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Effects of chitosan oligosaccharides on neutrophils from glycogen-induced peritonitis mice model

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ABSTRACT

To investigate the effects of chitosan oligosaccharides (COS) on the neutrophils from glycogen-induced peritonitis mice model, the production of superoxide and hydrogen peroxide, myeloperoxidase (MPO) release as well as apoptosis were measured. We found that 100 μg/ml COS supplementation could induce the production of superoxide and hydrogen peroxide by neutrophils, meanwhile, COS promoted the apoptosis of peritoneal neutrophils, whereas the MPO release was decreased. Furthermore, superoxide dismutase (SOD) administration could abolish the pro-apoptotic effects mediated by COS. These results demonstrated that COS exerted pro-apoptotic effects on neutrophils, and superoxide played an important role in neutrophil apoptosis caused by COS. By the use of inhibitors of PLD and PI3K respectively, administration of 1-butanol and wortamannin decreased the generation of superoxide stimulated by COS. The production of superoxide caused by COS resulted from the activation of PLD and PI3K to some extent.

1. Introduction

Recruitment and activation of neutrophils has been considered as one of the principal defense strategies of innate immunity, it represents a primary immunological response to invading pathogens and has also emerged as a hallmark of vascular inflammation. The inflammatory response usually accompanies with the recruitment of neutrophils from the blood stream to the venular endothelial cell surface and ultimately into the tissue interstitium. A massive and selective increase in PMN recruitment into the peritoneal cavity was observed 4 h after a single intraperitoneal oyster glycogen administration (Cockrell et al., 1999). It has been reported that the total number of leukocytes present in peritoneal lavage fluid increase remarkably, this is primarily reflected by an 8-folded increase in neutrophils (Ikeda et al., 2001). The oyster glycogen injection is a relatively modest stimulation, nevertheless, glycogen injection does induce the development of the inflammatory reactions. Neutrophils isolated from the lavage fluid are activated, based on cell morphological alterations and the reduced equivalents released into the extracellular space.

Neutrophils undergo spontaneous apoptosis, and it has been recognized as a crucial mechanism to eliminate the inflammation (Walker et al., 2005). However, during the peritonitis, the number of neutrophils within tissues is extremely high because of targeted influx from the circulation and because their constitutive apoptotic pathway is delayed with the involvement of local inflammatory mediators (Lee, Whyte, & Haslett, 1993). In addition, inflammatory neutrophils cause tissue damage via the release of inflammatory factors and production of granule enzymes such as myeloperoxidase (MPO) (Sendo et al., 1996), thus the elimination of these neutrophils becomes critically important to prevent tissue damage.

Previous studies demonstrated that suspension of chitin and chitosan particles (mean size of 1 μm) could attract canine neutrophils chemotactically (Usami et al., 1994). Moreover, the suspension of neutrophils incubated with chitin and chitosan contained higher concentrations of leukotriene B4 (LTB4) and prostaglandin E2 (PGE2) which could mediate canine PMN migration (Usami, Okamoto, Takayama, Shigemasa, & Minami, 1998). Chitin and chitosan promoted wound healing via production of osteopontin (Ueno et al., 2001). Chitosan administration stimulated the active oxygen generation in peritoneal exudate cells (Suzuki, Okawa, Hashimoto, Suzuki, & Suzuki, 1984). As the derivatives of chitosan, chitosan oligosaccharides (COS) have better biocompatibility and solubility (Zhang, Du, Yu, Mitsutomi, & Aiba, 1999). For these reasons, the biological activities of COS are of increasing interest in recent research. It has been reported that COS has anti-diabetic (Lee, Park, Choi, Yi, & Shin, 2003), antibacterial (Tsai, Wu, & Su, 2000), anti-fungal (Hadwiger, Ogawa, & Kuyama, 1994) and anti-tumor activities (HarishPrashanth & Tharanathan, 2005). Moreover, COS can protect liver damage induced by CCl4 (Yang, Liu, Han, Li, & Feng, 2006). In our previous study, the effects of COS on the rest neutrophils from rabbit peripheral blood were examined, we found that COS promoted O^{2-} generation in rest neutrophils and

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down-regulated the release of MPO in PMA-activated neutrophils significantly (Dou, Du, Bai, Wang, & Ma, 2007). However, the effects of COS on inflammatory neutrophils have not yet been investigated. In this study, we took the typical glycogen-induced peritonitis mice model in which we attempted to study the influences of COS on mice peritoneal exudate neutrophils.

