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Inhibition of inflammatory injure by polysaccharides from *Bupleurum chinense* through antagonizing P-selectin

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ABSTRACT

P-selectin-mediated adhesion between endothelium and neutrophils is a crucial process leading to acute inflammatory injure. Thus, P-selectin has been considered as promising target for therapeutics of acute inflammatory-related diseases. In the present study, the water-soluble polysaccharides (BCPs) were isolated from *Bupleurum chinense*, and we evaluated their therapeutical effects on acute inflammatory injure and antagonistic function against P-selectin-mediated neutrophil adhesion. Our results showed that BCPs significantly impaired the leukocyte infiltration and relieve lung injury in LPS-induced acute pneumonia model. BCPs significantly blocked the binding of P-selectin to neutrophils and inhibited P-selectin-mediated neutrophils rolling along CHO-P cell monolayer. The result from *in vitro* protein binding assay showed a direct evidence indicating that BCPs-treatment significantly eliminated the interaction between rhP-Fc and its physiological ligand PSGL-1 at protein level. Together, these results provide a novel therapeutical strategy for amelioration of inflammation-related disease processes by polysaccharides from *B. chinense*.

1. Introduction

Most bacteria are detected and destroyed within hours of entering the host by the defense mechanism of acute inflammation known as innate immunity (Nathan, 2006). Polymorphonuclear neutrophils (PMNs) are the predominant cell type involved in the acute inflammation after infection. Since excessive inflammation is harmful to normal tissues, once the pathogen has been removed the acute inflammation response is terminated (Yoshikai, 2001). However, under some special pathological circumstances, the improper recruitment and activation of neutrophils during acute inflammatory response contribute to organ dysfunction, serious tissue injury, and inflammation-related diseases, and a poorly controlled acute inflammatory response even leads to death (Grommes & Soehnlein, 2011; Segel, Halterman, & Lichtman, 2011; Tanya, George, & Naotake, 2009). Thus, the restriction of neutrophil recruitment into tissue compartments has been considered as an effective strategy in amelioration of the acute inflammatory response-related injure process (Ulbrich, Eriksson, & Lindbom, 2003).

The recruitment of neutrophils from blood to infected tissue is an important step of acute inflammation response, which is a dynamic and coordinated multi-step process regulated by a variety of adhesion molecules (Kelly, Hwang, & Kubes, 2007; Kubes & Ward, 2006; Simon & Green, 2005). P-selectin on activated endothelial cells, as a member of the selectin family of cell adhesion molecule, plays a key role in neutrophil recruitment during acute inflammatory response, and its main function is to regulate the initial attachment of neutrophil with the vessel endothelial cells (Geng, Chen, & Chou, 2004; Somers, Tang, Shaw, & Camphausen, 2000). Therefore, P-selectin has been considered as a promising therapeutic target for interfering and controlling acute inflammation-related pathological processes, and researchers focus on searching for high affinity glycoconjugate ligands from natural products for antagonists of P-selectin-mediated neutrophil initial recruitment (Barthel, Gavino, Descheny, & Dimitroff, 2007; Hackert, Büchler, & Werner, 2010; Woollard & Chin-Dusting, 2010). Among various natural carbohydrate compounds, polysaccharide extracted from medicinal herbs might prove to be one of the promising candidates in searching for high affinity natural products with antagonistic function against P-selectin (Fei et al., 2008; Tong et al., 2011).

In our previous study, we reported the physicochemical properties and structural characterization of the water-soluble polysaccharide from *B. chinense*, which is a heteropolysaccharide

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mainly composed of arabinose; galactose; glucose with a molar ratio of 2.1:2.5:1 (Sun et al., 2010). According to FT-IR, partial acid hydrolysis, periodate oxidation and Smith degradation, methylation and GC–MS analysis, we found this polysaccharide had a backbone of (1→5)-linked arabinose, (1→4)-linked galactose and (1→3)-linked galactose residues with occasionally branches at O-6, and the branches were composed of (1→4)-linked glucose, and terminated with galactose residues. In the present study, we further evaluated the effects of water-soluble polysaccharides from *B. chinense* on acute inflammation and antagonistic activity against P-selectin-mediated function by *in vivo* and *in vitro* model. Here, we identify a novel anti-P-selectin-mediated cell adhesion agent, and the previous finding and our present results provide theoretical support for those polysaccharides as anti-inflammatory drugs.

