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Inhibitory effect of CDP, a polysaccharide extracted from the mushroom *Collybia dryophila*, on nitric oxide synthase expression and nitric oxide production in macrophages

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Abstract

The effect of *Collybia dryophila* polysaccharide (CDP), a (1→3), (1→4)-β-D-glucan extracted from the mushroom *C. dryophila*, was evaluated on nitric oxide (NO) production induced by lipopolysaccharide (LPS) and gamma interferon (IFNγ) or by LPS alone in RAW 264.7 cells. CDP significantly inhibited NO production in a dose-dependent manner without affecting cell viability. The inhibition of NO by CDP was consistent with decreases in both inducible nitric oxide synthase (iNOS) protein and mRNA expression suggesting that CDP exerts its effect by inhibiting iNOS gene expression. In addition, CDP at concentrations of 400 and 800 μg/ml was shown to significantly increase prostaglandin E_2 (PGE_2) production in LPS- and IFNγ-induced macrophages when compared to the control.

Keywords: Nitric oxide; Inducible nitric oxide synthase (iNOS); β-D-glucan; Prostaglandin E_2 (PGE_2)

1. Introduction

Prostaglandins (thromboxane A_2, PGD_2, $PGF_{2\alpha}$, PGI_2, PGE_2) and nitric oxide (NO) are potent mediators of intercellular and intracellular communications, and they both play major roles in regulating inflammation, immune functions, blood vessel dilatation, and neurotransmission (Minghetti et al., 1996; Goodwin et al., 1999; Yun et al., 2003). NO is a highly reactive, diffusible and unstable radical (MacMicking et al., 1997; Sosroseno et al., 2002; Kim et al., 2005), produced from the oxidation of L-arginine, a reaction catalyzed by the enzyme nitric oxide synthase (NOS) (Song et al., 2002). NOS exists in two major isoforms: the constitutive form (cNOS) and the inducible form (iNOS). cNOS, which is an important regulator of homeostasis, is responsible for the release of physiological levels of NO (Minghetti et al., 1996; MacMicking et al., 1997; Aktan, 2004; Li and Poulos, 2005), while iNOS is expressed by many types of stimuli, including bacterial lipopolysaccharide (LPS) and pro-inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF-α) and gamma interferon (IFNγ) (Sosroseno et al., 2002; Pichika and Homandberg, 2004; Lee et al., 2005) in various cell types such as macrophages, neutrophils, mesangial cells, hepatocytes, and chrondrocytes (Lee et al., 2005). Excessive NO production by iNOS has been closely associated with pathogenesis in several inflammatory diseases including septic shock, rheumatoid arthritis, graft rejection and diabetes (Cuzzocrea et al., 2002; Song et al., 2002; Kim et al., 2005; Jantaratnotai et al., 2006). In this context, molecules able to decrease iNOS expression are of particular interest and could find application in the treatment of diseases that implicate overproduction of NO (Lee et al., 2005).

Recent work conducted with *Collybia dryophila* reported for the first time the presence in this mushroom of a (1→3), (1→4)-

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β-D-glucan (Pacheco-Sánchez et al., 2006). This glucan, named CDP (*C. dryophila* polysaccharide), was shown in the course of a preliminary experiment to inhibit nitric oxide production in activated macrophages. The objective of this work was to further investigate the effect of CDP on NO production to gain insight into the mechanism by which CDP affects NO production. The effect of CDP on PGE_2 formation in macrophages was also investigated.

2. Materials and methods

2.1. C. dryophila polysaccharide (CDP)

The water-soluble polysaccharide CDP was extracted from the fruiting bodies of *C. dryophila* and purified as previously described (Pacheco-Sánchez et al., 2006). CDP purity was ≥97% (as determined by HPLC analysis). Powdered, freeze-dried CDP was stored at 4 °C until use.

