Immunomodulatory Effects of Xylooligosaccharides

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Xylooligosaccharides (XOs) are considered as food ingredients, and exhibit the prebiotic effects related to gut modulation. However, no related research is available to explain their immunomodulatory effects. This report elucidated their immunomodulatory effects through the variations of proinflammatory mediators *in vitro*. We found that XOs (0.1–100 µg/mL) induced tumor necrosis factor alpha (TNF-α), IL-1β, IL-6 and nitric oxide (NO) production in un-stimulated macrophages, RAW264.7 cells. Furthermore, pre- and post-treated XOs (0.1–100 µg/mL) dose-dependently suppressed TNF-α, IL-1β, IL-6 and NO production and induced IL-10 production in lipopolysaccharide (LPS)-stimulated RAW264.7 cells without exerting cytotoxicity. Of note is that prostaglandin E₂ (PGE₂) production didn't change significantly through the XOs treatment. These data demonstrate that XOs potently down-regulates the LPS-induced inflammatory response. The *in vitro* assessment of inflammatory mediators should be useful in further characterization of the effects of XOs on immunomodulation.

Keywords: xylooligosaccharides, immunomodulatory, anti-inflammatory

Abbreviations: XOs, xylooligosaccharides; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor alpha; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; DMSO, Dimethyl sulfoxide

Introduction

Xylooligosaccharides (XOs) with degrees of polymerization from 2 to 6 are produced during the hydrolysis of xylan which is the major component of plant hemicellulose, a heteropolysaccharide with homopolymeric backbone of xylose units (Saha, 2003). XOs are neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract and they affect the host by selectively stimulating the growth or activity of probiotic bacteria in the colon and thus improve health to reveal prebiotic effects (Vazquez *et al.*, 2000). XOs have been shown to reduce cholesterol, maintain gastrointestinal health, and improve the biological availability of calcium (Campbell *et al.*, 1997). XOs are also known to reduce the risk of colon cancer in rats (Hsu *et al.*, 2004). Since they are moderately sweet, stable over a wide range of pH and temperatures, and they inhibit the starch retrogradation, they improve the nutritional and sensory properties of food (Voragen, 1998).

Macrophages play a major role in a host defense against

intracellular parasitic bacteria, pathogenic protozoa, fungi and helminthes as well as against tumors, especially metastasizing tumors. To execute these functions, macrophages classically produce and release pro-inflammatory cytokines and chemical mediators, such as NO and PGE₂ (Lorsbach *et al.*, 1993; Schmidt and Walter, 1994; Snyder and Bredt, 1992). Pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, are potentially capable of injuring host tissues to play key homeostatic functional roles (Laskin and Pendino, 1995).

NO and PGE₂ are generated by the inducible isoforms of NO synthase (iNOS) and cyclooxygenase-2 (COX-2) (Posadas *et al.*, 2000). NO is essential for host innate immune responses to pathogens, and for the regulation of other physiological functions including neurotransmission, vasodilatation, and neurotoxicity (MacMicking *et al.*, 1997). However, excessive NO production can result in the development of inflammatory diseases (O'Shea *et al.*, 2002). PGE₂ is a lipid mediator that has an important role in multiple physiologic processes, including kidney function, vascular homeostasis, bone remodeling, fever generation, gastrointestinal function, pregnancy, and acute inflammatory responses (Narumiya *et*

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al., 1999). Thus, variations in levels of these mediators can be thought of as a marker of immunomodulation.

XOs reveal many healthy effects, but not immunomodulatory and anti-inflammatory activities which have reported to be regulated by many saccharides, such as β -glucan, arabinogalactan, pectin, arabinan, heteroglycan, inulin, agaro-oligosaccharides and chitosan oligosaccharides (Chen and Yan, 2005; Yamada and Kiyohara, 2007; Yoon *et al.*, 2007). Our research will explore the immunomodulatory effects of XOs on a cellular level. First, we will evaluate the effect of XOs on the inflammatory mediators release in a murine macrophage cell line, RAW 264.7. In addition, we will try to find the effects of XOs on the anti-inflammatory cytokines and the inflammatory mediators release in RAW 264.7 stimulated with LPS.

