL) and densitometric analysis was performed with PDI Imageware System (Bio-Rad, Hercules, CA, USA). Cultured cells and aortic tissues were homogenized on ice in cell-lysis buffer. For immunoprecipitation, cell lysates were incubated with antibodies overnight at 4 °C. Antibody-protein complex was precipitated with protein A/G Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA).

2.6. Detection of O-GlcNAc-modified proteins in cell lysates and mouse aortic homogenates

The cell lysates or tissue homogenates were prepared in lysis buffer containing 25 mM HEPES (pH 7.0), 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.1% SDS, 1% protease inhibitor cocktail (Sigma, St. Louis, MO), 1% phosphatase inhibitor cocktail, and 1–100 μ M PUGNAc (O-GlcNAcase inhibitor). Proteins modified by O-linked GlcNAc were pulled down by using an Agarose bound Wheat Germ Agglutinin (WGA) Kit (Vector Laboratories, Burlingame, CA) as described previously (Zachara, Vosseller, & Hart, 2011: chap. 17). To confirm the WGA approach, an anti-O-GlcNAc antibody (CTD110.6) (Sigma, St. Louis, MO) was used to replace WGA for immunoprecipitation. The enriched proteins were subjected to Western blot analysis using antibodies of interest.

2.7. Inflammatory cytokine gene expression analysis

The total RNA extraction from cultured cells and lung tissues was achieved with Total RNA Kit I (#R683401, Omega Bio-Tek, Norcross, GA), reverse transcribed into cDNA, and the resulting cDNA was subjected to quantitative polymerase chain reaction (PCR) as previously described [40]. Forward and reverse specific primer sequences were: MCP-1 (human) (Meier et al., 2012), forward: 5'-CAA TAG GAA GAT CTC AGT GC AGA GG-3'; reverse: 5'-GGA ATC CTG AAC CCA CTT CTG C-3'. E-selectin (human) (Heo et al., 2013), forward: 5'-GCT CTG CAG CTC GGA CAT-3'; reverse: 5'-GAA AGT CCA GCT ACC AAG GGA AT-3'. β -actin (human) (Liu, Yu, Zhang, et al., 2012), forward: 5'-ACG GCA TCG TCA CCA ACT G-3'; reverse: 5'-GAG CCA CAC GCA GCT CAT T-3'. MCP-1 (mouse) (Jin et al., 2012), forward: 5'-CCT GGA TCG GAA CCA AAT GA-3'; reverse: 5'-CGG GTC AAC TTC ACA TTC AAA G-3'. E-selectin (mouse) (Sato et al., 2011), forward: 5'-CAT GGC TCA GCT CAA CTT-3'; reverse: 5'-GCA GCT CAT GTT CAT CTT-3'. GAPDH (mouse) (Pan et al., 2012), forward: 5'-TCC ATG ACA ACT TTG GCA TTG-3'; reverse: 5'-CAG TCT TCT GGG TGG CAG TGA-3'. iScript TM cDNA Synthesis Kit (170-8891), iQ TM SYBR Green Supermix (170-8880), and the Real Time Detection System were obtained from Bio-Rad (Hercules, CA). PCR specific primers were synthesized by Sigma (St. Louis, MO). PCR product was analyzed by running 6 μ l of each reaction mixture on a 1.5% agarose gel containing 1% GoldViewTM. Band intensity was quantified with ImageJ system (NIH, USA) and presented as a percentage of GAPDH expression.

2.8. Statistics

Values are expressed as mean $\pm$ SEM. Statistical comparisons were performed using SPSS 10.0 package (SPSS Inc., Chicago, IL, USA). One-way ANOVA and Student's *t*-test followed by a Bonferroni correction were carried out to determine statistical significance. Differences were considered significant at $P < 0.05$.

3. Results

3.1. COS block LPS-induced inflammatory cytokine gene expression in endothelial cells

To prepare a cell model in which LPS elicits inflammatory responses, we first monitored the NF- κ B dependent inflammatory cytokine gene expression in LPS-treated human vascular endothelial cells (EA.hy926). In this regard, we performed RT-PCR to detect LPS-altered mRNA expression levels of representative inflammatory cytokines. As depicted in Fig. 1A, in the presence of LPS, mRNA levels of respectively IL-1 β , MCP-1, and E-selectin were significantly increased compared with untreated controls (Fig. 1A). When pretreated with COS, the increase of mRNA levels was dramatically attenuated (Fig. 1A). These data were consistent with those reported in other endothelial cells (Liu, Li, et al., 2010; Liu et al., 2011), suggesting that the effect of COS was independent of endothelial cell type.

3.2. COS inhibit NF- κ B nucleus translocation in LPS-treated endothelial cells

NF- κ B is a fairly well studied transcriptional factor which is implicated both in health and disease in humans and experimental animals. It is widely recognized that increased NF- κ B nucleus translocation contributes to LPS-elevated gene expression of inflammatory cytokines in immune cells (e.g., macrophages) as well as vascular endothelial cells. To further confirm the anti-inflammatory capacity of COS, we examined intracellular locations of NF- κ B in LPS-treated endothelial cells (BAEC, a well established endothelial cell model). As expected, nuclear translocation of NF- κ B was enhanced by LPS, evidenced by increased Western blotting of NF- κ B in nuclear extraction from LPS-treated vs vehicle-treated control cells (Fig. 1B). However, NF- κ B protein staining in the nucleus was greatly reduced when cells were pretreated with COS (Fig. 1B). Similar results were obtained in cells subjected to immunohistochemical analysis which confirmed similar alterations in NF- κ B nuclear translocation (Fig. 1C). Given the importance of NF- κ B in the regulation of inflammatory gene expression, these results built an appropriate cell model for the rest of the study to evaluate LPS-mediated inflammatory response in endothelial cells.

