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Inhibitory Effect of Dibutyryl Chitin Ester on Nitric Oxide and Prostaglandin E_2 Production in LPS-stimulated RAW 264.7 Cells

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Inflammation is a highly complex process that protects against foreign challenge or tissue injury. The ester derivative dibutyryl chitin (DBC) reportedly accelerates wound healing and exerts an anti-inflammatory effect. However, little is known regarding the inhibitory effect of DBC in anti-inflammation. In this study, we investigated the effect of DBC on the inducible nitric oxide synthetase (iNOS) and cyclooxygenage-2 (COX-2) pathways and pro-inflammatory cytokine production in lipopolysaccharide (LPS)-treated RAW 264.7 macrophages. Our results demonstrate that DBC (MW 3,772) significantly inhibits overproduction of NO and PGE_2 as well as pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1β, in LPS-stimulated RAW 264.7 macrophages. Inhibition of NO and PGE_2 overproduction in LPS-stimulated RAW 264.7 macrophages by DBC was mediated through the down-regulation of iNOS and COX-2 expression. These results demonstrate that DBC efficiently inhibits inflammation and has potential as an effective anti-inflammatory and wound healing agent.

Key words: Dibutyl chitin ester, Nitric oxide, Prostaglandin E_2, Pro-inflammatory cytokines, Anti-inflammatory effect

INTRODUCTION

Chitin is the most widespread amino polysaccharide in nature and a major structural constituent of the exoskeleton of crustaceans and insects. Chitin is used in food, agriculture, textile, polymers, wastewater treatment, and pharmaceutical industries due to its specific physiochemical and biological properties (Austin et al., 1981; Muzzarelli et al., 2005; Muzzarelli, 2010). For use in pharmaceutical and biomedical materials, research has focused on the wound-healing effect of chitin and its application as an artificial skin substitute and sutures (Austin et al., 1981; Su et al., 1997). Additionally, chitin has antimicrobial activity and improves immune dysfunction (Seferian and Martinez, 2000). However, the low solubility of chitin in common solvents has restricted its technological application. An ester derivative, dibutyryl chitin (DBC), is a technologically friendly polymer (Blasinska and Drobnik, 2008). Good solubility of DBC in several organic solvents (ethanol, dimethyl sulfoxide (DMSO), acetone, etc.) results from the presence of bonding butyryl groups at positions C-3 and C-6. After subcutaneous implantation of DBC to rats, the inflammatory reaction was lower than that observed for chitin (Paluch et al., 2000). However, the anti-inhibitory mechanism remains unclear.

Nitric oxide (NO) is a highly reactive free radical and is an important second messenger in many cell types (Lowenstein and Snyder, 1992). Cyclooxygenase (COX) catalyzes the conversion of arachidonic acid to prostaglandin H2, a precursor of a variety of biologically active

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mediators such as prostaglandin E_2 (PGE_2), prostacyclin, and thromboxane A_2 (Smith et al., 1996). Under normal conditions, COX-2 and NO synthase (NOS) are undetectable or detectable only at low levels in most tissues. Large amounts of NO derived from NOS and PGE_2 derived from COX-2, which is induced by many pro-inflammatory mediators, including tumor necrosis factor-α (TNF-α) interleukin-1β (IL-1β), and lipopolysaccharide (LPS), have been implicated in the pathogenesis of inflammation (Hammond et al., 1999). However, this strong inflammatory response to foreign cells may also induce further damage to neighboring cells and tissues around the wound area, thus inhibiting the healing process (Bauer et al., 1998). Previous studies have reported that decreasing NO and PGE_2 production by adding NOS and COX-2 inhibitors may protect against some forms of injury (Mulligan et al., 1992; Mack Strong et al., 2001). It is unknown whether the anti-inflammatory effect of DBC is associated with the pathway of iNOS and COX-2.

In this study, we examined the effect of DBC on LPS-induced NO and PGE_2 production in murine RAW 264.7 macrophages. We found that DBC suppressed the production of NO, PGE_2, and pro-inflammatory cytokines in RAW 264.7 macrophages activated by LPS526. These results indicate that DBC can be used as a therapeutic agent for treating wound-related inflammation.