2. Materials and methods

2.1. Materials and chemicals

The water-soluble *B. chinense* polysaccharides (BCPs) were isolated and characterized as previously described (Tong et al., 2013). Recombinant human P-selectin/Fc chimera protein (rhP-Fc) and blocking mAb to P-selectin (9E1) were obtained from R&D Systems (Minneapolis, MN, USA). The monoclonal antibodies for P-selectin (P8G6) and PSGL-1 (PL1) were obtained from Santa Cruz Biotechnology. A non-blocking mAb to P-selectin (AC1.2) was purchased from BD PharMingen (Franklin Lakes, New Jersey, USA). Goat anti-human fluorescein-isothiocyanate (FITC)-labeled immunoglobulin G (IgG) and goat anti-mouse IgG were purchased from Jackson Immuno-Research Laboratories (West Grove, Pennsylvania, USA). The negative isotype controls, depending on the species and subclasses of the primary antibodies used, were obtained from Santa Cruz Biotechnology. Calcein acetoxymethylester (Calcein-AM) was purchased from Invitrogen. All other chemical reagents used were analytical grade.

CHO cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Science (Shanghai, China). CHO-P cells stably expressing human P-selectin were obtained by transfecting full-length human P-selectin vector into CHO cells. All cells were grown in IMDM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. Neutrophil isolation

Human neutrophils were freshly isolated from whole blood from healthy human volunteers using the density gradient technique (Filippi, Szczur, Harris, & Berclaz, 2007; Nauseef, 2007). Remaining erythrocytes were removed by hypotonic lysis. More that 95% of the cells isolated were neutrophils as assessed by Wright-Giemsa staining, and the viability was determined to be >95% by trypan blue exclusion test. The isolated neutrophils were kept on ice and used within 4 h.

2.3. LPS-induced acute pneumonia model and HE staining

Male BALB/c mice (18–22 g) were purchased from Animal Experimental Center of Jilin University. The mice were housed in plastic cages and kept under standardized conditions at a temperature of 22–24 °C, and 20% humidity with a 12 h light/dark cycle, and free access to tap water and food. They were allowed to acclimatize for 3 days before the experiments started. Mice were intravenously administrated saline solution with or without BCPs (50 and 200 mg/kg). 30 min after BCPs administration, LPS was administered intranasal for pulmonary delivery. The animals were suspended vertically. LPS (50 μg in 50 μl saline) was instilled into the nares. The mice were maintained in vertical orientation for 5 min to allow full penetration of the LPS into the lungs. 4 h after LPS treatment, the mice were anesthetized with isoflurane and the lungs were removed for further histologic analysis. Normal control mice were produced with the treatment of saline.

For HE staining, lung tissues were perfused with a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.0), and incubated overnight at 4 °C. After washing three times, the tissues were processed in preparation for paraffin embedding and cut into 5-μm-thick sections. Sections were air-dried on gelatin-coated slides, immersed in hematoxylin for 5 min and checked for complete staining in tap water. Eosin staining was performed for 3 min. Sections were dehydrated through a graded series of alcohols (70–100% ethanol, 3 min each), cleared in xylene and mounted on coverslips. The stained sections were photographed using a Nikon microscope. Five sections from each sample were evaluated.

2.4. Flow cytometry

For the cell surface P-selectin binding assay, neutrophils were washed twice with PBS, then incubated with 2 μg/ml recombinant human P-selectin/Fc chimera protein (rhP-Fc) or human IgG at 4 °C for 30 min. After washing, the cells were suspended in 100 μl PBS containing FITC-labeled goat anti-human IgG (2 μg/ml) and incubated at 4 °C for 30 min. Cells were washed twice and 10 000 cells were counted by flow cytometric analysis with a Beckman FACScan. For inhibition assay of P-selectin binding, rhP-Fc was pre-incubated with mAb 9E1 (positive control) or AC 1.2 (negative control), or various concentrations of BCPs at 37 °C for 30 min.