3.3. LPS enhances O-GlcNAc modification of NF- κ B/p65

Increasing evidence support the notion that post-translational modulation of NF- κ B is crucial in promoting inflammatory gene expression. Recent studies demonstrate that O-GlcNAc modification of NF- κ B alters downstream gene expression. O-GlcNAc modification or O-GlcNAcylation is an essential cycling modification consisting of a single O-linked N-Acetylglucosamine sugar attached to the serine or threonine residues of nuclear and cytoplasmic proteins (Hart, Housley, & Slawson, 2007). We wondered whether LPS affected O-GlcNAc modification of NF- κ B in cultured vascular endothelial cells (BAEC). To detect O-GlcNAcylation, we followed the WGA Kit protocol (Zachara et al., 2011) to enrich the O-GlcNAcylated proteins which were then probed with an NF- κ B antibody recognizing specifically one of the subunits p65. The Western blotting showed that administration of LPS increased the staining for O-GlcNAcylated NF- κ B/p65 (Fig. 2A), without changing total levels of NF- κ B/p65 protein in these endothelial cells (Fig. 2A). To confirm results obtained from the WGA approach, we used an anti-O-GlcNAc antibody (CTD110.6) to harvest O-GlcNAc-modified proteins. As presented, a dramatic increase of NF- κ B/p65 staining was found in O-GlcNAc coimmunoprecipitates prepared from LPS-treated cells (Fig. 2B), similar to those with the WGA

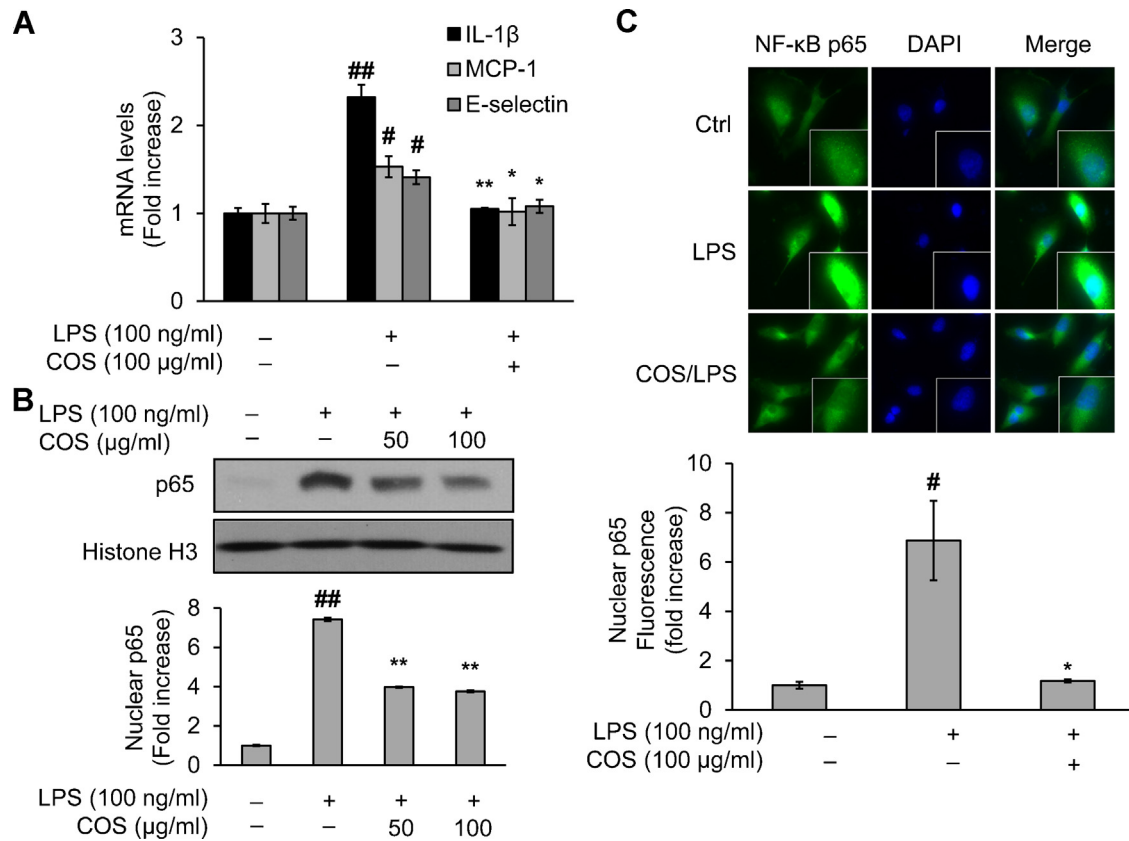


Fig. 1. COS inhibit NF-κB nucleus translocation and inflammatory cytokine gene expression in LPS-treated endothelial cells. EA.hy926 or BAEC were pretreated with COS (up to 100 μg/ml) for 4 h followed by LPS (100 ng/ml) incubation for 4 h. Then the cells were subjected to the assessment of (A) mRNA levels of inflammatory cytokines including IL-1β, MCP-1, and E-selectin, using RT-PCR as described in detail in Materials and Methods (*Inflammatory cytokine gene expression analysis*) in EA.hy926, and in BAEC: (B) Western blotting of NF-κB in the nucleus fraction prepared by NE-PER[®] Nuclear and Cytoplasmic Extraction Reagents, and (C) immunofluorescent staining of NF-κB or DAPI with a kit including ProLong[®] Gold and SlowFade[®] Gold Antifade. The secondary antibody was conjugated with a fluorescent green dye (Alexa Fluor 488). Nuclear p65 fluorescence was quantified. All images shown are representative of three independent experiments. Data are expressed as means ± SEM. ^{##}*P* < 0.01 compared to the vehicle-treated control group; ^{*}*P* < 0.05, ^{**}*P* < 0.01 compared to the LPS-only group.