MATERIALS AND METHODS

Reagents

RPMI 1640, fetal bovine serum (FBS), and antibiotics were purchased from Gibco BRL. Antibodies against iNOS and COX-2 were obtained from Santa Cruz Biotechnology. ELISA kits for PGE_2, TNF-α, IL-1β, and IL-6 were obtained from R&D systems. LPS and other reagents were purchased from Sigma.

Chitin dibutyrate preparation

Chitin dibutyrate was prepared as previously described in the literature (Batt et al., 2011). Briefly, 37.0 mL of butyric acid was mixed with 56.0 mL of Trifluoroacetic anhydride (TFAA) followed by addition of 3.4 mL of 85% phosphoric acid; the samples were kept in ice. After mixing the solution, 10.0 g of chitin (TCI, MW: 3,772) was added to solution. The reaction mixture was stirred for 72 h below 5°C. It was then mixed with 300 mL of ethyl alcohol and filtered to collect the precipitate. The product was obtained by washing the precipitate several times with diethyl ether and water. The sample was dried in a hood for 3 days and then in an oven at 60°C for 6 h (Fig. 1).

Cell line and culture

RAW 264.7 cells, murine macrophages, were obtained from American Type Culture Collection (ATCC). Cells were cultured with RPMI 1640 medium containing 10% heat-inactivated FBS, penicillin G (100 IU/mL), and streptomycin (100 μg/mL) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

MTT assay

The effect of DBC against LPS-induced cytotoxicity was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (Sun et al., 2005). RAW 264.7 macrophages (1×10^6 cells/well) were seeded into 6-well plates in 1 mL of medium containing 10% FBS. After 24 h, the cells were washed and incubated with 1 mL RPMI 1640 medium containing LPS (1 μg/mL) in the presence of varying doses of the DBC or a control vehicle (pH 7.2 PBS containing 0.01% DMSO). After 48 h, MTT was added to each well and formazan crystals were solubilized using DMSO. Next, absorbance was measured at 570 nm using a microplate reader. All experiments were performed in triplicate.

Measurement of nitrite

NO production was quantified spectrophotometrically by measuring the accumulation of nitrite in the culture media using the Griess reagents and sodium nitrite as a standard (Ignarro et al., 1993). For nitrite assays, cells were subcultured into 96-well tissue culture plates at 1×10^5 cells/well and incubated for 24 h. Cells were treated with LPS (1 μg/mL) in the presence or absence of varying doses of the DBC or the control vehicle and cultured for an additional 24 h. Nitrite concentration

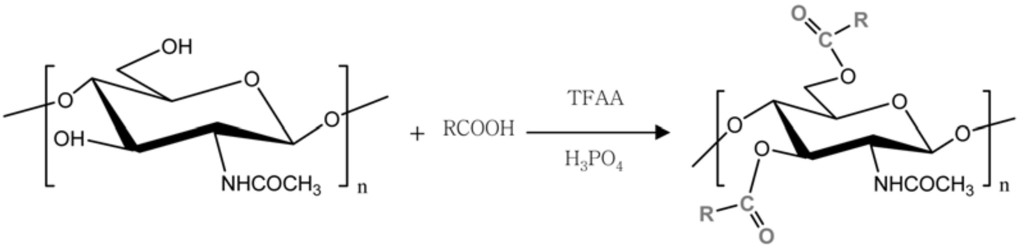

Fig. 1. Synthesis of dibutyryl chitin (DBC); R: CH_3-CH_2-CH_2-.

from the cell supernatant was determined by measuring absorbance at 540 nm using a microplate reader (Molecular Devices).

ELISA assay

For the inflammatory mediator assay, RAW 264.7 macrophages (1×10^5/well) were seeded into 24-well plates in 1 mL medium containing 10% FBS. After 5 h, the cells were washed and incubated with 1 mL RPMI 1640 medium containing LPS (1 μg/mL) in the presence or absence of varying doses of DBC or the control vehicle. Cell supernatants were obtained after 24 h and analyzed for the presence of PGE_2, TNF-α, IL-1β, and IL-6 using ELISA kits following the manufacturer's instructions.