Materials and Methods

Chemicals and reagents XOs were purchased from Suntory Co, Ltd (Xylooligo 95P, Osaka, Japan). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, trypsin-EDTA, streptomycin stock solution, trypan blue, griess reagent 1 and 2 were obtained from Gibco Laboratories (Chagrin Falls, IL). LPS, sodium bicarbonate, sodium phosphate, 3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) DMSO (Dimethyl sulfoxide) and sodium nitrite were from Sigma Chemical Company (St. Louis, MO). RAW264.7 cells were obtained from ATCC (No. TIB-71, Rockville, MD).

Cell culture RAW 264.7 was grown in DMEM supplemented with 10% (v/v) FBS, 1 mM sodium pyruvate, 1% (v/v) streptomycin (100 mg/mL) and penicillin (100 U/mL). All cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Cells were grown to confluence in sterile tissue culture dishes and gently detached by repeated pipetting. For experiments, cells were cultured in triplicate at a density of 5×10^5 cells per mL in 96-well flat-bottomed tissue culture plates (Costar, Cambridge MA). Cultures containing XOs with and without LPS were incubated and analyzed for cell viability, NO, TNF- α , IL-1 β , IL-6 and IL-10.

Cell viability Cell viability was assessed using a modified MTT assay (Mosmann, 1983). Briefly, cells (2.5×10^4 cells/well) were seeded in a 96-well plate and treated with XOs for 24h. Following treatment, 100 μ L of a MTT solution (2mg/mL in phosphate buffered saline) was added to each well and further incubated for 4 h at 37°C. Subsequently, 100 μ L of DMSO was added to each well to solubilize any deposited formazon. The optical density (OD) of each well was measured at 570 nm with a microplate reader (EL311s, BIO-TEK Instruments, INC).

Cytokines (TNF- α , IL-1 β , IL-6 and IL-10) and PGE₂ determination After pre-incubation of RAW264.7 cells (1×10^6 cells /well) for 24 h, XOs, either with or without LPS (0.2 μ g/mL), was incubated for 24 h. Cytokines (TNF- α , IL-1 β , IL-6 and IL-10) and PGE₂ were in the culture supernatants were measured by ELISA kits (R&D systems).

NO determination After pre-incubation of RAW264.7 cells (2×10^5 cells /well) for 3 h, XOs, either with or without LPS (0.2 μ g/mL), was incubated for 24 h. The nitrite in culture supernatants were measured by adding 50 μ L of Griess reagent 1 and 50 μ L of Griess reagent 2. After 5–10 min at room temperature the optical density at 540 nm (OD₅₅₀) was measured by a microplate reader. Samples of the culture media incubated without macrophage were assayed for background levels of nitrite and these values were subtracted from the values measured in the culture supernatant. Sodium nitrate (4 ~ 500 μ M) was used as nitrite standards and nitrite was linear over this concentration range.

Statistic analysis All data were expressed as mean $\pm$ SD. The data analysis was performed by ANOVA test and Tukey-Kramer multiple comparison test. A P value of < 0.05 was considered significant. The comparison was carried out with control or with reagent-treated data.

Results

Effects of XOs on cell viability RAW 264.7 macrophage viability in the presence of XOs is shown in Table 1. Cytotoxicity of XOs (up to a concentration of 100 μ g/mL) was not obvious after 24 h incubation. With this result, concentrations of 0.1 to 100 μ g/mL of XOs were chosen for subsequent experimentation.

Effects of XOs on cytokines, nitrite and PGE₂ secretion from RAW 264.7 cells To evaluate the potential immunomodulatory effect of XOs on un-stimulated RAW 264.7, the secreting profiles of inflammatory mediators, including pro-inflammatory cytokines, NO and PGE₂, were assessed. As shown in Table 1, XOs (1–100 μ g/mL) stimulated RAW264.7 macrophages to produce dose- dependently substantial amounts of pro-inflammatory cytokines, TNF- α , IL-1 β and IL-6. Moreover, the similar increasing secreting quantity of NO was induced by XOs among the used doses (0.1–100 μ g/mL). However, no significant alteration in PGE₂ production could be found. The magnitudes of mediators, except PGE₂, induced by XOs were less than those by LPS stimulation.