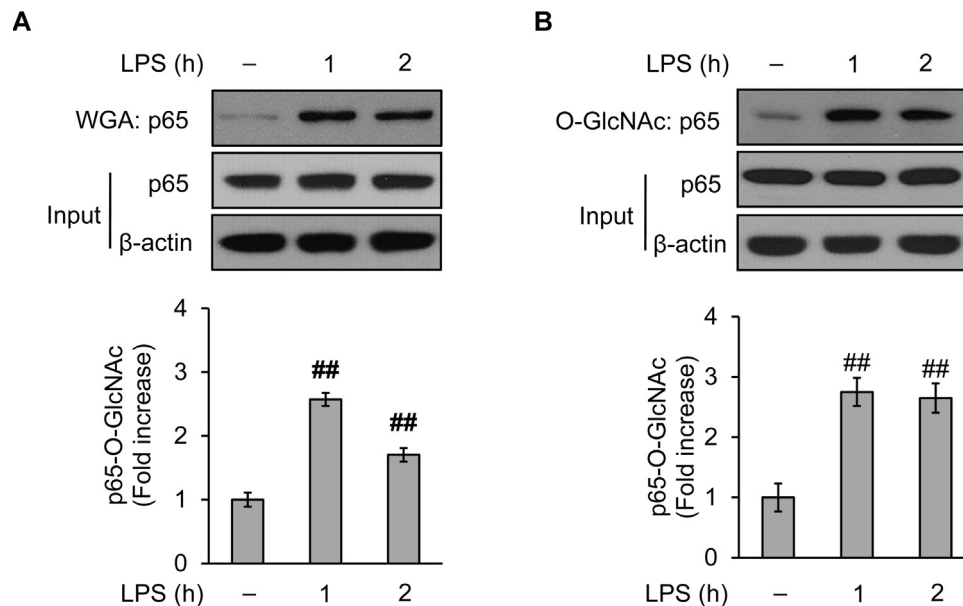


Fig. 2. LPS augments O-GlcNAc modification of NF-κB/p65 in vascular endothelial cells. BAEC were pretreated with (100 ng/ml) for up to 2 h and the collected cells were subjected to Western blotting after the enrichment of O-GlcNAc modified protein either by (A) WGA protocol or (B) anti-O-GlcNAc antibody (CD110.6). The whole cell lysates were used to assess the protein input as a loading control. Data are expressed as means ± SEM (*n* = 3). ^{##}*P* < 0.01 compared to the vehicle-treated control group.

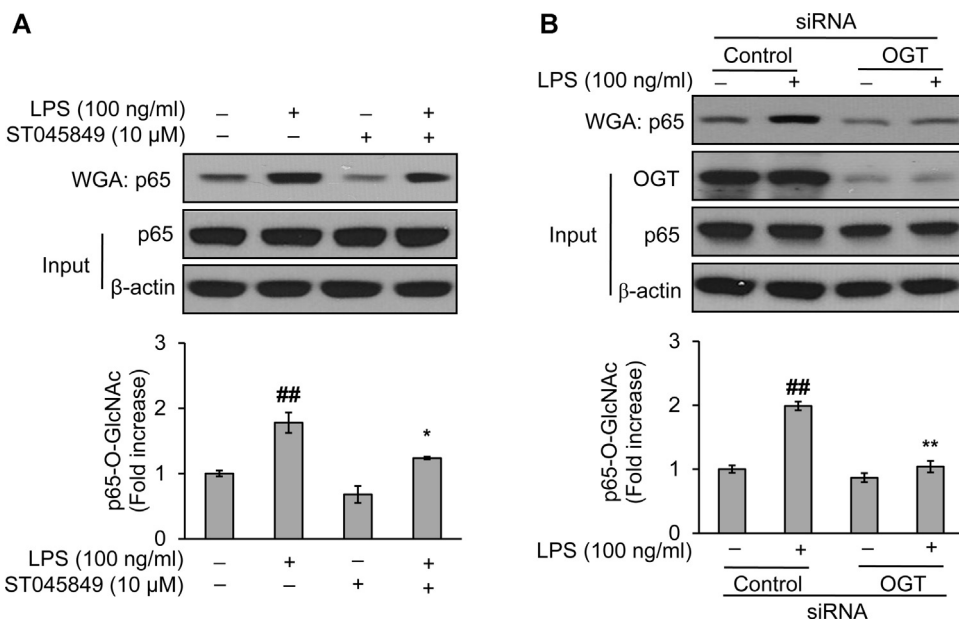


Fig. 3. OGT mediates LPS-induced O-GlcNAcylation of NF-κB/p65. Western blotting of indicated proteins of either the whole cell lysates (used to assess the protein input as a loading control) or WGA-enriched O-GlcNAc modified proteins, isolated respectively from endothelial cells being treated (A) either with vehicle (culture medium) or ST045849 (10 μM), a potent OGT inhibitor, for 1 h followed by LPS (100 ng/ml) challenge for 1 h (in EA.hy926) and (B) either with control or OGT siRNA for 48 h followed by LPS (100 ng/ml) challenge for 1 h (in EA.hy926). Data are expressed as means ± SEM ($n=3$). ^{##} $P<0.01$ compared to the vehicle-treated control group; ^{*} $P<0.05$, ^{**} $P<0.01$ compared to the LPS-only group or the group of LPS-only with control siRNA treatment.

approach (Fig. 2A). Together, LPS increased the O-GlcNAcylation of NF-κB/p65 in endothelial cells. In rest of the study, the WGA approach was adopted to enrich O-GlcNAc-modified proteins.

3.4. OGT mediates LPS-increased O-GlcNAc modification of NF-κB/p65

O-GlcNAc transferase (OGT) is a major dynamic enzyme that directly controls O-GlcNAc modification. Specifically, OGT catalyzes O-GlcNAcylation. Increasing evidence support a crucial role of OGT in the regulation of intracellular protein O-GlcNAcylation. We wondered whether OGT was required for the LPS-mediated NF-κB O-GlcNAcylation. To test this, we took pharmacological loss-of-function approach with ST045849, a potent OGT inhibitor (Lima et al., 2011). BAEC were pretreated either with ST045849 or vehicle (culture medium) before LPS challenge. As depicted, LPS reproducibly increased the levels of NF-κB/p65 O-GlcNAcylation in vehicle-treated cells, which was significantly attenuated in ST045849-treated cells (Fig. 3A), although levels of total p65 protein remained unchanged (Fig. 3A). To exclude potential off-target effects of OGT inhibitor, we took genetic loss-of-function approach with siRNA treatment in EA.hy926. As anticipated, OGT-siRNA treatment significantly reduced the protein levels of OGT compared with control-siRNA treatment (Fig. 3B). Incubation of LPS with control siRNA-treated cells increased the O-GlcNAcylation of NF-κB/p65 (Fig. 3A), confirming the effects of LPS in non-siRNA treated cells (Figs. 2 and 3A). However, the otherwise increased levels of NF-κB/p65 O-GlcNAcylation were largely reduced in OGT-siRNA treated cells (Fig. 3B). These data suggested that OGT mediated the O-GlcNAc modification of NF-κB/p65 induced by LPS.