2.2. Cell culture

RAW 264.7, a murine macrophage cell line (TIB71) from the American Type Culture Collection (Rockville, MD, USA), was cultured in phenol red-free Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Burlington, ONT, Canada) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% heat-inactivated fetal bovine serum (Bio Media Canada, Drummondville, QUE, Canada). Cells were grown at 37 °C under an atmosphere of 5% CO_2. Unless stated otherwise, DMEM was amended with 0.05 μg/ml LPS (Sigma, St. Louis, MO, USA) and 0.1 ng/ml IFNγ (Sigma) to promote iNOS synthesis and NO production.

2.3. Effect of CDP on cell viability

The cytotoxicity of CDP was evaluated using the alamarBlue™ test. This test incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. Briefly, RAW 264.7 cells (5×10^5 cells/well in 24-well culture plates) were incubated at 37 °C (5% CO_2) with different concentrations of CDP (0 [control], 50, 250, 500 and 1000 μg/ml] and 10% of alamarBlue. After different incubation periods, 100 μl of each supernatant was removed and fluorescence was measured at 535 nm with an automated plate reader (Tecan Genios, Gröding/Salzburg, Austria).

2.4. Effect of CDP on NO production

The effect of CDP on NO production was evaluated in the course of four separate experiments. RAW 264.7 cells (5×10^5 cells/well in 24-well culture plates) were incubated at 37 °C (5% CO_2) with LPS and IFNγ or LPS alone and different concentrations of CDP (0 [control]–800 μg/ml) for 48 h. After the incubation period, NO production was determined with a colorimetric test as described below. In the first experiment, cells were incubated in culture medium containing LPS, IFNγ and different concentrations of CDP (0 [control], 25, 50, 100, 200, 400 and 800 μg/ml) for 48 h. In the second experiment, cells were incubated in a culture medium containing different concentrations of CDP (0, 200 and 400 μg/ml) for 2 h. Afterwards, the culture medium containing CDP was aspirated and fresh medium containing LPS and IFNγ was added to the cells, which were incubated for 46 h. In the third experiment, cells were incubated in a culture medium containing LPS and IFNγ for 4 h. The culture medium containing LPS and IFNγ was subsequently aspirated and fresh medium containing different concentrations of CDP (0, 200 and 400 μg/ml) was added to the cells, which were incubated for 44 h. In the fourth experiment, cells were incubated for 48 h in a culture medium containing LPS and different concentrations of CDP (0, 200, 400 and 800 μg/ml). Each experiment was performed according to a completely randomized design consisting of nine (first experiment) or three (second, third and fourth experiments) replicates. Cells incubated in culture medium containing no LPS, no IFNγ and no CDP were used as blanks.

Released nitrite (NO_2^-) in the culture medium was measured as an indicator of NO production according to the colorimetric test based on the Griess reaction (Green et al., 1982). Briefly, 150 μl of cell supernatant was mixed with 150 μl of Griess reagent (1% sulphanilamide and 0.1% naphthyl-ethylenediamide in 5% phosphoric acid) at room temperature for 30 min. The nitrite concentration was determined by measuring the absorbance at 548 nm in an automated plate reader (Tecan Genios) using standard curve of $NaNO_2$. The results were expressed as percentage of NO production compared to the control as follows: [Nitrite concentration released in presence of CDP/Nitrite concentration released in absence of CDP (control)]×100.

2.5. Effect of CDP on iNOS expression (Western blot analysis)

RAW 264.7 cells (5×10^5 cells/well in 24-well culture plates) were incubated with LPS, IFNγ and different concentrations of CDP (0, 200, 400 and 800 μg/ml) at 37 °C (5% CO_2) for 24 h. Cells incubated in culture medium containing no LPS, no IFNγ and no CDP were used as blanks. After incubation, the cells were washed twice with ice-cold phosphate buffer saline (PBS), and lysed in 250 μl of ice-cold buffer containing 62.5 mM Tris (pH 6.8), 2% sodium dodecyl sulphate (SDS), 10% glycerol, 5% 2-mercaptoethanol and protease inhibitors. The supernatant was centrifuged (13,000 rpm; 4 °C) for 10 min. Total cellular