2. Materials and methods

2.1. Materials

1-Butanol, catalase, chelerythrine, cytochlasm B, cytochrome *c*, dihydrorhodamine 123 (DHR123), oyster glycogen, propidium iodide (PI), RPMI1640, superoxide dismutase (SOD), tetramethy ibenzidine (TMB) and wortmannin were purchased from Sigma; Dimethyl Sulfoxide (DMSO) and Nitro Blue Tetrazolium Chloride (NBT) were purchased from Amresco, fetal bovine serum (FBS) from Gibco, Percoll was purchased from Phamacia, chitosan oligosaccharides (COS) are the products of our lab, polymerization: 2–10.

2.2. Animal model

Balb/c mice were injected intraperitoneally 3 ml of 1% glycogen sterile solution, 4 h later 1 ml of PBS was injected to dilute the neutrophils in the peritonea then the mice were decaptioned and the lavage fluids were collected (Hidemura et al., 2003).

2.3. Isolation of peritoneal neutrophils

The lavage fluids were double diluted with PBS, then centrifuged for 20 min at 4 °C (400 g), erythrocytes were removed by hypotonic lysis. The cell suspension was purified by percoll (1.089 g/L) gradient. The purity of neutrophils was greater than 95% as determined by Wright–Gimesa staining. Neutrophils were suspended in RPMI1640 medium containing 10% fetal bovine serum, 200 U/ml penicillin and 100 μg/ml streptomycin.

2.4. Estimation of superoxide (O_2^-) and hydrogen peroxide (H_2O_2)

To assess the oxidative burst, production of O_2^- and H_2O_2 was estimated. NBT reduction assay and cytochrome *c* reduction test were used to detect the production of O^{2-}, DHR123-labeled flow cytometric analysis was used to determine the generation of H_2O_2. The O_2^- production of pre-treated neutrophils was measured by adding NBT (0.2%) (Liao, Lou, Ma, & Wu, 2005). After incubation for 30 min at 37 °C, cells were collected by centrifugation and DMSO was added to dissolve formazan producing in the 30-min incubation. Then colorimetric analysis was carried out at 570 nm to determine O_2^- production. In addition, the method of cytochrome *c* reduction was also used to confirm the generation of superoxide (Goldstein & Czapski, 1991). For H_2O_2 assessment, the cells were incubated with 1 μmol L^{-1} DHR123 as a H_2O_2 capture for 40 h (Ischiropoulos, Nelson, Duran, & Al-Mehdi, 1996). Blank control was set in which DHR123 was omitted. After incubation with DHR123, cells were harvested before an immediate detection of fluorescence intensity by flow cytometry FACS scan. Propidium iodide (PI) was used to distinguish dead cells.

2.5. Flow cytometric analysis to assess neutrophils apoptosis

Neutrophil apoptosis was measured by flow cytometry with the PI apoptosis assay kit (Pellicciari, Manfredi, Bottone, Schaack, & Barni, 1993). The experiment was performed by following the manufacturer's instructions with minor changes. Neutrophils were stained for 30 min with propidium iodide (PI: 50 μg/ml containing RNase type A and detergent), and analyzed by flow cytometry. Apoptotic neutrophils typically showed sub-G1 DNA contents. Compared to non-apoptotic neutrophils, sub-G1 cells had lower values of low-angle (FSC) scatter and higher values of right-angle (SSC) scatter, in dual parameter cytograms of FSC versus SSC, two distinct cell populations were apparent, and were separated by flow sorting.