2.5. Static adhesion assay

CHO-P or CHO cells were seeded in 24-well plates overnight to form monolayers. For inhibition experiments, CHO-P monolayers were pre-incubated with mAb 9E1 or AC 1.2, or various concentrations of BCPs at 37 °C for 30 min. Neutrophils were fluorescently labeled with 5 μM Calcein-AM for 30 min at 37 °C in IMDM. The fluorescently labeled neutrophils were added to CHO-P or CHO monolayers at room temperature for 1 h. After gently washing with PBS, fluorescence was measured by a Molecule Devicer CytoFluor II plate reader using 485 nm excitation and 530 nm emission filters.

2.6. Parallel-plate flow chamber assay

Dynamic interaction between neutrophils and CHO-P cell monolayers were analyzed in a parallel plate flow chamber as described (Tong et al., 2011). CHO and CHO-P cells were seeded in 35 mm culture dishes, respectively, and incubated overnight to form cell monolayers. Blocking mAb (9E1), non-blocking mAb (AC1.2) and BCPs were added onto the cell monolayers, respectively, then incubated at 37 °C for 30 min. The culture dishes were assembled in a parallel-plate flow chamber (GlycoTech, Rochville, MD, USA) and mounted onto an inverted microscope. After washing with PBS, 2×10^6 cells/ml neutrophils were perfused through the flow chamber at the shear stress of 1.2 dyn/cm^2 driven by a syringe pump. The observation fields were randomly selected and recorded for 3 min *via* a CCD-camera (Panasonic, Yokohama, Japan). The number of neutrophils rolling on CHO or CHO-P cells and the relative rolling speed were calculated by NIH ImageJ software from three independent experiments.

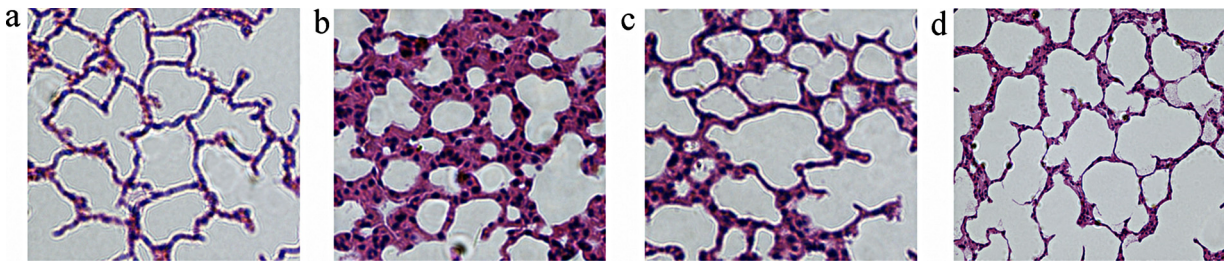

Fig. 1. Effects of BCPs on LPS-induced lung histopathogic changes. (a) Control mice; (b) LPS induced acute pneumonia model mice; (c and d) acute pneumonia model mice administered with 50 or 200 mg/kg BCPs, respectively.

2.7. In vitro protein binding assay between P-selectin and its native ligands PSGL-1

Recombinant human P-selectin/Fc chimera protein (rhP-Fc, 2 μg) was incubated with 20 μl of Protein G-Sepharose beads (50% slurry) for 1 h at 4 °C, and then the beads were washed three times. Neutrophils (1×10^7 per sample) were lysed in lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM NaF, 1 mM Na_3VO_4, 1 mM β-glycerophosphate, 20 μg/ml aprotin/leupeptin, and 1 mM PMSF) on ice for 15 min. After centrifuged at 15000 rpm for 25 min, the supernatants of cell lysates were incubated with 20 μl Protein G-Sepharose beads coated with rhP-Fc for 2 h. The beads were collected by centrifugation and washed four times with ice-cold lysis buffer. After boiling in Laemmli sample buffer, the binding proteins, PSGL-1, were detected by immunoblotting. Chemiluminescent detection was performed by using ECL Plus Western blotting reagents according to the manufacturer's protocol (Amersham Pharmacia Biotech). Quantification was performed using the ImageJ software.