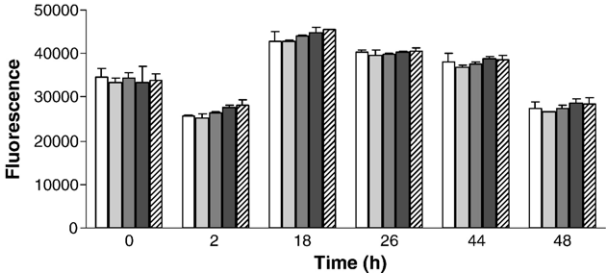

Fig. 1. Time course of RAW 264.7 cell viability following exposure to different concentrations of CDP. Cell viability was determined using the alamarBlue™ test. Cells not exposed to CDP (control, 0 μg/ml of CDP, □), and exposed to 50 (▒), 250 (▒), 500 (▒), and 1000 (▒) μg/ml. Each value represents the mean± S.E.M. of triplicate determinations.

proteins were separated by 8% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. For immunoblotting, the membrane was incubated with 3% bovine serum albumin in PBS supplemented with 0.01% Tween-20 at 4 °C for 1 h. This solution, which served as the blocking solution, was also used for all incubation steps. After blocking, membranes were incubated overnight at 4 °C with anti-iNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:500. After washes, a biotinylated anti-mouse IgG_1 (BD Biosciences, Mississauga, ONT, Canada) was added for 90 min and followed by peroxidase-conjugated extrAvidin (Sigma). The blots were developed using an ECL detection kit (Roche Applied Sciences, Laval, QUE, Canada). The experiment was conducted twice.

2.6. Effect of CDP on iNOS mRNA expression

RAW 264.7 cells (1×10^6 cells/well in 24-well culture plates) were incubated at 37 °C (5% CO_2) with LPS, IFNγ and different concentrations of CDP (0 [control], 200, 400 and 800 μg/ml) for 6 h, according to a completely randomized design consisting of three replicates. After incubation, the cells were collected and total RNA was extracted using RNAquous™ kit (Ambion, Austin, TX, USA), following the manufacturer's instructions. Total RNA was treated with DNAse and then quantified using RiboGreen™ RNA quantification reagent (Molecular Probes, Eugene, OR, USA). Single-strand cDNA was synthesized from 2.5 ng of total RNA of each sample using the SuperScript II reverse transcriptase system kit (Invitrogen Canada, Burlington, ONT, Canada) according to the manufacturer's protocol. Reverse transcription was performed in a Mastercycler (Eppendorf, Westbury, NY, USA) for 50 min at 42 °C, and 15 min at 70 °C. The PCR amplification was performed in an Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA), in combination with the *Taq*Man chemistry using the comparative method; β-actin was used as the housekeeping gene. The PCR reaction was carried in a 25 μl final volume containing: 1.2 ng/μl cDNA sample, 300 nM probe, 200 nM of forward and reverse primers, 3.5 mM $MgCl_2$, and 1 U TaqGold enzyme (Integrated DNA Technologies, Coralville, IA, USA). Primers and probes used for real-time RT-PCR were supplied by Integrated DNA Technologies. The specific primers used were: 5′-CAGCTGGGCTGTACAAACCTT (forward) and 5′-CATTGGAAGTGAAGCGTTTCG-3′ (reverse) for iNOS; 5′-AGAGGGAAATCGTGCGTGAC-3′ (forward) and 5′-CAATAGTG ATGACCTGGC CGT-3′ (reverse) for β-actin. The probes used were: 5′FAM™CGGGCAGCCTGTGAGACCTTTGABHQ-1™3′ for iNOS and 5′Cy5™CACTGCCGCATCCTCT TCCCBHQ-2™3′ for β-actin. After an initial denaturation step at 95 °C for 10 min, temperature cycling was initiated. Each cycle consisted of 95 °C for 15 s and 60 °C for 60 s. Forty cycles were performed and the fluorescence was read at the end of each cycle. PCR amplification was performed in triplicate.