2.6. Assessment of MPO release

Exocytosis of MPO was observed in triple as index of neutrophil degranulation (Suzuki, Ota, Sasagawa, Sakatani, & Fujikura, 1983). Pre-treated neutrophils were suspended in D-Hank's balance salt solution, supernatant was collected after 30-min incubation at 37 °C to measure MPO activity with 2.5 mM fresh TMB as the substrates. Colorimetric analysis was carried out at 450 nm. Neutrophils were lysed with 0.2% CTAB to determine total MPO activity.

2.7. Statistical analysis

Data are expressed as means ± SD. Differences between data sets were evaluated by performing an unpaired Student's *t*-test. A level of $p < .05$ was accepted as statistical significance.

2.8. Ethics

All mice were maintained in specific pathogen-free facility and were housed in microisolator cages containing sterilized feed, autoclaved bedding, and water. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

3. Results and discussion

3.1. Superoxide and hydrogen peroxide generation by neutrophils

As shown in Fig. 1, the generation of superoxide was determined by the reduction assays of NBT and horse heart ferricytochrome *c*. We found that administration of COS (100 μg/ml) increased the production of O_2^- significantly. To detect the H_2O_2 production after incubation for 40 h, we labeled the cells with DHR123, meanwhile, PI staining was used to distinguish the dead neutrophils from the live ones (Fig. 2). PI positive represented the dead cells, DHR123 positive indicated the cells generated H_2O_2. Incubation with 100 μg/ml COS for 40 h resulted in more DHR123 and PI double positive cells (from 40% to 65.69%) compared with control group. This suggested that COS stimulated the generation of H_2O_2 which thus led to a higher rate of cell death.

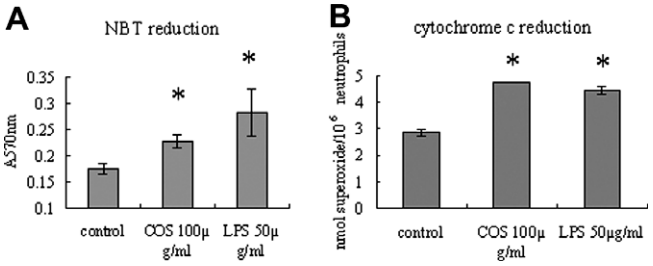

Fig. 1. The generation of O^{2-} was detected in neutrophils. LPS (50 μg/ml) was as positive control. (A) The generation of superoxide was determined by the method of NBT reduction. (B) The generation of superoxide was determined by the reduction of cytochrome *c*. Each data point represents mean ± SEM of three independent determinations from three experiments. $^*p < .05$.