Anti-inflammatory activity of XOs TNF- α , IL-1 β , IL-6, NO and PGE₂ are known to be pro-inflammatory mediators that posses a multitude of biological activities linked to the immunopathology of acute or chronic inflammatory diseases such as septic shock and rheumatoid arthritis, and autoim-

Table1. Effects of XOs on RAW264.7 cell viability, and cytokines, nitrite and PGE₂ production from RAW264.7 cells.

	Control	Control + LPS	XOs (μg/mL)			
			0.1	1	10	100
Cell viability (%)	100 ± 0 ^b	114 ± 1 ^a	102 ± 0 ^b	101 ± 1 ^b	103 ± 2 ^b	102 ± 3 ^b
TNF-α (pg/mL)	44 ± 9 ^d	448 ± 13 ^a	18 ± 5 ^e	79 ± 4 ^e	98 ± 2 ^c	122 ± 4 ^b
IL-1β (pg/mL)	2 ± 0 ^d	61 ± 7 ^a	3 ± 1 ^{cd}	7 ± 3 ^{cd}	12 ± 3 ^c	34 ± 1 ^b
IL-6 (pg/mL)	13 ± 5 ^d	263 ± 7 ^a	6 ± 5 ^d	23 ± 3 ^d	53 ± 2 ^c	89 ± 13 ^b
NO (μM)	13 ± 0 ^c	30 ± 1 ^a	21 ± 0 ^b	20 ± 0 ^b	21 ± 0 ^b	21 ± 1 ^b
PGE ₂ (pg/mL)	13 ± 8 ^b	1956 ± 64 ^a	16 ± 14 ^b	15 ± 11 ^b	17 ± 4 ^b	45 ± 23 ^b

Means of 3 replicates ± standard deviations; means followed by different letters in a row differ significantly ($P < 0.05$).

Table 2. Effect of XOs on cytokines, nitrite and PGE₂ secretion from RAW 264.7 cells stimulated with LPS in the pre-treatment model.

	Control	Control + LPS	XOs (μg/mL)			
			0.1	1	10	100
TNF-α (pg/mL)	24 ± 1 ^c	1056 ± 64 ^a	1054 ± 58 ^a (-0.2)	744 ± 13 ^b (-30)	549 ± 30 ^c (-48)	351 ± 11 ^d (-66)
IL-1β (pg/mL)	4 ± 1 ^d	58 ± 0 ^a	59 ± 1 ^a (0)	41 ± 1 ^b (-29)	39 ± 1 ^{bc} (-32)	38 ± 1 ^c (-35)
IL-6 (pg/mL)	25 ± 8 ^e	331 ± 31 ^a	338 ± 11 ^a (0)	286 ± 7 ^b (-14)	263 ± 5 ^{cd} (-20)	244 ± 10 ^d (-26)
IL-10 (pg/mL)	2 ± 0 ^f	11 ± 1 ^c	12 ± 0 ^d (+4)	14 ± 1 ^c (+27)	18 ± 0 ^b (+58)	20 ± 1 ^a (+73)
NO (μM)	14 ± 0 ^b	17 ± 1 ^a	14 ± 0 ^b (-16)	14 ± 1 ^b (-20)	12 ± 1 ^c (-30)	11 ± 0 ^c (-37)
PGE ₂ (pg/mL)	16 ± 3 ^b	2073 ± 42 ^a	2059 ± 144 ^a (0)	2124 ± 123 ^a (0)	2146 ± 145 ^a (0)	2027 ± 68 ^a (-2)

Means of 3 replicates ± standard deviations; means followed by different letters in a row differ significantly ($P < 0.05$). The figures in parentheses represent inhibition (-) or increment (+) ratio: ((sample / control + LPS) - 1)*100.