3.5. NF-κB/p65 O-GlcNAcylation is associated with LPS-induced NF-κB nuclear entry

We next determined whether O-GlcNAc modification of NF-κB/p65 resulted in NF-κB nuclear entry, a hallmark as well as an initiate step in LPS-activated NF-κB signaling pathway. To do

this, we performed the same experiments in Fig. 3 but isolated the nucleus fraction with a subcellular fractionation kit. Challenge with LPS augmented the NF-κB/p65 staining in nucleus fraction (Fig. 4A), consistent with previous results (Fig. 1A), indicating enhanced nuclear entry of NF-κB/p65. However, this entry was reduced in OGT-knockdown cells (Fig. 4A). Clearly, these changes were associated with those of the NF-κB/p65 O-GlcNAcylation (Fig. 3). We further assessed the functional outcome of NF-κB/p65 O-GlcNAcylation by evaluating NF-κB dependent inflammatory gene expression. As shown, LPS treatment elevated mRNA levels of IL-1β, MCP-1, and E-selectin in vehicle but not in OGT inhibitor-treated cells (Fig. 4B).

3.6. COS abolish LPS-induced NF-κB/p65 O-GlcNAcylation in endothelial cells

We next decided whether COS would prevent NF-κB/p65 from LPS-induced O-GlcNAcylation. In this regard, we incubated 100 μg/ml of COS for 1 h before endothelial cells were challenged with LPS. As shown, LPS reproducibly induced NF-κB/p65 O-GlcNAcylation compared with the controls (Fig. 5A). Such an effect was abolished when the cells were pretreated with COS (Fig. 5A). Apparently, the effects of COS on levels of NF-κB/p65 O-GlcNAcylation were closely related to COS-mediated downregulation of NF-κB/p65 nucleus translocation (Fig. 1B/C) and mRNA levels of the inflammatory cytokine gene expressions (Fig. 1A) stimulated by LPS. To gain further mechanistic insights, we assessed the levels of NF-κB/p65 O-GlcNAcylation in the nucleus. This was achieved by Western blotting of O-GlcNAc-modified proteins enriched by WGA from nuclear extracts of the cells. As it turned out, NF-κB/p65 O-GlcNAcylation in the nucleus was significantly augmented by LPS. In contrast, when COS was present, the induced O-GlcNAc modification of NF-κB/p65 was greatly attenuated (Fig. 5B). We next measured DNA binding activity of NF-κB with an NF-κB p50/p65 transcription factor assay kit. It was achieved by quantification of the binding of NF-κB (from the enriched nuclear extracts as above) to the DNA

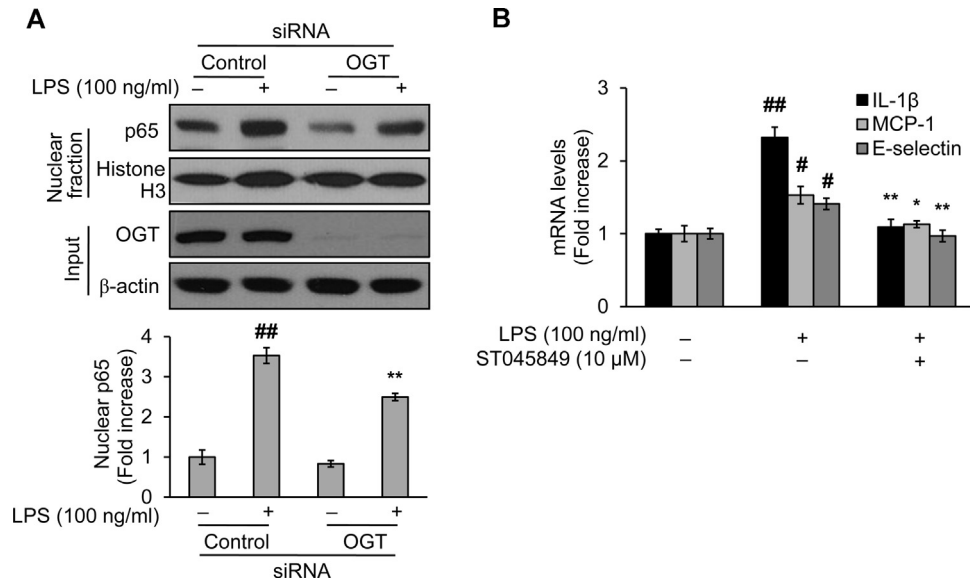


Fig. 4. OGT mediates LPS-induced NF-κB/p65 nucleus entry and activation. (A) Western blotting of the nucleus fraction proteins from EA.hy926 treated either with control or OGT siRNA for 48 h followed by LPS (100 ng/ml) challenge for 1 h. (B) mRNA levels of inflammatory cytokines of EA.hy926 either treated with vehicle (culture medium) or ST045849 (10 μM), a potent OGT inhibitor, for 1 h, followed by incubation with LPS (100 ng/ml) for 4 h. The mRNA levels IL-1β, MCP-1, and E-selectin were determined by using RT-PCR as described in detail in Materials and Methods (Inflammatory cytokine gene expression analysis). Data are expressed as means ± SEM (n = 3). *P < 0.05, **P < 0.01 compared to the vehicle-treated control group; *P < 0.05, **P < 0.01 compared to the LPS-only group or the group of LPS-only with control siRNA treatment.

containing NF-κB response elements (immobilized on a 96-well plate from the kit). As anticipated, LPS enhanced DNA binding activities of NF-κB (Fig. 5C); however, the presence of COS significantly reduced such activities (Fig. 5C). These data suggested that O-GlcNAc modification of NF-κB may underlie the effect of COS.