Western blotting analysis

RAW 264.7 macrophages were cultured, harvested, and lysed in 1× SDS sample buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, 2 mM β-mercaptoethanol, 1 mM dithiothreitol (DTT), bromophenol blue (BPB), and xylene cyanol]. Cell lysates were electrophoresed on a 12% SDS polyacrylamide gel and proteins were transferred to polyvinylidene difluoride (PVDF) membranes at 300 mA for 1 h. After briefly rinsing with PBS containing 0.1% Tween (PBST), the blots were blocked for 1 h at room temperature in blocking buffer (PBST containing 4% non-fat dried milk). Primary antibodies that had been diluted in blocking buffer (1:1,000) were added to the blots and the blots were incubated for 1 h at room temperature or overnight in a refrigerator. After washing three times with PBST, the blots were incubated with HRP-conjugated secondary antibodies (1:2,000 dilutions in blocking buffer) for 1 h at room temperature. The blots were washed three times in PBST and developed using super signal enhanced chemiluminescence (ECL) substrate solution (Pierce) according to the manufacturer's instructions. Signals were visualized using X-ray film.

Statistical analysis

Differences in the data among groups were analyzed using one-way analysis of variance (ANOVA), and all values are expressed as the mean ± S.D. Differences between groups were considered to be significant when $p < 0.05$.

RESULTS

Synthesis of DBC

Synthesized DBC was identified using infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy as follows (Batt et al., 2011): IR (KBr Pallet, cm^{-1}); N-H (3,100), C-H (3,000-2,890), C=O (1,740), O=CNH (1,685 and 1590), $-CH_2$ (1,395), $-CH_3$ (1,340), C-O (1,200 and 1,080). H^1-NMR (DMSO-d_6, δ ppm); CON*H* (7.92, br), C_1H-C_6H (3.30-5.20, m), O (CO)CH_2 on the C-3 and C-6 ester groups, respectively (2.14 and 2.30, br), NH(CO)CH_3 (1.70, s, br), O(CO)CH_2CH_2 at the C-3 and C-6 ester groups, respectively (1.60 and 1.48, s), O(CO)CH_2CH_2CH_3 at the C-3 and C-6 ester groups, respectively (0.90 and 0.80, 2 peaks, br) (Fig. 1).

Protective effect of DBC on LPS-induced cytotoxicity

The protective effect of DBC was examined based on LPS-induced cytotoxicity of murine macrophage RAW 264.7 cells. After 48 h of incubation, the effect of DBC against LPS-induced cytotoxicity was assayed using the MTT test. As shown in Fig. 2, LPS (1 μg/mL) treatment significantly induced cytotoxicity compared to that observed for unstimulated control cells. However, the growth of LPS-treated RAW 264.7 cells was significantly enhanced by DBC in a dose-dependent manner. Moreover, 25-200 μg/mL concentrations of DBC had a protective effect on LPS-induced cytotoxicity in RAW264.7 cells. DBC did not significantly diminish cell respiration at the concentrations used in this study (25-200 μg/mL) (data not shown).

Inhibitory effect of DBC on of NO and PGE_2 production

Murine macrophage RAW 264.7 cells were shown to produce a large amount of NO and PGE_2 when incubated with LPS (Patel et al., 1999). In RAW 264.7 cells, incubation with LPS for 24 h significantly enhanced