Table 3. Effect of XOs on cytokines, nitrite and PGE₂ secretion from RAW 264.7 cells stimulated with LPS in the post-treatment model.

	Control	Control + LPS	XOs (μg)			
			0.1	1	10	100
TNF-α (pg/mL)	23 ± 2 ^d	719 ± 16 ^a	731 ± 13 ^a (0)	702 ± 15 ^a (-2)	584 ± 6 ^b (-19)	454 ± 14 ^c (-37)
IL-1β (pg/mL)	5 ± 2 ^d	54 ± 3 ^a	53 ± 3 ^{ab} (2)	47 ± 2 ^b (-13)	39 ± 3 ^c (-29)	38 ± 1 ^c (-29)
IL-6 (pg/mL)	6 ± 2 ^c	287 ± 6 ^a	299 ± 20 ^a (0)	295 ± 3 ^a (0)	267 ± 7 ^{ab} (-7)	245 ± 27 ^b (-15)
IL-10 (pg/mL)	3 ± 0 ^b	11 ± 1 ^b	8 ± 7 ^b (0)	12 ± 7 ^b (+9)	14 ± 4 ^{ab} (+22)	27 ± 5 ^a (+139)
NO (μM)	12 ± 0 ^d	19 ± 0 ^a	16 ± 0 ^b (-13)	15 ± 1 ^{bc} (-19)	15 ± 0 ^{bc} (-22)	14 ± 0 ^c (-27)
PGE ₂ (pg/mL)	17 ± 4 ^c	1851 ± 42 ^{ab}	1929 ± 109 ^a (0)	1906 ± 12 ^{ab} (0)	1884 ± 37 ^{ab} (0)	1784 ± 31 ^b (-4)

Means of 3 replicates ± standard deviations; means followed by different letters in a row differ significantly ($P < 0.05$). The figures in parentheses represent inhibition (-) or increment (+) ratio: ((sample / control + LPS) - 1)*100.

mune diseases (Eigler *et al.*, 1997).

To determine the anti-inflammatory effects of XOs on cytokines, NO and PGE₂ production in RAW 264.7 cells, the cells were pre-treatment with various concentrations of XOs and then stimulated with LPS for 24 h. The control group was not treated with LPS or XOs. Supernatant from cell culture media was collected to measure the amount of cytokines, NO and PGE₂. LPS-stimulated pro-inflammatory cytokines and NO productions were significantly decreased by XOs in a dose-dependent manner. Pre-treating 100 μg/mL of XOs significantly inhibited TNF-α, IL-1β, IL-6 and NO production by 66%, 35%, 26% and 37%, respectively. Meanwhile, anti-inflammatory cytokine IL-10 was only modestly

increased compared to LPS-stimulated cells. In addition, XO didn't decrease PGE₂ production in LPS-stimulated cells (Table 2).

Furthermore, Table 3 revealed the anti-inflammatory effects of XOs on cytokines, NO and PGE₂ production in RAW 264.7 cells, the cells were stimulated with LPS and treated with various concentrations of XOs for 24 h, which called the post-treatment model. In consistent with the inhibitory effect seen when added before stimulation, XOs also inhibited LPS-induced NO and PGE₂ production in a dose-dependent manner (Table3). XOs at 100 μg/mL inhibited TNF-α, IL-1β, IL-6 and NO production by 37%, 29%, 15% and 27%, respectively. The magnitudes of mediators inhibited by post-

treated of XOs were less than those by pre-treated of XOs.

Compare to the LPS-stimulated cells, XOs also increased the quantity of IL-10 modestly, and didn't change the PGE₂ production significantly.