3.7. Blockade of MAP kinase inhibits NF-κB/p65 O-GlcNAcylation and nucleus translocation

MAPK mediated COS-exerted anti-inflammatory effects both in immune cells and endothelial cells treated with LPS. We wondered whether MAPK mediated O-GlcNAc modification of NF-κB/p65

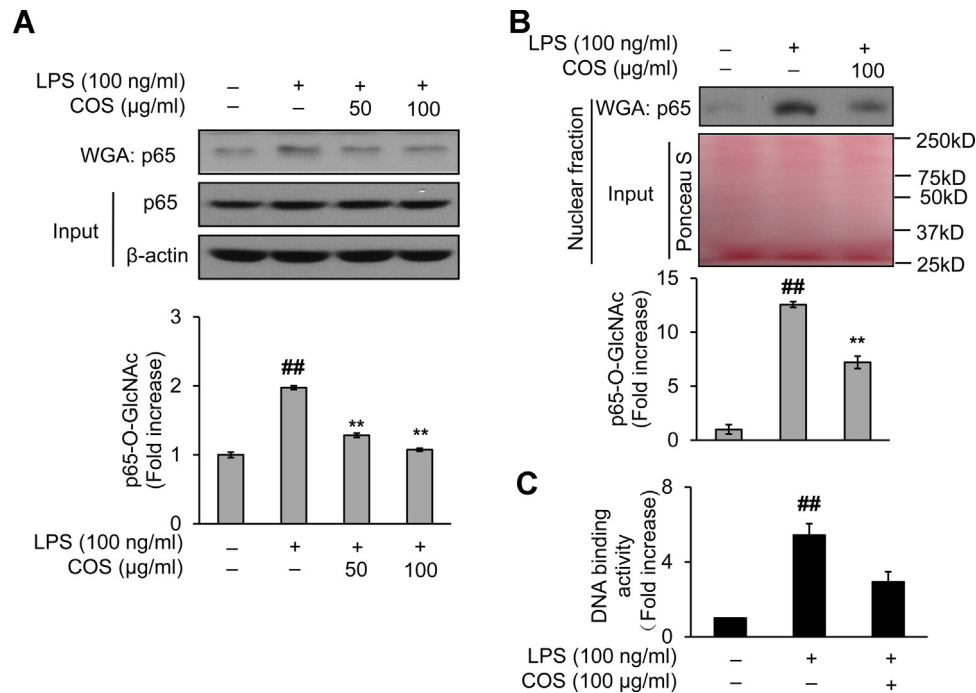


Fig. 5. COS abolishes LPS-induced NF-κB/p65 O-GlcNAcylation in endothelial cells. BAEC were pretreated with COS (up to 100 μg/ml) for 1 h and then exposed to LPS (100 ng/ml) for 1 h. Western blotting was performed with O-GlcNAc-modified proteins enriched by WGA either from (A) whole cell lysates (the protein loading or input was assessed by Western blotting on whole cell lysates) or (B) nuclear fraction isolated with NE-PER® Nuclear and Cytoplasmic Extraction Reagents (the protein loading or input was assessed by Ponceau S staining of the blot). (C) DNA binding activity was measured by following the instruction of the NF-κB p50/p65 Transcription Factor Assay Kit provided by the manufacture (Abcam). It was achieved by quantification of the binding of NF-κB (from WGA-enriched nuclear extracts) to the DNA containing NF-κB response elements (immobilized on a 96-well plate from the kit). Data are expressed as means ± SEM (n = 3). **P < 0.01 compared to the LPS-only group; **P < 0.01 compared to the LPS-only group.

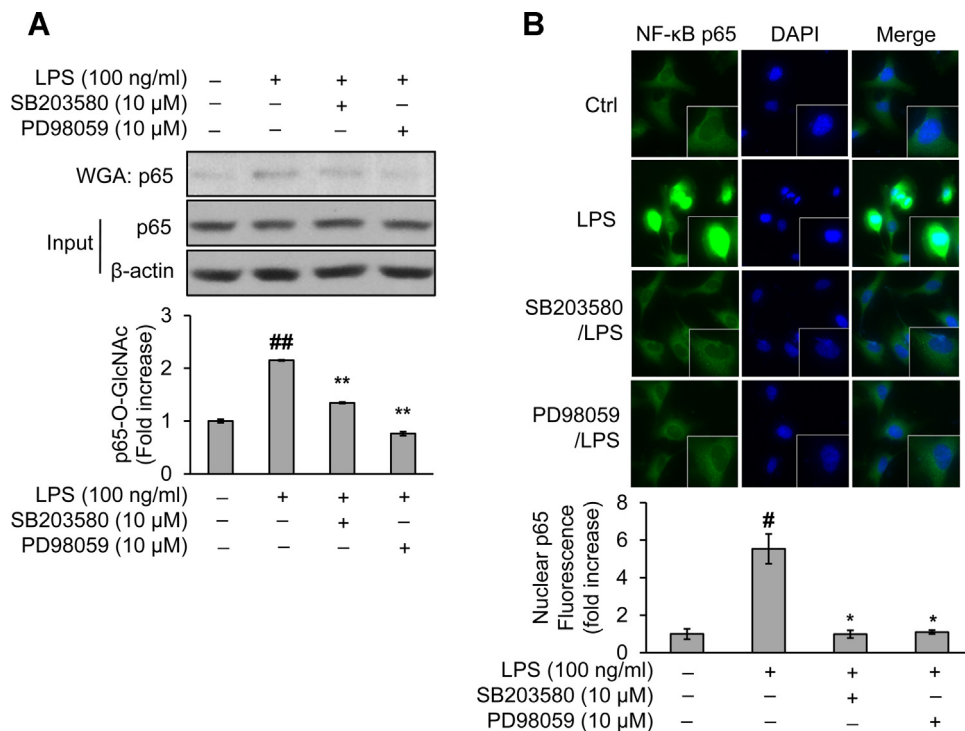


Fig. 6. Blockade of p38 MAPK pathway inhibits O-GlcNAcylation and activation of NF-κB/p65 in LPS-treated endothelial cells. BAEC were pretreated either with SB203580 (10 μM, a specific inhibitor of p38 MAPK) or PD98059 (10 μM, a selective inhibitor of MAPK kinase or MEK) for 1 h followed by LPS (100 ng/ml) incubation for 1 h. The treated cells were subjected to (A) Western blotting of proteins either from WGA-enriched O-GlcNAc modified proteins or whole cell lysates (to assess protein input as a loading control); (B) immunofluorescent staining of NF-κB or DAPI with a kit including ProLong® Gold and SlowFade® Gold Antifade. The secondary antibody was conjugated with a fluorescent green dye (Alexa Fluor 488). Nuclear p65 fluorescence was quantified. All images shown are representative of three independent experiments. Data are expressed as means ± SEM (n = 3). *P < 0.05, **P < 0.01 compared to the vehicle-treated control group; *P < 0.05, **P < 0.01 compared to the LPS-only group.

induced by LPS. To this end, endothelial cells were pretreated with SB203580, a specific MAPK inhibitor. Compared with vehicle-treated controls, LPS treatment increased O-GlcNAc modification of NF-κB/p65 (Fig. 6A); however, the enhancement was drastically diminished by MAPK inhibitor (Fig. 6A). Notably, the changes in levels of NF-κB/p65 O-GlcNAcylation were mirrored by those of the NF-κB nucleus translocation under same conditions (Fig. 6B). Similar results were obtained when the MAPK inhibitor was replaced by PD98059, a selective inhibitor of MAPK kinase, which significantly reduced LPS-elicited NF-κB/p65 O-GlcNAcylation (Fig. 6A) and nuclear entry (Fig. 6B). In sum, these data implicated that MAPK modulated NF-κB/p65 O-GlcNAcylation and the consequent nucleus translocation.