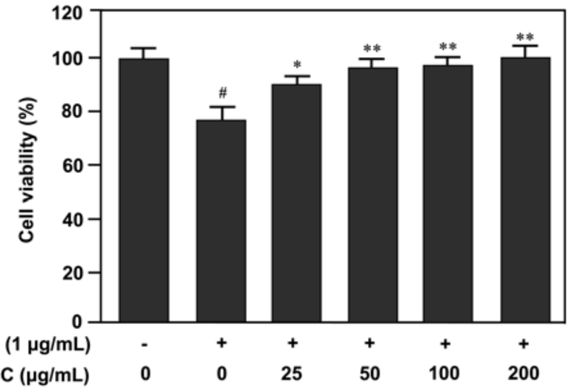

Fig. 2. Effect of DBC on viability of RAW 264.7 macrophages. Cells were incubated with medium alone or with LPS (1 μg/mL) in the absence or presence of the indicated concentrations of DBC. Cell viability was measured using the MTT assay after 48 h incubation. Data are presented as the mean ± S.D. of three independent experiments. $^{\#}p < 0.001$ *vs* untreated cells. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs* LPS alone.

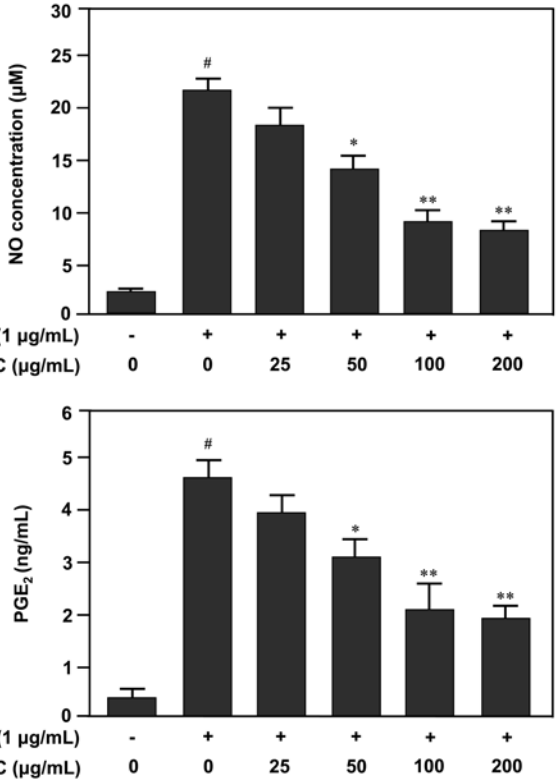

Fig. 3. Effect of DBC on NO and PGE_2 production in activated RAW 264.7 cells. Cells were incubated with medium alone or with LPS (1 μg/mL) in the absence or presence of the indicated concentrations of DBC. Nitrite concentration in the culture medium was quantified after incubation for 48 h. PGE_2 level was analyzed using ELISA kits after 24 h. Data are presented as the mean ± S.D. of three independent experiments. $^{\#}p < 0.001$ *vs* untreated cells. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs* LPS alone.

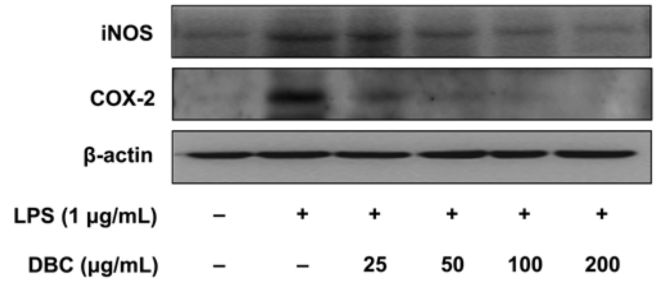

Fig. 4. Effect of DBC on iNOS and COX-2 expression in activated RAW 264.7 cells. Cells were incubated with medium alone or with LPS (1 μg/mL) in the absence or presence of the indicated concentrations of DBC. iNOS and COX-2 expression were determined using Western blot analysis with specific antibodies after 18 h.

production of NO and PGE_2 compared to that in unstimulated cells (Fig. 3). However, NO and PGE_2 production of LPS-treated RAW 264.7 cells was significantly inhibited by DBC in a dose-dependent manner. The maximum inhibitory effect was observed at a DBC concentration of 100 μg/mL, which caused approximately 57% and 67% reductions in NO and PGE_2 levels, respectively (Fig. 3). LPS induces production of NO and PGE_2 in RAW 264.7 cells by activating iNOS and COX-2 (Clancy et al., 1998). Therefore, we analyzed the effect of DBC on iNOS and COX-2 expression stimulated using LPS. In parallel with protein secretion, DBC treatment suppressed LPS-induced expression of iNOS and COX-2 in RAW 264.7 cells (Fig. 4). These results demonstrate that DBC suppresses LPS-induced production of NO and PGE_2 in RAW 264.7 cells.