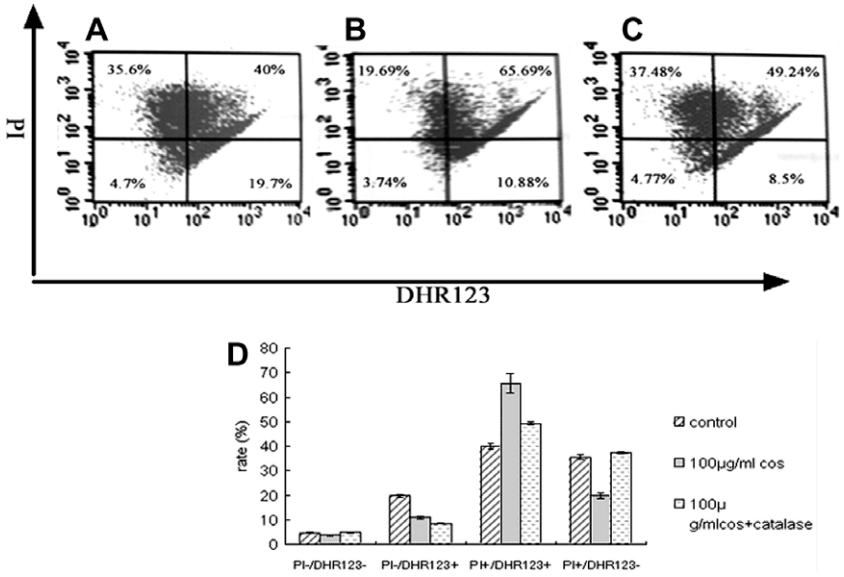

Fig. 2. The generation of H_2O_2 was determined by DHR123 using flow cytometry in neutrophils, PI was used to distinct the dead cells. (A) Control, (B) neutrophils were treated with 100 μg/ml COS for 40 h, (C) 20 μM catalase was co-added with COS to neutrophils. (D) The experiment was repeated twice.

Supplementation of 20 μM catalase in the COS-treated group decreased the percentage of DHR123 and PI double positive cells (from 65.69% to 49.24%); however, the percentage of total dead cells did not change (from 85.38% to 86.72%). These results demonstrated that though the production of H_2O_2 was diminished, there were some other mediators responsible for the death of neutrophils induced by COS.

3.2. Apoptosis of neutrophils from glycogen-induced peritonitis induced by COS

Apoptosis and necrosis were two well known patterns of cell death, so we further detected the effect of COS on the neutrophils apoptosis. The apoptosis was examined after 24 h and 40-h incubation using flow cytometry by PI staining, 100 μg/ml of COS supplementation promoted the apoptosis of peritoneal neutrophils obviously, the percentage of apoptosis increased along with time. After incubation with COS for 24 h, the percentage of neutrophils apoptosis was up-regulated to 40.90% from 24.89%, furthermore, the percentage increased from 29.33% to 59.43% after 40-h incubation, shown in Fig. 3.

3.3. PLD and PI3K were associated with the generation of O_2^-

As an inhibitor of the generation of phosphatidic acid (PA) by PLD pathway, 1.5% 1-butanol decreased the production of O_2^- stimulated by COS, this suggested that COS might stimulate the O_2^- production by the generation of PA through PLD activation. In addition, wortmannin, the inhibitor of PI3K, and chelerythrine, the inhibitor of PKC α, β were used to address the roles of PI3K and PKC in the production of O_2^- in neutrophils after COS incubation. When wortmannin (1 μM) was added to the medium, the O_2^- generation stimulated by COS was down-regulated, but the addition of chelerythrine (0.66 μM) showed no affect. These results suggested that PI3K rather than PKC α, β played an important role in O_2^- generation induced by COS (Fig. 4).

3.4. O_2^- play an essential role in neutrophils apoptosis induced by COS

It has been reported that reactive oxygen species can trigger cell apoptosis (Herrera et al., 2001). To characterize the role of O^{2-} in the apoptosis of neutrophils induced by COS, the superoxide scanvenger SOD was used. The release of O^{2-} occurred after incubation

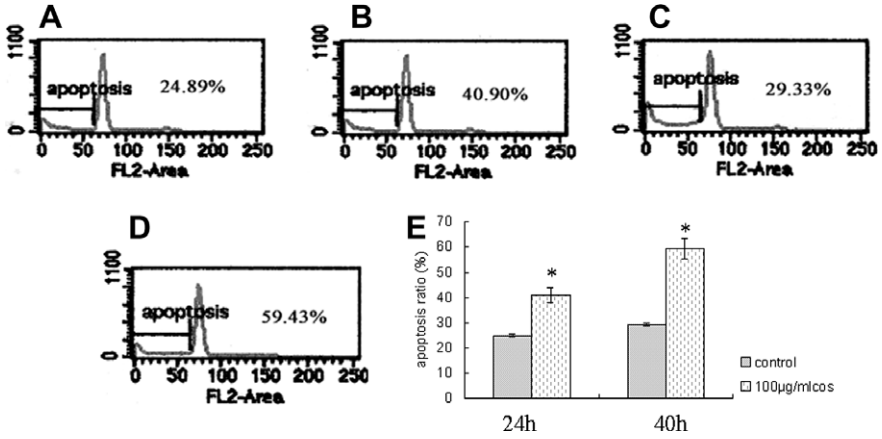

Fig. 3. Neutrophils apoptosis was determined by PI staining using flow cytometry. (A) Control 24 h, (B) neutrophils were treated with 100 μg/ml COS for 24 h, (C) control 40 h, (D) neutrophils were treated with 100 μg/ml COS for 40 h. (E) The experiment was repeated twice. $^*p < .05$