3.8. Superoxide scavenger blocks NF-κB/p65 O-GlcNAcylation and nucleus translocation

LPS is well known for its capacity to induce superoxide in endothelial cells (Al Ghoulé & Magder, 2008), whereas superoxide has been shown to activate MAPK (Chan et al., 2005). We wondered whether superoxide contributed to LPS-induced O-GlcNAc modification of NF-κB/p65. To test this, we pretreated endothelial cells with mTempol (1 mM for 1 h), a mitochondria-targeted antioxidant with superoxide scavenging properties (Dikalova et al., 2010), before LPS challenge. As predicted, LPS activated MAPK as shown by increased expression of phosphorylated MAPK (Fig. 7A), which was significantly less activated in the presence of mTempol (Fig. 7A). Repeatedly, LPS treatment increased O-GlcNAc modification of NF-κB/p65 (Fig. 7B); such an elevation was reduced when mTempol was pre-incubated (Fig. 7B), in a similar trend to the changes of MAPK phosphorylation (Fig. 7A).

Importantly, preincubation with mTempol significantly reduced LPS-mediated NF-κB nucleus translocation (Fig. 7C), further supporting a potential cause-and-effect relationship between NF-κB modification and nuclear entry which was modulated by superoxide.

3.9. Administration of COS inhibits vascular inflammatory response in LPS-treated mice

To extend our findings to physiologically relevant setting, we sought to test whole animal in vivo. In this regard, we selected a LPS mouse model, which has long been used to evaluate acute pro-inflammatory response to LPS either in immune cells (Xu et al., 2003) or beyond (Goldblum, Brann, Ding, Pugin, & Tobias, 1994; Kisseleva et al., 2006). We chose to test vascular tissues in which aberrant inflammatory response has been attributed to the pathogenesis of atherosclerotic cardiovascular diseases (Packard & Libby, 2008). Indeed, mice received LPS injection (3 mg/kg, i.p.) upregulated the gene expression of NF-κB-dependent inflammatory cytokines, as evidenced by the mRNA levels of these genes in vasculature-rich tissues (lung, which met the minimum requirement of weight for assessment of tissue mRNA levels) isolated from the treated mice 24 h after LPS injection (Fig. 8A). However, such an upregulation was absent in mice receiving COS administration for 2 weeks before LPS injection (COS: 1 mg/ml in normal drinking water, for 2 weeks) (Fig. 8A). Of note, administration of COS alone for the same duration (COS: 1 mg/ml in normal drinking water, for 2 weeks) did not invoke the inflammatory response (Fig. 8A), similar to the effects of vehicle-treated controls (normal drinking water without COS) (Fig. 8A).

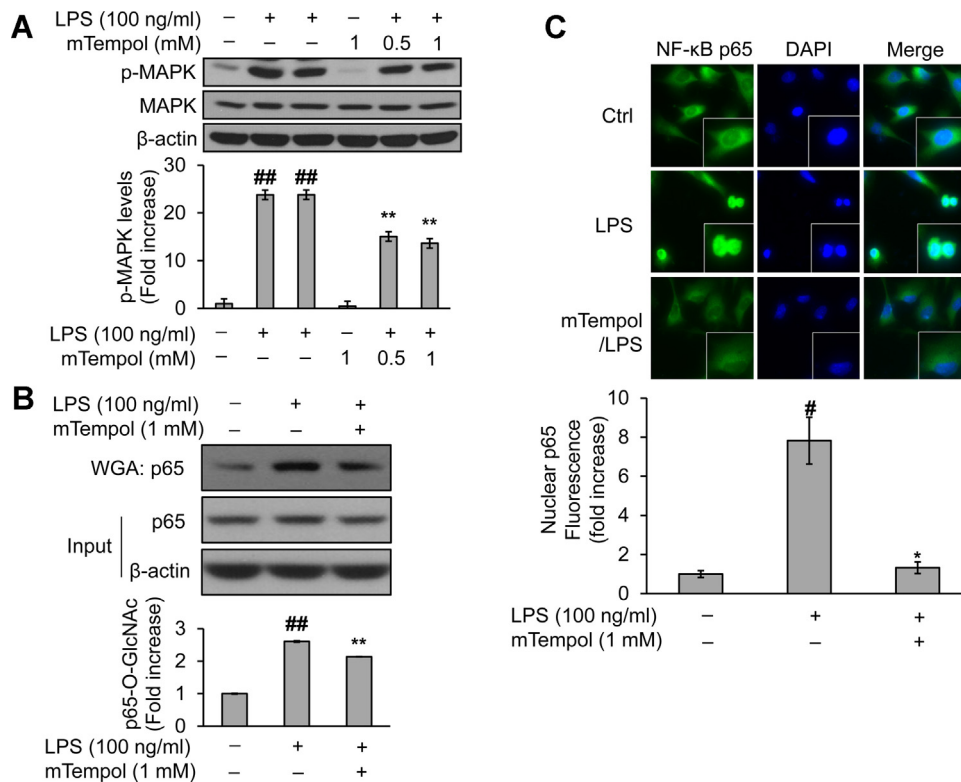


Fig. 7. Scavenging superoxide obliterates the O-GlcNAc modification and nucleus translocation of NF-κB/p65 in LPS-challenged endothelial cells. BAEC were pretreated either with vehicle (culture medium) or mTempol (up to 1 mM, a mitochondria-targeted antioxidant with superoxide scavenging properties) for up to 1 h followed by incubation with LPS (100 ng/ml) for 1 h. The treated cells were subjected to (A) assessment of the role of superoxide scavenger on LPS-induced MAPK activation by Western blot; (B) Western blotting of proteins either from WGA-enriched O-GlcNAc modified proteins or whole cell lysates (to assess protein input as a loading control); (C) immunofluorescent staining of NF-κB or DAPI with a kit including ProLong[®] Gold and SlowFade[®] Gold Antifade. The secondary antibody was conjugated with a fluorescent green dye (Alexa Fluor 488). Nuclear p65 fluorescence was quantified. All images shown are representative of three independent experiments. Data are expressed as means ± SEM ($n = 3$). [#] $P < 0.05$, ^{##} $P < 0.01$ compared to the vehicle-treated control group; ^{*} $P < 0.05$, ^{**} $P < 0.01$ compared to the LPS-only group.