Inhibitory effect of DBC on LPS-induced pro-inflammatory cytokine production

LPS also induces pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (Ishii et al., 2003). Therefore, we examined the effect of DBC on production of pro-inflammatory cytokines. RAW 264.7 cells were incubated in the absence or presence of DBC for 2 h and then treated with LPS for 24 h. Production of pro-inflammatory cytokines was examined using ELISA kits against TNF-α, IL-1β, and IL-6. As shown in Fig. 5, LPS (1 μg/mL) treatment significantly induced production of TNF-α, IL-1β, and IL-6 compare to that in unstimulated control cells. However, DBC significantly inhibited production of TNF-α, IL-1β, and IL-6 in LPS-stimulated RAW 264.7 cells in a dose-dependent manner. In parallel with NO and PGE_2 inhibition, DBC treatment suppressed LPS-induced pro-inflammatory cytokine production in RAW 264.7 cells (Fig. 5). These results demonstrate that DBC suppresses LPS-induced production of inflammatory mediators in RAW 264.7 cells.

DISCUSSION

Chitin is a component of the invertebrate skeleton as well as fungal cell walls and shows good biocompatibility and positive effects on wound healing (Okamoto et al., 1993). Chitin accelerates the repair of various tissues, facilitates contraction of wounds, and regulates secretion of inflammatory mediators such as IL-8, PGE_2, and IL-1, among others (Muzzarelli, 2010). Although chitin has been applied in pharmaceutics due to its specific physiochemical and biological properties (Austin et al., 1981; Su et al., 1997), the low solubility of chitin has restricted its technological application. Recently, DBC, a modified chitin, has been examined due to its solubility in common solvents such as ethanol. DBC also retains the filmogenic property of standard chitin; thus, dibutyryl chitin can be used to manufacture threads, filaments, and non-woven materials (Van de Velde and Kiekens, 2004; Blasinska and Drobnik,