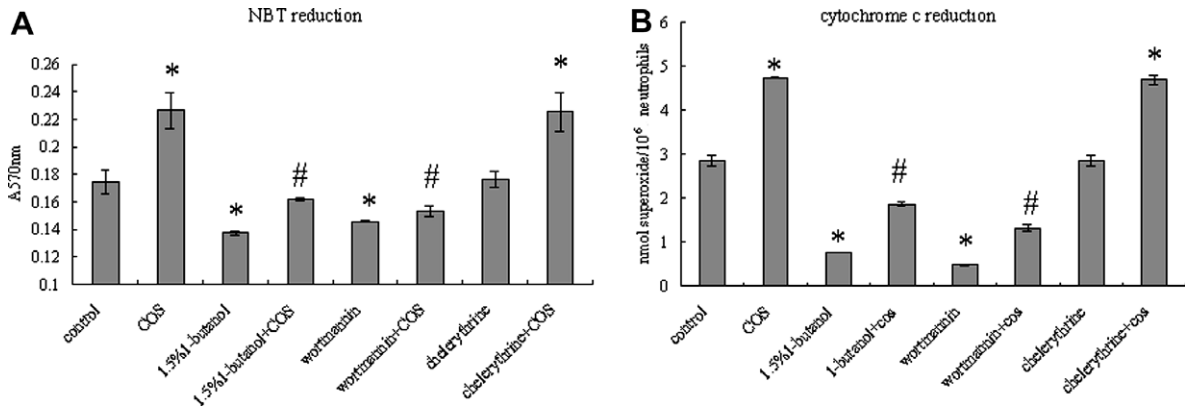

Fig. 4. The participation of PLD and PI3K was determined. (A) NBT reduction; (B) cytochrome *c* reduction. Each data point represents mean ± SEM of three independent determinations from three experiments. $^{*}p < .05$ versus control, $^{\#}p < .05$ versus COS-treated group.

with COS for 30 min, we chose 16 h as the time dot to assess the importance of O^{2-}. The administration of SOD (25 U/ml) diminished the pro-apoptotic effect of COS (Fig. 5), indicating that the neutrophil apoptosis induced by COS was caused by the generation of reactive oxidative stimuli.

3.5. Inhibition of PLD and PI3K did not decrease the neutrophil apoptosis

As mentioned above, PLD and PI3K were found to associate with the O_2^- production induced by COS. To assess the roles these two enzymes in neutrophil apoptosis, 1.5% 1-butanol and 1 μM wortmannin were used. Though these two inhibitors down-regulated superoxide generation, their supplementation did not reduce the neutrophil apoptosis, in contrast, the percentages of neutrophils apoptosis were increased to some extent, 1-butanol addition elevated the percentage of neutrophils apoptosis from 11.19% to 46.51%, administration of wortmannin improved the rate of apoptosis to 20.51% (Fig. 6).

3.6. Decrease of myeloperoxidase (MPO) release of neutrophils after incubation with COS

Co-incubation of neutrophils with 100 μg/ml of COS for 30 minutes attenuated the release of MPO significantly. 1-butanol also decreased the release of MPO, in accordance with previous studies' demonstrating activation of PLD is important to the release of MPO, however, 1-butanol did not further change the MPO release caused by COS. As we know, Ca^{2+} was required for the excytosis of MPO, so EDTA was used as a positive control. As shown in Fig. 7, the addition of EDTA lessened the release of MPO in control group, furthermore, EDTA further inhibited the MPO release induced by COS treatment.