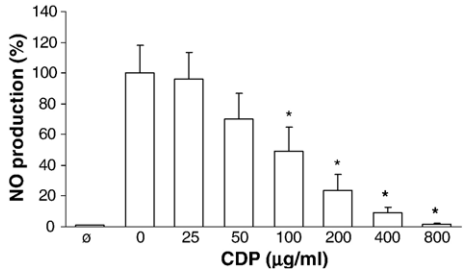

Fig. 2. Effect of CDP on NO production in RAW 264.7 cells. Cells were not exposed (ø) or exposed simultaneously to LPS, IFNγ and different concentrations of CDP. Each value represents the mean±S.E.M. of nine replicates. Asterisks indicate significant difference ($P<0.05$) when compared to LPS- and IFNγ-stimulated cells not exposed to CDP (control, 0 μg/ml of CDP).

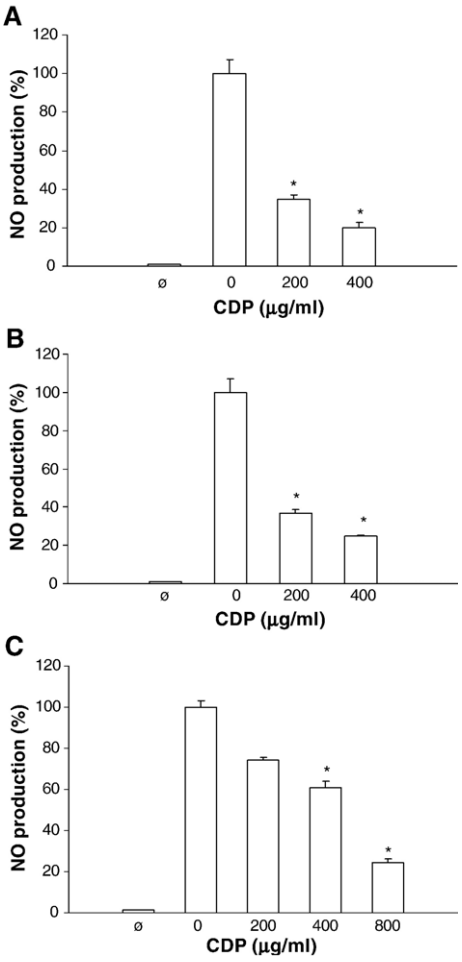

Fig. 3. Effect of CDP on NO production in RAW 264.7 cells. Cells were primed with different concentrations of CDP 2 h before stimulation with LPS and IFNγ (A); cells were primed with LPS and IFNγ 4 h before CDP addition (B); cells were treated simultaneously with LPS and CDP (C). Each value represents the mean±S.E.M. of three replicates. Asterisks indicate significant difference ($P<0.05$) when compared to LPS- and IFNγ-stimulated cells (A, B) or LPS-stimulated cells (C) not exposed to CDP (controls, 0 μg/ml of CDP). (ø) Cells not exposed to LPS, IFNγ and CDP.

2.7. Effect of CDP on PGE_2 production

RAW 264.7 cells (5×10^5 cells/well in 24-well culture plates) were incubated at 37 °C (5% CO_2) in a culture medium containing LPS, IFNγ and different concentrations of CDP (0 [control], 200, 400 and 800 μg/ml) for 24 h, according to a completely randomized design consisting of three replicates.

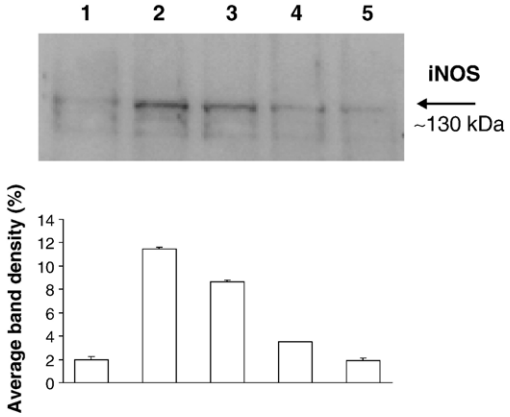

Fig. 4. Effect of different concentrations of CDP on LPS- and IFNγ-induced iNOS expression in RAW 264.7 cells. Cells were not exposed to LPS, IFNγ and CDP (lane 1); LPS- and IFNγ-stimulated cells were treated with 0 (control, lane 2), 200 (lane 3), 400 (lane 4), and 800 μg/ml (lane 5) of CDP. Values in the histogram represent the mean of two replicates±S.E.M.