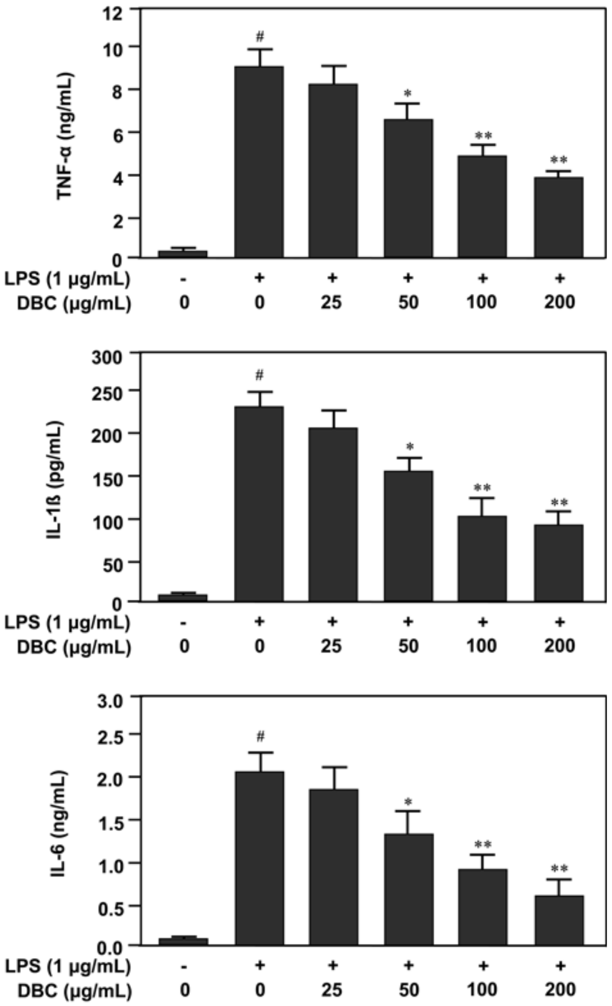

Fig. 5. Effect of DBC on TNF-α, IL-1β, and IL-6 in activated RAW 264.7 cells. Cells were incubated with medium alone or with LPS (1 μg/mL) in the absence or presence of the indicated concentrations of DBC. Levels of TNF-α, IL-1β, and IL-6 in the medium were measured using an ELISA kits. Resting cells were used as the basal group. Data are presented as the mean ± S.D. of three independent experiments. $^{\#}p < 0.001$ *vs* untreated cells. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs* LPS alone.

2008; Batt et al., 2011). Biochemical data indicate that DBC is not cytotoxic for fibroblasts and keratinocytes. The role of DBC appears to be confined to imparting better handling and mechanical resistance; DBC has no known relevant role in promoting the ordered regeneration of wounded tissues due to its resistance against enzymatic hydrolysis by lysozyme, lipase, collagenase, or amylase (Muzzarelli et al., 2005). However, the mechanism of DBC's biological activity remains unclear. Therefore, the anti-inflammatory action of the synthesized DBC should be evaluated (Fig. 1).

In the present study, we investigated the effects of DBC on the production of inflammatory mediators, including NO, PGE_2, TNF-α, IL-1β, and IL-6, in RAW 264.7 macrophages activated by LPS in order to clarify the anti-inflammatory activity of DBC. DBC suppressed production of NO and PGE_2 in LPS-stimulated RAW 264.7 macrophages by inhibiting iNOS and COX-2 expression. Additionally, DBC also inhibits production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6.

During inflammation and infection, activated macrophages that have been attracted to the site of inflammation produce a large amount of NO around the wounded tissues (DeGeorge et al., 1997). NO is a highly reactive oxidant that is produced through the action of iNOS and participates in diverse biological processes such as regulation of inflammation. NO is thought to be a major destructive factor in the wound healing process (Rubbo et al., 1995), although some reports showed that the presence of small amounts of NO may help wound repair during the early phase of healing (Schaffer et al., 1999). It is well-known that overproduction of inflammatory prostaglandins derived from COX-2 is an important pathophysiological factor contributing to inflammation (Patel et al., 1999). Our results suggest that DBC inhibit LPS-induced NO and PGE_2 production through suppression of iNOS and COX-2 expression in RAW 264.7 macrophages. However, further investigation of the mechanism of DBC action is necessary.

Macrophages, as critical factors in inflammation, directly counteract these harmful stimuli. In response to LPS, they also mediate the inflammatory response by secreting pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 (Lin and Karin, 2007). Overproduction of these mediators results in excessive inflammatory responses (Lawrence et al., 2002). Thus, inhibition of pro-inflammatory mediator release may be beneficial in attenuating the inflammatory response. Furthermore, RAW 264.7, a murine macrophage cell line, is an excellent model for anti-inflammatory drug screening and for subsequently evaluating inhibitors of pathways leading to induction of pro-inflammatory cytokines. In this study, DBC suppressed production of TNF-α, IL-1β, and IL-6 in RAW 264.7 macrophages stimulated by LPS. Our results demonstrate that DBC suppresses production of inflammatory mediators in RAW 264.7 cells.

In conclusion, this study demonstrates that DBC significantly inhibits overproduction of NO and PGE_2 as well as pro inflammatory cytokines such as TNF-α, IL-1β, and IL-6 production in LPS-stimulated RAW 264.7 macrophages. Inhibition of NO and PGE_2 overproduction in LPS-stimulated RAW 264.7 macrophages by DBC is mediated through down-regulation of iNOS and COX-2. Our results suggest mechanisms by which DBC exerts its beneficial effect by accelerating anti-inflammation.

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