4. Discussion

Neutrophils have been well documented to exhibit three different phenotypes: resting, primed and activated. In our previous studies, we observed that COS administration stimulated the superoxide generation in rest neutrophils from peripheral blood of rabbit. At the site of infection, neutrophils are on the stage of activation to phagocytose inflammation triggers. When recruited into tissues, neutrophils move from normoxic to hypoxic conditions, and hence decreased O_2 tensions at inflammatory sites may extend their lifespan. The prolonged survival of inflammatory neutrophils cause chronic tissue damage, therefore, apoptosis of these cells and the subsequent engulfment by phagocytes are important to the rapid elimination of inflammations. Oxidative metabolites are such critical mediators of the apoptosis in various types of cells. It has been suggested that reactive oxygen metabo-

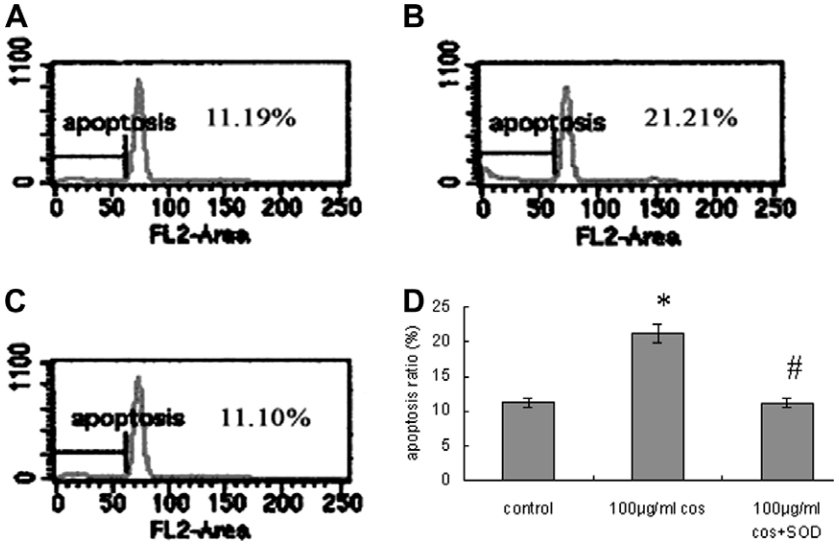

Fig. 5. Superoxide anion play an important role in the neutrophils apotosis induced by COS. (A) Control 16 h; (B) neutrophils were treated with 100 μg/ml COS for 16 h; (C) neutrophils were co-treated with 20 U/ml SOD and 100 μg/ml COS, 16 h; (D) the experiment was repeated twice. $^{*}p < .05$ versus control, $^{\#}p < .05$ versus COS-treated group.

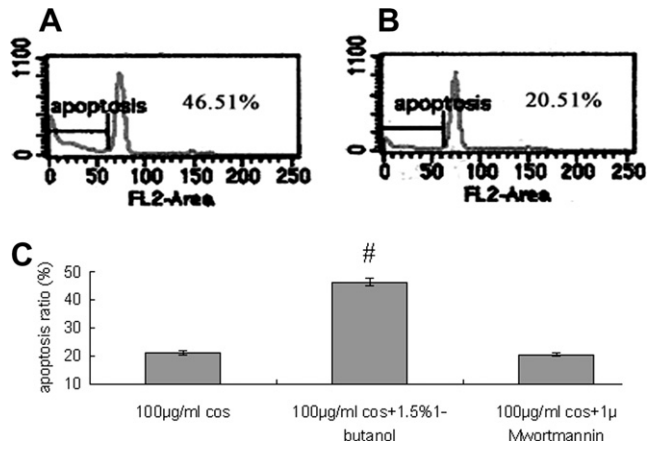

Fig. 6. Effects of PLD and PI3K in the neutrophils apoptosis induced by COS. (A) Neutrophils were co-treated with 1.5% 1-butanol and 100 μg/ml COS for 16 h, (B) neutrophils were co-treated with 1 μM wortmanin and 100 μg/ml COS for 16 h, (C) the experiment was repeated twice. $^{\#}p < .05$ versus COS-treated group.