2.8. Statistical analysis

Data shown represent mean ± SEM. Statistical significance was determined by Student's *t*-test. Statistical analysis was performed using SPSS version 13.0. $p < 0.05$ was considered statistically significant.

3. Results

3.1. BCPs impaired the in vivo leukocyte infiltration

Severe systemic acute inflammation can be induced and perpetuated by diverse insults, such as the administration of toxic bacterial products (*e.g.*, lipopolysaccharide, LPS) (Chow et al., 2005). In the present study, we first probe whether BCPs, the water-soluble polysaccharide from *B. chinense*, inhibited *in vivo* leukocyte infiltration in LPS-induced acute pneumonia model. As shown in Fig. 1, the HE staining of the lung sections from LPS-induced acute pneumonia mice showed interstitial thickening, alveolar hemorrhage, and marked augmentation of leukocyte infiltration in both interstitial and alveolar compartments, as compared to saline-treated normal control mice. BCPs pretreatment significantly decreased all of these markers of lung injury with a concentration-dependent manner.

3.2. BCPs inhibit the interaction between P-selectin and endogenous ligands

Flow cytometry was performed to evaluate the inhibitory effect on P-selectin binding to endogenous ligands on neutrophils. As shown in Fig. 2, the binding specificity was confirmed by using P-selectin antibodies, 9E1 (P-selectin blocking mAb) significantly

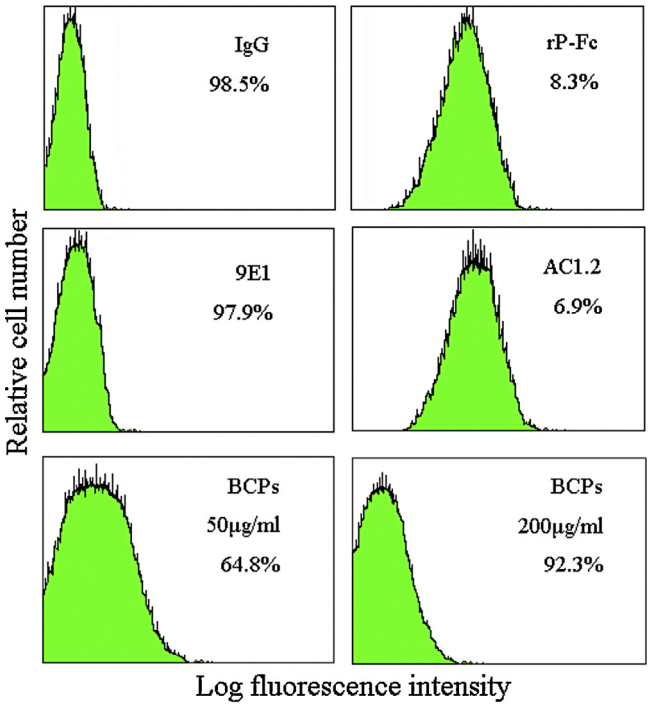

Fig. 2. Inhibitory effects of BCPs on P-selectin-mediated-binding to neutrophils evaluated by flow cytometry. The inhibition values of P-selectin binding to neutrophils were indicated in each histogram. Data were representative of three independent experiments.

reduced the binding of P-selectin to neutrophils, while AC1.2 (non-blocking P-selectin mAb) did not impact the binding. Compared with the positive control group, the BCPs-treatment significantly reduced the percentage of neutrophils binding to P-selectin by 64.8% and 92.3%, respectively, at the concentrations of 50 and 200 μg/ml.

3.3. BCPs inhibit P-selectin-mediated adhesion under static conditions

BCPs were tested for their ability in inhibiting the adhesion of neutrophils to CHO-P cells (CHO cells stably transfected with P-selectin) under static condition. CHO-P or CHO cells were seeded in 24-well plates to form monolayers, and then pre-incubated with mAb 9E1, AC 1.2, or BCPs. Calcein-AM fluorescently labeled neutrophils were added onto CHO-P or CHO monolayers incubated for 1 h. After gentle washing, fluorescence intensity of each well was measured by Molecule Devicer CytoFluor II plate reader. As shown in Fig. 3, BCPs markedly blocked the adhesion between neutrophils and CHO-P cells at the concentration of 50 and 200 μg/ml, and the inhibitory rates reached 49.3% and 85.0%, respectively. However, P-selectin blocking mAb (9E1) showed a more marked inhibitory