Cells incubated in culture medium containing no LPS, no IFNγ and no CDP were used as blanks. After incubation, the level of PGE_2 released into the culture medium was determined using a competitive enzyme immunoassay (EIA) according to the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI, USA).

2.8. Statistical analysis

Analysis of variance was carried out with the GLM (General Linear Models) procedure of SAS (SAS Institute, Cary, NC). When significant ($P<0.05$), treatment means were compared using Dunnett's *t*-test. Each experiment was repeated at least three times.

3. Results

3.1. Effect of CDP on cell viability

The effect of CDP on RAW 264.7 cell viability was determined with the alamarBlue™ test. As shown in Fig. 1, none of the concentrations of CDP tested (50 to 1000 μg/ml) exhibited cytotoxic effects on the macrophages on a period of 48 h, when compared to the untreated cells (control).

3.2. Effect of CDP on NO production

To determine the effect of CDP on LPS- and IFNγ-induced NO production, RAW 264.7 cells were treated with different concentrations of CDP (0 to 800 μg/ml). As shown in Fig. 2, LPS and IFNγ strongly stimulated NO production in macrophages. CDP applied simultaneously with LPS and IFNγ was shown to significantly reduce NO production in a dose-dependent manner. The addition of 25, 50, 100, 200, 400 and 800 μg/ml of CDP resulted in a reduction of 3.7, 29.9, 50.9, 76.1, 91.0 and 98.5% in NO release, respectively, when compared to the cells that were not exposed to CDP (control). Applied 2 h prior to (Fig. 3A) or 4 h after (Fig. 3B) exposure to LPS and IFNγ, CDP at concentrations of 200 and 400 μg/ml significantly inhibited NO production when compared to the control. In addition, the results showed that CDP at concentrations of 400 and 800 μg/ml significantly reduced NO production in macrophages treated with LPS only (Fig. 3C).

3.3. Effect of CDP on iNOS expression

To determine whether the reduced NO production by CDP in activated RAW 265.7 cells was due to a decrease of the iNOS expression, LPS- and IFNγ-induced cells were treated with different concentrations of CDP and iNOS expression was subsequently analyzed by Western blotting. As shown in Fig. 4, LPS and IFNγ induced a high level of expression of the iNOS protein in macrophages. The addition of different concentrations of CDP (400 and 800 μg/ml) markedly decreased iNOS expression induced by LPS and IFNγ in a dose-dependent manner.

3.4. Effect of CDP on iNOS mRNA expression

LPS- and IFNγ-induced RAW 264.7 cells expressed high level of iNOS mRNA after 6 h of incubation (Fig. 5). CDP

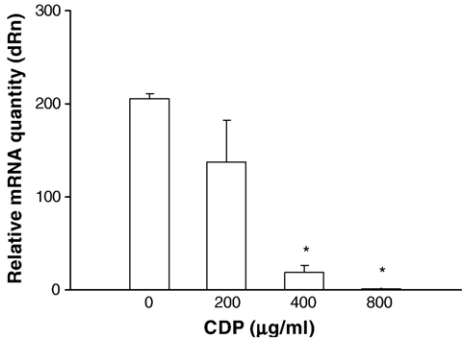

Fig. 5. Effect of different concentrations of CDP on LPS- and IFNγ-induced iNOS mRNA expression in RAW 264.7 cells. iNOS mRNA expression was determined by comparative real-time RT-PCR. Each value represents the mean± S.E.M. of three replicates. Asterisks indicate significant difference ($P<0.05$) when compared to cells not exposed to CDP (control, 0 μg/ml of CDP).

