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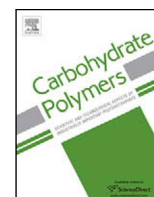

Chitosan oligosaccharides block LPS-induced O-GlcNAcylation of NF-κB and endothelial inflammatory response

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ABSTRACT

It is known that chitosan oligosaccharides (COS) suppress LPS-induced vascular endothelial inflammatory response by mechanism involving NF-κB blockade. It remains unknown how COS inhibit NF-κB. 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-κB-dependent inflammatory gene expression was suppressed by COS, which was associated with reduced NF-κB nucleus translocation. LPS enhanced O-GlcNAc modification of NF-κB/p65 and activated NF-κB 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-κB O-GlcNAcylation. Consistently, aortic tissues from LPS-treated mice presented enhanced NF-κB/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-κB 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; Tavaria 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-κB-dependent inflammatory gene expression. Indeed, pre-treatments with COS significantly abolish the otherwise increased inflammatory cytokines by LPS, such as IL-1β (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-κB activation induced by LPS.

NF-κB 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 IκB. Treatment with LPS or other activating agents stimulate IκB kinase, which phosphorylates IκB and thereby induces its degradation. The degradation of IκB leads to dissociation and translocation of NF-κB into the nucleus and activation of target genes. In this study, we sought to provide evidence to test a potentially novel hypothesis involving NF-κB 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 amebocyte 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-κB/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-kB 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 μg/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 μg/ml). All cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2. 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% CO_2 + 95% O_2 at 37 °C.

2.4. Immunofluorescent staining of endothelial cells for NF-κB 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-κB 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 × 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× loading buffer (125 mM Tris·HCl, pH 6.8, 10% SDS, 8% dithiothreitol, 50% glycerol and 0.5% bromchlorphenol 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

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