Discussion

Murine macrophage-like cell line, RAW 264.7, has been used to characterize the immunomodulatory action of various components at the molecular level previously (Choi *et al.*, 2001; Wu *et al.*, 2005). It releases several mediators, including inflammatory cytokines, TNF- α , IL-1 β and IL-6, and NO (Nathan, 1987). These mediators induce the activation and differentiation of lymphocytes, the proliferation of granulocytes, support enhanced cytotoxicity against tumor cells and accelerate immunoreactivity *in vivo* (MacMicking *et al.*, 1997). TNF- α , IL-1 β , considered 'early response cytokines' that are produced rapidly by macrophages in response to inflammatory stimuli, upregulate expression of adhesion molecules on endothelial cells thus facilitating phagocyte margination and emigration to sites of tissue injury (Laskin and Laskin, 2001). In addition, TNF- α exhibits tumor necrosis activity and has been recognized as an important host regulatory molecule (Vilcek and Lee, 1991). IL-6 plays an essential role in the host immune response, acute protein synthesis and the maintenance of homeostasis (Liu *et al.*, 2007). NO is involved in the destruction of tumor cells by activated macrophages (Moncada *et al.*, 1991). Therefore, variations in levels of mediators can be thought of as a marker of immunomodulation, and has been used for assessing the immunomodulatory activity of tested sample in the previous literatures (Hu *et al.*, 2008; Liu *et al.*, 2007). In this study, we demonstrated that XOs (0.1–100 μ g/mL) are able to activate RAW264.7, resulting in increased TNF- α , IL-1 β , IL-6 and NO production, and exhibited the immunomodulatory activities in the innate immunity.

The release of inflammatory mediators is essential for host survival from infection, and is also required for the repair of tissue injury (Glauser, 1996). However, large amounts of macrophage-derived mediators can also cause collateral damage to normal cells and are potentially lethal when the release is sufficient to cause systemic exposure (Glauser, 1996). Therefore, the anti-inflammatory activity of XOs was also assessed. Our study demonstrated that XOs could significantly suppress TNF- α , IL-1 β , IL-6 and NO productions in LPS-stimulated RAW264.7 cells in a dose-dependent manner, and the inhibitory effect in the pre-treatment model is stronger than the post-treatment model. Table 1 showed that XOs did not affect the cell viability of RAW 264.7 cells at the used concentrations (0.1–100 μ g/mL) to inhibit cytokines and NO production. Thus, the inhibitory effects were

not attributable to cytotoxic effects.

The inhibitory effect of XOs on TNF- α is stronger than the other mediators in the pre-treatment and the post-treatment models, which suggesting that XOs exerted the major effect via the inhibition of TNF- α production in the inflammatory cytokine network.

IL-10, an anti-inflammatory cytokine secreted predominantly by macrophages, inhibits the transcription of many pro-inflammatory cytokines, chemokines and inflammatory enzymes, and this appears to be mediated, at least in part, via an inhibitory effect on NF- κ B (Wang *et al.*, 1995). Anti-inflammatory cytokines (e.g. IL-10) tend to suppress the inflammatory responses (Marshall *et al.*, 1997). Although highly regarded as an immunosuppressive and anti-inflammatory cytokine, IL-10 possesses immuno-stimulatory properties, including the ability to activate T cell, B cells, NK cells, and mast cells (Mocellin *et al.*, 2004). The stimulation of immune-related cells may be related to the inflammatory process. In the present study, we found that XOs induced LPS-induced IL-10 production in a dose-dependent manner. Further investigation is necessary to determine whether XOs suppresses or stimulates inflammation via the activation of IL-10 production.

In conclusion, this research provides the first evidence that XOs induces TNF- α , IL-1 β , IL-6 and NO productions in un-stimulated macrophages and inhibits their production in a dose-dependent manner in LPS-stimulated macrophages. These effects may account for the immunomodulatory activities of XOs. Except for food ingredients or modulating the intestinal function, the consumption of XOs may be able to enhance innate immunity, and to protect against cardiovascular and chronic inflammatory diseases. Of note, XOs failed to inhibit the PGE₂ production in LPS-stimulated macrophages. The further researches are necessary to focus on the activation and inhibition mechanisms of XOs to reveal the immunomodulatory activities, and the characterization on gastrointestinal and systemic immunity.

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