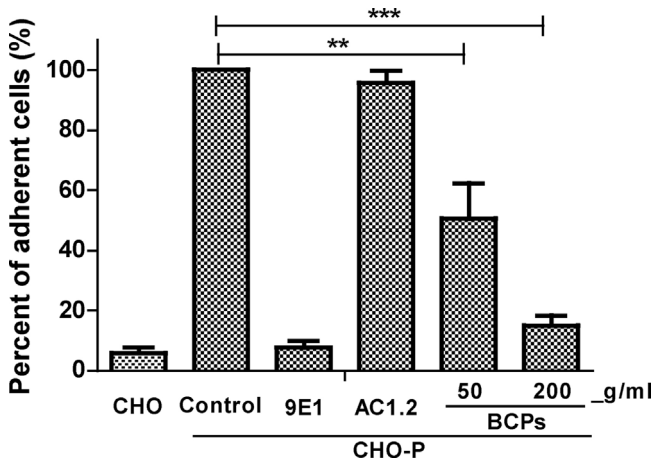

Fig. 3. BCPs inhibit the adhesion of neutrophils to CHO-P cells under static conditions. Values were calculated as percentage of the positive control. All results were representative of three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$.

effect, and the inhibitory rate reached 92.4%, significantly better than that of BCPs ($p < 0.05$).

3.4. P-selectin-mediated adhesion was blocked by BCPs under flow conditions

Next, we investigated the inhibitory capacity of BCPs in blocking P-selectin mediated neutrophils adhesion to CHO-P cells under dynamic condition by parallel plate flow chamber assay. The initial adhesion and rolling of neutrophils on CHO-P cells were observed and recorded under an inverted microscope with CCD-camera, when the neutrophils run through the surface of CHO-P monolayers. As shown in Fig. 4A, BCPs-treatment significantly reduced the percentage of neutrophil rolling on CHO-P cell monolayers compared with the positive control group by 51.7% and 90.0%, respectively, at the concentrations of 50 and 200 μg/ml. The inhibitory effect of BCPs at 200 μg/ml was similar to that treated with P-selectin blocking mAb 9E1 ($p > 0.05$). In addition, the relative rolling speed of neutrophil was calculated with NIH ImageJ software (Fig. 4B). Due to the inhibition of initial adhesion and rolling of neutrophils to CHO-P cell monolayers, the relative rolling speed of neutrophils treated with BCPs was significantly increased compared with positive control group.

3.5. BCPs directly blocked the interaction between P-selectin and PSGL-1

P-selectin glycoprotein ligand-1 (PSGL-1), a heavily glycosylated sialomucin specifically expressed on leukocytes, is a vital and high affinity ligand for P-selectin mediated initial adhesion and rolling of leukocyte on the endothelium (Bernimoulin et al., 2003; Carlow et al., 2009; Zarbock, Ley, McEver, & Hidalgo, 2011). In order to provide the direct evidence which BCPs block the binding of P-selectin and its physiological ligand PSGL-1, an *in vitro* protein binding assay was carried out to evaluate the blocking effects. The Protein G-Sepharose beads were coated with rhP-Fc. After treated with or without BCPs, the coated beads were incubated with lysates of neutrophils. Then the binding protein PSGL-1 was detected by immunoblotting. As shown in Fig. 5, rhP-Fc showed a high affinity to PSGL-1, however, BCPs-treatment significantly eliminated the interaction between rhP-Fc and PSGL-1, the blocking effects reached 75% and 85%, respectively, at the concentrations of 50 and 200 μg/ml. Those results suggest BCPs inhibit P-selectin-mediated neutrophil adhesion through directly blocking the interaction between P-selectin and PSGL-1.

