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Chitosan nanoparticles reduce LPS-induced inflammatory reaction via inhibition of NF-κB pathway in Caco-2 cells

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ABSTRACT

Chitosan nanoparticles (CNP), an extensively oral-administered drug carrier, was investigated for the anti-inflammatory effects on LPS-inflamed Caco-2 cells and the relate mechanisms. CNP could alleviate the decrease of transepithelial electrical resistance (TEER) induced by LPS in Caco-2 monolayer, and significantly inhibit LPS-induced production of TNF-α, MIF, IL-8 and MCP-1 in a dose-dependent manner. PCR array assay revealed that CNP down-regulated the mRNA expression levels of TLR4 in LPS-inflamed Caco-2 cells. CNP was further showed to reduce cytoplasmic IκB-α degradation and nuclear NF-κB p65 levels in LPS-inflamed Caco-2 cells. These results suggested that CNP suppressed LPS-induced inflammatory response by decreasing permeability of intestinal epithelial monolayer and secretion of pro-inflammatory cytokine in Caco-2 cells, which were partially mediated by NF-κB signaling pathway.

1. Introduction

Chitin, the second most abundant polysaccharide in nature, is commonly found in lower organisms of fungi, crustaceans and insects, and is known to affect a variety of biological responses, especially the complex and size-dependent effects on immune response [1]. Chitosan is a relatively reactive derivative converted from chitin by deacetylation and can be produced in various forms. Over the past several decades, chitosan and its derivative have gained intensive attention due to the excellent biological properties related to well-documented biocompatibility, biodegradability, low toxicity, low immunogenicity and diverse biological activities such as antiviral activity, anticancer and anti-inflammatory activity [2–6]. However, the high molecular mass and poor solubility of these polysaccharides have hampered their applications especially as therapeutic agents. Recently, improved biodistribution, increased specificity and sensitivity are achieved by combining chitosan with nanostructured materials, making them perfect polymeric platforms for the development of new pharmacological and therapeutic drug delivery systems [7–11].

Accordingly, recent studies in our laboratory have focused on preparation and biological activities of various chitosan microsphere (chitosan nanoparticles and the nanoparticles loaded different metal ions) since they can form physically stable nanosuspensions and exhibit higher oral absorption due to their mini size and high zeta potential. Previous studies in our laboratory demonstrated chitosan nanoparticles (CNP), formed inexpensively by the ionotropic gelation of cationic chitosan with polyanion sodium tripolyphosphate, possess diverse biological activities, including anti-bacterial, anti-tumor, anti-angiogenesis and immunological adjuvant activity [12–15]. Recently, the anti-inflammatory effects of chitosan and its derivative have also been reported [16,17]. However, as one of most extensively oral-administered drug carrier, the effects of CNP on inflammation-associated intestinal damages have not been explored yet.

Inflammatory bowel disease (IBD) including Crohn's disease (CD) and ulcerative colitis (UC) is characterized by mucosal damage of the intestine due to chronic intestinal inflammation [18]. Lipopolysaccharide (LPS), a main component of the outer membrane of Gram-negative bacteria, is considered as one of the most potent stimuli of inflammation in gut and hypothesized to form an important risk factor of IBDs [19]. It is capable of activating several cell types including macrophages, lymphocytes, granulocytes, endotheliocyte, epithelium and smooth muscle cells [20]. As the ligand of toll-like receptor 4 (TLR4), LPS can initiate a cascade of

Abbreviations: CNP, chitosan nanoparticles; LPS, lipopolysaccharide; IBD, inflammatory bowel diseases; IEC, intestinal epithelial cell; TEER, transepithelial electrical resistance; TLR4, toll like receptor 4; NF- κB, nuclear factor-kappa B; IκB, inhibitor of NF-κB; TNF, tumor necrosis factor; MIF, macrophage migration inhibitory factor; MCP-1, monocyte chemoattractant protein-1.

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signal transduction by binding to the extracellular domain of TLR4, which enhances pro-inflammatory cytokine transcription through the NF-κB signaling pathway [21].

Intestinal epithelial cells (IECs) are considered as essential components of the intestinal mucosal barrier preventing the entry of pathogens and taking actively part in both innate and acquired immune responses through the secretion of a variety of inflammatory mediators, most of which have the potential effect to initiate and perpetuate inflammation [22]. Caco-2 cells are the most commonly used *in vitro* model for studies of structural and functional properties of human differentiated IECs [23,24]. The current experiments were designed to investigate the potential anti-inflammatory effects of CNP on inflamed Caco-2 cells induced by LPS, by determining the effects on the tight-junction (TJs) permeability, pro-inflammatory cytokines production, and explore its relate mechanisms by examining the TLR4 expression and activation of NF-κB pathway.