3.10. LPS enhances aortic NF-κB/p65 O-GlcNAcylation, which is prevented in COS-treated mice

We further evaluated the levels of O-GlcNAc modification of NF-κB/p65 in aortic tissues. In line with the results obtained from cultured cells, acute administration of LPS (3 mg/kg, i.p.) induced a 2 fold increase of O-GlcNAcylation levels of NF-κB/p65 (Fig. 8B). However, pretreatment with COS for 2 weeks (3 mg/kg in drinking water) markedly reduced the levels of NF-κB/p65 O-GlcNAcylation in LPS-treated mice (Fig. 8B). Importantly, the changes in levels of NF-κB/p65 O-GlcNAcylation were closely associated with the activation of NF-κB pathway, evidenced by the alteration in NF-κB-dependent inflammatory gene expression in aortic tissues in response to administration of LPS and/or COS (Fig. 8A). In all experiment groups, the expression levels of total NF-κB/p65 protein remained similar (Fig. 8B). Taken together, LPS increased O-GlcNAcylation of NF-κB/p65 and enhanced inflammatory cytokine gene expression, which could be prevented by administration of COS in mouse model in vivo.

4. Discussion

The present study provided evidence to support the hypothesis that COS exert an anti-inflammatory effect through posttranslational modulation of NF-κB, the transcriptional factor key to gene expression of major inflammatory cytokines. Mechanistically, LPS induced OGT-mediated NF-κB O-GlcNAcylation in cultured cells, which was closely associated with enhanced NF-κB nucleus translocation and the resultant upregulation of inflammatory gene expression. Importantly, this connection was recapitulated in LPS

mouse model in vivo. Given the indispensable role of OGT in this mechanism, this study identified a potentially new mechanism by which COS confer a resistance to inflammation in vascular endothelial cells (Fig. 8C).

An important finding in this study was the demonstration both in the cell and mouse model that LPS-induced O-GlcNAc modification NF-κB/p65 was linked to NF-κB pathway activation in vascular endothelial cells. NF-κB serves as a critical regulator of cytokine production, cell activation, and proliferation both in immune cells (Li & Verma, 2002; Vallabhapurapu & Karin, 2009) and vascular endothelial cells (Pober & Sessa, 2007). NF-κB activation represents the major mechanisms by which LPS upregulates NF-κB-dependent inflammatory gene expressions (Dumitru et al., 2000; Natoli, 2004, 2012). Activation of NF-κB requires posttranslational modifications, such as phosphorylation and acetylation. NF-κB O-GlcNAcylation-induced activation of NF-κB-dependent genes has been found in T lymphocytes (Golks, Tran, Goetschy, & Guerini, 2007), high glucose-treated vascular smooth muscle cells (Yang et al., 2008) and Mesangial cells (James et al., 2002), and recently in HEK cells (Allison et al., 2012). Our study extends the findings to vascular endothelial cells both in the cell and mouse models that LPS-induced O-GlcNAc modification of NF-κB/p65 is associated with activation of NF-κB pathway, likely through a similar mechanism involving disruption of the interaction with IκB, although direct involvement in nuclear translocation of NF-κB is also possible (Ozcan, Andrali, & Cantrell, 2010). The finding has also provided a testable mechanism by which COS affords the anti-inflammatory function.

Another interesting finding in the present study was the involvement of OGT in LPS-induced O-GlcNAc modification (Fig. 3), nuclear