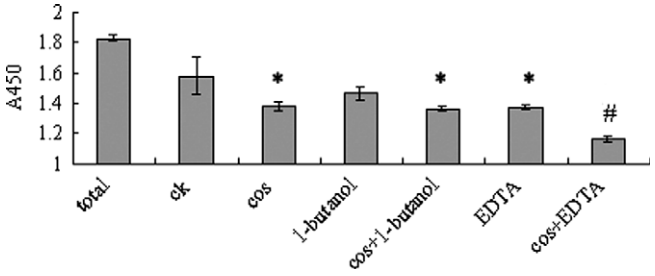

Fig. 7. Release of myeloperoxide was determined in neutrophils. Each data point represents mean ± SEM of three independent detections from three experiments. $^{*}p < .05$ versus control, $^{\#}p < .05$ versus COS-treated group.

lites including O_2^- and H_2O_2 accelerate the apoptosis of neutrophils (Yamamoto, Taniuchi, Tsuji, Hasui, & Kobayashi, 2002). So we proposed that the inflammatory neutrophils can be activated to secrete a large amount of reactive oxygen metabolites which then promote apoptosis of neutrophils and facilitate subsequent removal by other professional phagocytes. This may pose an important feedback loop for neutrophil life and death.

In the present study, we found 100 μg/ml of COS supplementation increased the generation of O_2^- and H_2O_2. Though the administration of COS increased the percentage of the H_2O_2-generating neutrophils and resulted in more dead cells, the H_2O_2 generation was not necessary to the cell death, total percentage of dead cells was not changed obviously after the catalase administration compared with COS-treated group, maybe this effect was accomplished by other oxygen mediators. Patterns of cell death have been traditionally divided into apoptosis and accidental necrosis. To further study the effects of COS on inflammatory neutrophils, their apoptosis was measured. COS promoted the neutrophil apoptosis significantly, however, the pro-apoptotic capacity diminished when SOD was added, suggesting that the pro-apoptotic role of COS in neutrophils depended on the production of O_2^-.

Neutrophils generate the O^{2-} directly from molecular oxygen through the NADPH oxidase enzyme. Activation of NADPH oxidase requires additional phosphorylation, mainly on the serine and threonine residues of p47phox, p67phox, and p40phox, via kinases such as p38MAPK, PKC, PI3K or PA-activated protein kinase, followed by translocation to the membrane. It has been suggested PLD plays an important role in the generation of reactive oxygen species. PA is generated by the PLD activation, and PA is known to trigger a number of cellular events (Bauldry, Bass, Cousart, & McCall, 1991). PA generation and superoxide release through NADPH oxidase in both cell-free systems and intact neutrophils under physiological stimulation are associated with PLD activity (English & Taylor, 1991). Specifically, PA appears to activate certain isoforms of PKC and NADPH oxidase directly (Tokumura, Moriyama, Minamino, Hayakawa, & Tsukatani, 1997). When PLD is stimulated in the presence of 1-butanol, transphosphatidylation results in the production of phosphatidylalcohol instead of PA. However, phosphatidylalcohol does not affect the generation of O^{2-} in neutrophils. In the present study, we found that supplementation of 1.5% 1-butanol abolished the release of O^{2-} induced by COS. This finding demonstrated that PA was required to the activation of NADPH oxidase caused by COS. PI3K has been well known for its roles in phagocytosis (Hannigan, Huang, & Wu, 2004) and superoxide generation stimulated by some chemicals in neutrophils (Hsu, Lee, Lee, & Lin, 2003). Wortmannin is a cell-permeable, potent, and selective inhibitor of PI3K and pleckstrin phosphorylation. The production of O^{2-} induced by COS could be blunted by administration of 1 μM wortmannin, which suggested the ability of COS induced O^{2-} release was associated with the activation of PI3K. Chelerythrine competes for the conserved catalytic sites of PKC and seems to be a potent inhibitor of the groups α and β. Though several kinds of stimuli activate the NADPH oxidase through PKC, supplementation of chelerythrine does not change the generation of O^{2-} caused by COS, demonstrating that the activation of NADPH by COS is PKC α, β independent.