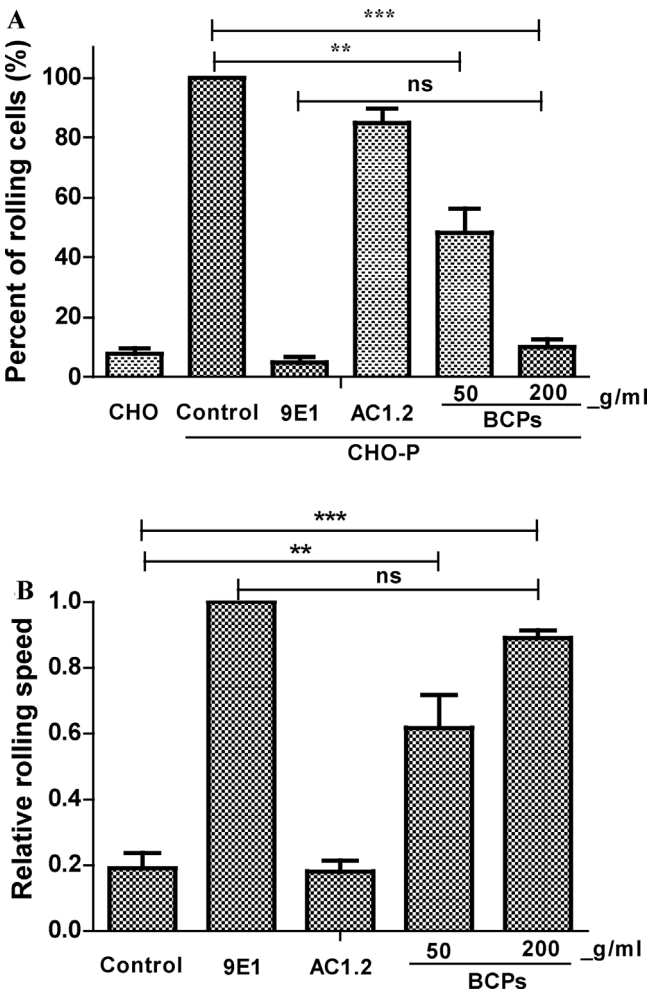

Fig. 4. BCPs inhibit the interaction between neutrophils and CHO-P cells evaluated by parallel plate flow chamber. (A) The relative percent of rolling neutrophils on CHO-P cell monolayers. (B) The relative rolling speed of neutrophil on CHO-P cell monolayers. All results are representative of three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$; ns, non-significant.

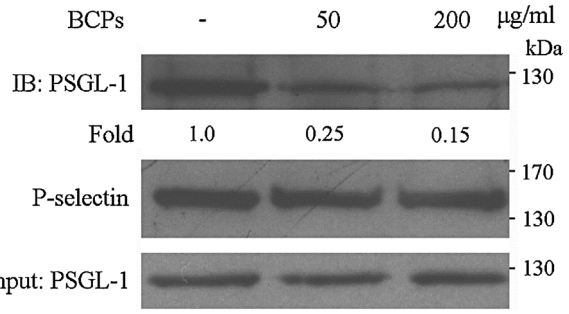

Fig. 5. BCPs inhibit the *in vitro* protein binding between P-selectin and its native ligands PSGL-1. Data were representative of three independent experiments.

4. Discussion

The rapid recruitment of neutrophils from the peripheral blood into infected tissues is essential for the development of acute inflammatory response. However, under certain pathological conditions, the excessive and improper recruitment of neutrophils often leads to serious tissue damages (Geng, 2001). Therefore, the recruitment of neutrophils into tissue compartments should be

tightly controlled, and that has been considered as an effective strategy in relieving those disease processes (Tong et al., 2013).