2. Materials and methods

2.1. Materials

Chitosan was obtained from the Chitosan Company of Pan'an, Zhejiang, China (degree of deacetylation, 95%; average molecular weight, 220 kDa). 3-(4,5-Dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and LPS (*Escherichia coli* 055:B5) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Monoclonal antibody against laminb and actin, and polyclonal antibodies against NF-κB p65 (C-20) and IκB-α (C-21) were acquired from Abcam (Cambridge, MA, USA). Iscove's modified Dulbecco's medium (IMDM), fetal bovine serum (FBS), nonessential amino and trypsin-EDTA were obtained from Invitrogen-Gibco (Grand Island, NY, USA). RNAiso Plus, the PrimeScript® RT reagent Kit and SYBR Premix Ex Taq II (2×) were bought from Takara Biotechnology (Dalian, China). Human cytokine ELISA kits were from Boster Biological Technology (Wuhan, China).

2.2. Preparation and characterization of chitosan nanoparticles (CNP)

Chitosan nanoparticles were prepared and characterized as described previously [25]. Briefly, chitosan was dissolved at 0.5% (w/v) with 1% (v/v) acetic acid (HOAc) and then raised to pH 4.6–4.8 with 10 N NaOH. CNP were formed by coacervation between negatively charged sodium tripolyphosphate (0.25%, w/v) and positively charged chitosan (0.5%, w/v) and purified by centrifugation at 9000 *g* for 30 min. Supernatants were discarded and nanoparticles were extensively rinsed with distilled water to remove any NaOH residues, and freeze-dried before further use or analysis. Particle size distribution and the zeta potential of CNP were determined using Zetasizer Nano-ZS90 (Malvern Instruments). The analysis was performed at a scattering angle of 90° at a temperature of 25 °C using samples diluted to different concentration with de-ionized distilled water. Atomic force microscopy (AFM, SPM-9500J3) was used for visualization of CNP deposited on silicon substrates operating in the contact mode. AFM imaging was performed using Si_3N_4 probes with a spring constant of 0.06 N/m.

CNP with a mean particle diameter of about 64.02 nm and a positive surface charge of about 30.5 mV, regularly formed and well distributed in HAC/sodium tripolyphosphate solution were used in this study (Fig. 1).

2.3. Caco-2 cell culture

Caco-2 cells were obtained from American Type Culture Collection (ATCC, MA, USA). The cells were maintained in the logarithmic phase of growth in IMDM supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml of streptomycin and 1% nonessential amino acids at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4. Cell viability assay

The effect of CNP on the viability of Caco-2 cells was determined by MTT method. Briefly, cells were seeded at 1×10^4 cells/well in a 96-well plate (Nunc). After incubation for 24 h, the cells were treated with different concentrations of CNP for 44 h. After that, MTT solution (2 mg/ml in serum-free medium, 50 μl/well) was added to each well, the cells were cultured for additional 4 h at 37 °C. Finally, the MTT medium was removed, 150 μl DMSO was added into each well, and then the optical density was measured at 570 nm with an automated micro-plate reader (Bio-Rad, CA, USA).

2.5. Lactate dehydrogenase (ldh) release

Inflammatory damage induced by 20 μg/ml LPS in the absence or presence of different concentrations of CNP (6, 12 and 24 μg/ml) for 24 h was assessed by LDH leakage into the culture medium. The activity of LDH in the medium was determined using a commercially available kit (Jiancheng Biochemical, Jiangsu, China). The LDH assay is based on the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD to NADH. The change in the absorbance was recorded at 340 nm using automatic biochemistry analyzer (Hitachi, Tokyo, Japan). LDH activity was expressed as IU/L of media.

2.6. Electrical resistance measurements

The tight-junction (TJs) permeability of the Caco-2 monolayer was evaluated by measuring transepithelial electrical resistance (TEER) which was monitored using the Millicell-ERS system (Millipore, Manassas, USA). The cut-off point of TEER was selected as 450 Ω/cm^2 indicating development of functional polarity and an intact monolayer, which is referred to as integrity of intestinal barrier. TEER was measured before the addition of LPS with or without CNP (12 μg/ml) (time zero) and then at various time intervals (5 min, 3 h, 9 h and 24 h) and expressed as the ratio of TEER at time in relation to the initial value (at time zero).

2.7. Analysis of cytokine production

Cells were treated with LPS (20 μg/ml) in the presence or absence of CNP for 24 h. Then the TNF-α, MIF, IL-8 and MCP-1 levels in the cell supernatants were analyzed with ELISA kits according to the manufacturer's instructions.

2.8. Real-time RT-PCR analysis

After treated with LPS (20 μg/ml) in the presence or absence of CNP for 6 h, cells were harvested and total RNA isolation and cDNA synthesis were conducted according to the manufacturer's instructions. All of the cDNA samples were stored at −20 °C until use. The real-time quantitative PCR mixture system was set up as follows: 12.5 μL of SYBR Premix Ex Taq II (2×), 1 μL of a forward primer (10 μM), 1 μL of a reverse primer (10 μM), 1 μL of cDNA, and 9.5 μL of double-distilled water. The reaction protocol was as follows: 95 °C for 3 min; 40 cycles at 95 °C for 15 s, 60 °C for 25 s. All of the samples were run in triplicate, and the average cycle threshold values were calculated using the $2^{-\triangle\triangle Ct}$ method as previously described [26].

The primers used for amplification were as follows: TLR4 (NCBI reference sequence NM_138554.4) (forward) 5′-CTGGAAATATGACCACAGTCAGAA-3′