In addition to stimulating the activation of NADPH oxidase, PLD and PI3K are shown to be linked with the apoptosis of neutrophils. The apoptotic rate of neutrophils was found to be increased by 1-butanol and decreased by the exogenous addition of PLD (Lee et al., 2004; Park, Lee, Lee, Jung, & Kwak, 2002). TLR agonists are demonstrated to delay apoptosis and extend the functional life span of neutrophils. This effect correlated with a PI3K-dependent phosphorylation of the heat shock protein 27, which has been reported to play a pivotal role in neutrophils survival (Francois et al., 2005). Though the production of superoxide depends on PLD and PI3K, the supplementation of inhibitors of these two enzymes does not down-regulate the apoptosis rate in neutrophils induced by COS, on the contrary, the percentages of apoptosis are slightly increased, this maybe due to the anti-apoptotic roles of PLD and PI3K play in neutrophils (Franke, Hornik, Segev, Shostak, & Sugimoto, 2003; Nozawa, 2002).

MPO is primarily hosted in neutrophils and is one of the most abundant proteins in these cells. Increased intracellular MPO is recognized as a hallmark of systemic inflammatory disease. The granules of neutrophils distribute randomly in cytoplasma at resting stage, excytosis of granules happens following the increase of Ca^{2+} concentration in cytoplasma when cells are activated. It was proposed that MPO evolved as an acceptor for the high concentrations of hydrogen peroxide and superoxide generated in the phagosome which needed to activate membrane immobilized proteases, the combination ultimately may be responsible for bacterial killing and tissue damage (But, Fomina, Murav'ev, & Rogovin, 2003). MPO-compound I, formed by the reaction between MPO and H_2O_2, generates the highly reactive species HOCl in the presence of chloride ion. HOCl is the major strong oxidant generated by neutrophils. On the other hand, MPO can also react with superoxide anion to form compound ||, the predominant form found in activated neutrophils (Lau et al., 2005). High myeloperoxidase content results in the subsequent production of HOCl, chloramines and singlet oxygen. Furthermore, it has been recently shown that MPO elicited pro-inflammatory, cell-activating properties which proved to be independent of the catalytical properties of this enzyme (Faurschou & Borregaard, 2003). COS addition decreased the release of MPO then the compound with reactive oxygen metabolites would

be reduced, hence the tissue injury was lessened. Moreover, the decrease of itself lessened the inflammatory responses. It had been reported that activation of PLD promoted the release of MPO, and in front study we had known PA was associated with the production of O^{2-} so we used 1-butanol to assess the role of PA on the decreased release of MPO induced by COS, the result demonstrated PA had not obvious impact on the effect of COS, however, the release of MPO was further reduced by the addition of EDTA, this is in accordance with excytosis of MPO needs high concentration of Ca^{2+}. The ability of chitosan and its derivatives to bind cations is well known. So we supposed that this ability had some relationship with the decrease of the MPO release caused by COS.

Our present study showed that COS displayed the pro-apoptotic capacity on the neutrophils from glycogen-induced peritonitis mice model, the COS-mediated superoxide generation led to the neutrophil apoptosis. PLD and PI3K were associated with the superoxide production, but inhibition of these two enzymes did not decrease the neutrophil apoptosis rate. One explanation may be that PLD and PI3K also achieve certain anti-apoptotic roles in neutrophils under physiological or pathological circumstances. Moreover, we found that COS administration could reduce the MPO release. Taken together, our results suggested that COS held great potential in easing the inflammation.

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