Polysaccharides have been proved to be promising candidates with significant anti-inflammatory effect (Ramberg, Nelson, & Sinnott, 2010). Some natural polysaccharides downregulate the expression of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α and INF-γ, whereas the expression of anti-inflammatory cytokine, such as IL-10 and MIP-1β, was markedly upgraded, suggesting that the anti-inflammatory potential of polysaccharides might be *via* modulating pro-/anti-inflammatory cytokine secretion profiles (Liu & Lin, 2012; Ostergaard et al., 2000). Sulfated polysaccharide fraction from *Ecklonia cava* significantly inhibited the production of NO and prostaglandin-E2 (PGE2), and suppressed inducible iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells (Kang et al., 2011). Polysaccharides from *Padina tetrastromatica* significantly reduced the activity of lipoxygenases (LOX) and cyclooxygenase (COX), and inhibited the nitric oxide synthase (iNOS) and COX-2 expression in peripheral blood mononuclear cells (PBMC), whereas the concentration of serum ceruloplasmin and myeloperoxidase (MPO) was markedly increased (Mohsin & Kurup, 2011). Therefore, natural polysaccharides affect multiple targets in the inflammatory progression. Recently, one of the interests in natural polysaccharides deemed as anti-inflammatory agents is more and more evidence illustrating their ability to interfere with the migration of leukocytes to inflammatory sites (Jiao, Yu, Zhang, & Ewart, 2011). As an important cell adhesion molecule, P-selectin is involved in neutrophil initial attachment and rolling on activated vascular endothelial cells, and it plays a critical role in the development of acute inflammatory diseases. Therefore, intervention in P-selectin-mediated adhesion is an attractive therapeutic strategy for acute inflammation (Woollard & Chin-Dusting, 2010). The binding of P-selectin to its ligands on leukocyte could be blocked by several natural carbohydrates, for example, PPS-2 (a polysaccharide fraction from *Physalis alkekengi*), p-PGBL (purified polysaccharides from *Ginkgo biloba* leaves), and fucoidan (sulfated polysaccharide found mainly in various species of brown algae and brown seaweed), they effectively reduce leukocyte recruitment to peritoneum in a model of acute peritoneal inflammation (Fei et al., 2008; Preobrazhenskaya et al., 1997; Tong et al., 2011), and the authors presumed that these anti-inflammatory activity were ascribed to the polysaccharides that mimic the selectin ligands to block the interaction of P-selectin and its native ligands.

In the present study, we found BCPs, the polysaccharide from *B. chinense*, inhibited *in vivo* leukocyte infiltration and relieve lung injury in LPS-induced acute pneumonia model. We further evaluated the inhibitory capacity of BCPs in P-selectin-mediated neutrophil adhesion by flow cytometry, static adhesion assay and laminar flow assay. These findings from flow cytometry and static adhesion assay indicated that BCPs exhibited significant and dose-dependent blocking capacity on the interaction between P-selectin and its native ligands under static conditions. In addition, the results from laminar flow assay suggest that the interruption of the P-selectin-mediated interaction between neutrophils and CHO-P cells by BCPs may suffice to dissociate neutrophil from CHO-P monolayers, and BCPs can act as an effective P-selectin antagonist, which markedly inhibited P-selectin-mediated neutrophil rolling and adhesion under physiological condition. Furthermore, we established an *in vitro* protein binding assay based on ameliorated method of co-immunoprecipitation, compared with previous findings, we obtained direct evidence that BCPs block the interaction of P-selectin and its native ligand PSGL-1 at the protein level. Given *in vivo* and *in vitro* data, it showed that BCPs impacted the recruitment of neutrophil during acute inflammatory response through antagonism of P-selectin-mediated neutrophil adhesion.

Recently, more and more evidences show that BCPs are the key factors in *B. chinense* for anti-inflammatory activity. Xie et al. (2012) found BCPs could attenuate LPS-induced acute lung injury (ALI) in mice, and the effect of BCPs against ALI might be related with inhibitory effect on excessive activation of complement and on the production of pro-inflammatory mediators, such as MOP, TNF-α, and NO. In addition, our previous study found that BCPs impact the recruitment and migration of neutrophils *via* blocking fMLP chemoattractant receptor-mediated functions, including regulation of affinity and avidity of β2 integrin, actin polymerization, and Vav1 and Rac1 activation (Tong et al., 2013). Taken together, those results indicate BCPs posses anti-inflammatory effects with distinct mechanism, they block the inflammatory response during different stage.

Although NSAIDs (Nonsteroidal anti-inflammatory drugs) have been widely used for treating inflammation diseases, they exhibited strong side-effects (Varas-Lorenzo et al., 2011). As natural, non-toxic substance, BCPs have been demonstrated their effects in inhibiting neutrophil transmigration and inflammatory response. More importantly, BCPs could be considered as promising candidate for amelioration of the acute inflammation-related disease with higher efficiency and lower side-effects.

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