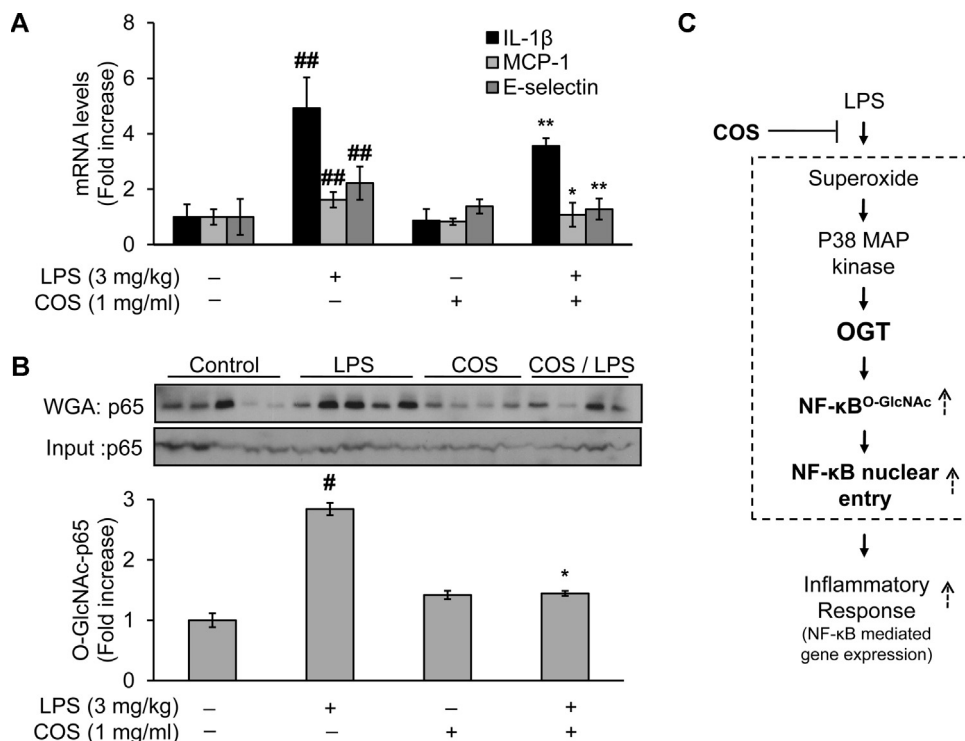


Fig. 8. Administration of COS inhibits vascular inflammatory response in LPS-treated mice, likely through reduction of LPS-induced NF- κ B O-GlcNAcylation. Male C57BL/6J mice (10 weeks of age, 20–30 g) were randomly assigned into two major groups: the COS and the non-COS treatment groups (COS-group: 1 mg/ml in drinking water, 2 weeks; non-COS group: normal drinking water). Each major group was further divided into two subgroups treated either with LPS or vehicle (LPS from *E. coli*: 3 mg/kg, i.p.; vehicle: PBS). All mice were euthanized 24 h after the acute injection of either LPS/vehicle for tissues collection. Aortic tissues were then subjected to evaluation of (A) mRNA levels of inflammatory cytokines including IL-1 β , MCP-1, and E-selectin; and (B) Western blotting of proteins either from WGA-enriched O-GlcNAc modified proteins or whole cell lysates (to assess protein input as a loading control). (C) Proposed mechanism by which COS exerts its anti-inflammatory effects induced by LPS: COS counteracts LPS-elicited vascular endothelial inflammatory response through a mechanism involving OGT-mediated O-GlcNAcylation, nucleus translocation, and activation of NF- κ B/p65, both in cultured endothelial cells and experimental mouse model. Text in bold highlighted the new findings in the present study. Data are expressed as means $\pm$ SEM. [#] $P < 0.05$, ^{##} $P < 0.01$ compared to the vehicle-treated control group; ^{*} $P < 0.05$, ^{**} $P < 0.01$ compared to the LPS-only group.

entry (Fig. 4A), and activation nuclear entry (Fig. 4B) of NF- κ B. This finding is in line with a recent report that LPS enhances OGT-mediated O-GlcNAcylation by activating OGT (Ryu & Do, 2011). Furthermore, increased O-GlcNAcylation has been shown contributable to insulin resistance and hence the development of diabetes (McClain & Crook, 1996), elevated vascular contractility in response to endothelin-1 (Lima et al., 2010), and DOCA-salt hypertension (Lima et al., 2009). However, acute incubation ex vivo with rat aortas of O-GlcNAc-increasing agents such as glucosamine prevented inflammation-induced vascular dysfunction (Hilgers et al., 2012). Similarly, increased O-GlcNAc levels have been shown to reverse NF- κ B activation in macrophage (Zou et al., 2009) and inhibit inflammatory responses to acute arterial injury (Xing et al., 2008). The discrepancies and even contradictions in these and perhaps other similar studies might attribute to a major difference in modes of OGT activation, durations of GlcNAc modulation, and even the cell types and animal models adopted. However, the dynamics of O-GlcNAc modification are complex (Hart & Copeland, 2010; Hart et al., 2007; Wells, Vosseller, & Hart, 2001) and so is OGT-mediated O-GlcNAc modification of transcriptional factors (Ozcan et al., 2010). It needs further studies to define exactly how OGT regulates NF- κ B-mediated inflammatory response.

The most important finding in the current study was the identification of the mechanism by which COS abolished LPS-induced activation of NF- κ B pathway. Specifically, administration of COS both in the cell and mouse models lowered the levels of NF- κ B/p65 O-GlcNAcylation induced by LPS. The downregulation in NF- κ B O-GlcNAcylation may contribute to the reduction in NF- κ B/p65 nucleus translocation and activation of NF- κ B pathway,

because treatment with COS diminished LPS-elicited inflammatory response, evidenced by reduced gene expression of inflammatory cytokines both in cultured cells and mice in vivo. It is yet unknown exactly how COS regulate NF- κ B/p65 O-GlcNAcylation; however, it is known that COS inhibit LPS-mediated inflammatory response (Liu, Li, et al., 2010) and hydrogen peroxide-induced apoptosis (Liu, He, et al., 2010) through suppression of MAPK, an important kinase known to be activated by superoxide (Chan et al., 2005). Interestingly, inhibition of either MAPK (or MAPK kinase) (Fig. 6) or superoxide (Fig. 7) abrogated LPS-induced O-GlcNAc modification of NF- κ B and nuclear translocation. Given the requirement of OGT in LPS-induced NF- κ B O-GlcNAcylation (Fig. 3) and the resultant activation of NF- κ B pathway (Figs. 4B, 5B and C), it is likely that COS interrupt NF- κ B/p65 O-GlcNAcylation by blocking the impact of either MAPK or superoxide on OGT. Such an impact has been demonstrated: the activation of MAPK increased OGT-mediated O-GlcNAc modification in TGF- β -treated retinal pigment epithelial cells (Chung, Chun, Jung, Cho, & Lee, 2011), although modulation of OGT could affect MAPK in high glucose-treated renal cells (Goldberg, Whiteside, & Fantus, 2011) and in mouse embryonic neural precursor cells (Yanagisawa & Yu, 2009). These results implicate that COS-suppressed O-GlcNAc modification of NF- κ B is an important integrated component of the mechanism by which COS inhibit NF- κ B pathway. It warrants further investigation on how COS modulate the complex crosstalk among MAPK, superoxide, and OGT to exert its anti-inflammatory function. It is worth noting that as a logical extension to the published studies, the present paper focused only on intracellular effects of COS; however, it does not exclude the extracellular impacts from COS as found in cultured

immune cells (Qiao et al., 2010). It also merits further investigation on the role of COS in not only the prevention but also the treatment of inflammation.

Taken together, LPS enhances gene expression of the inflammatory cytokines in vascular endothelial cells through a mechanism involving OGT-mediated O-GlcNAcylation and nucleus translocation of NF- κ B; mechanistically, these are mediated by MAP kinase and superoxide generation activated by LPS. However, administration of COS, both to cultured endothelial cells and experimental mouse model, attenuates these impacts. We conclude that COS suppress vascular endothelial inflammatory response induced by LPS through a mechanism involving MAP kinase, superoxide, and OGT-mediated O-GlcNAc modification and nucleus translocation of NF- κ B. These insights may help develop COS-based pharmacological strategies to regulate inflammation.

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