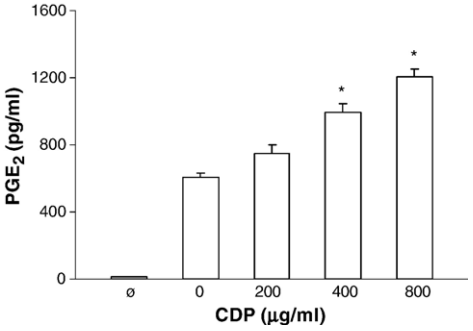

Fig. 6. Effect of different concentrations of CDP on LPS- and IFNγ-induced PGE_2 production in RAW 264.7 cells. Each value represents the mean±S.E.M. of three replicates. Asterisks indicate significant difference ($P<0.05$) when compared to cells not exposed to CDP (control, 0 μg/ml of CDP). (ø) Cells not exposed to LPS, IFNγ and CDP.

significantly reduced iNOS mRNA expression in induced cells in a dose-dependent manner. The iNOS mRNA expression was almost totally inhibited with 800 μg/ml of CDP. The amount of β-actin mRNA, an internal control, was not affected by CDP treatment.

3.5. Effect of CDP on PGE_2 production

Treatment of macrophages with LPS and IFNγ markedly increased PGE_2 production (Fig. 6). CDP at concentrations of 400 and 800 μg/ml significantly increased the production of PGE_2 to 992.8 and 1203.4 pg/ml, respectively, in LPS- and IFNγ-induced macrophages when compared to the control (Fig. 6).

4. Discussion

Several bioactive (hypoglycemic, immunomodulatory, anti-inflammatory, anti-tumoral, antiviral, antibacterial or antiparasitic) compounds such as polysaccharides, polysaccharides–peptides, nucleosides and triterpenols, have been identified in numerous mushroom species (Wasser and Weis, 1999). Among them, numerous polysaccharides having unique structures revealed immunomodulatory activity.

The principle upon which immunomodulator compounds operate is primarily based on the modulation of different immune cells that may result in several therapeutic properties (Pugh et al., 2001). In some cases, the therapeutic property is attributable to an effect on NO and/or prostaglandin production by activated macrophages. For example, several natural compounds extracted from different plants including *Cinnamomum cassia* Nees ex Blume (Lee et al., 2005), *Platycodon grandiflorum* (Jacq.) A. DC. (Ahn et al., 2005) and *Kaempferia pandurata* Roxb. (Yun et al., 2003) were shown to possess therapeutic properties resulting from their effect on NO and/or prostaglandin biosynthesis by macrophages. Among mushrooms, *Lentinus edodes* (Berk.) Sing. has been reported to contain polysaccharides displaying effects on NO and/or prostaglandin production in macrophages (Minato et al., 2001; Murata et al., 2002). In this study, the effect of CDP, a (1→3), (1→4)-β-D-glucan recently isolated from the mushroom *C. dryophila*, was investigated on NO and PGE_2 production in RAW 264.7 cells.

The results obtained showed that CDP applied either simultaneously with LPS and IFNγ or with LPS alone markedly reduced macrophage NO production in a dose-dependent manner without producing toxic effects on cells. CDP also showed the ability to decrease iNOS mRNA expression leading to reduced iNOS protein expression, suggesting that down-regulation of NO production by CDP is at the transcriptional level.

The interactions between NO and prostaglandin biosynthesis pathways are complex and remain unclear (Salvemini, 1997; Goodwin et al., 1999). While several studies have demonstrated that NO increases prostaglandin synthesis (Salvemini et al., 1993; Salvemini, 1997; Hughes et al., 1999), others have reported its negative effect on prostaglandin production (Stadler et al., 1993; Vane et al., 1994; Minghetti et al., 1996; Habib et al., 1997). In this study, CDP was shown to increase PGE_2 levels. The repressive effect on NO production combined with the enhancing effect on PGE_2 production may confer a particular interest in CDP.

Overall, the results presented herein demonstrated the ability of CDP (i) to inhibit NO production through the suppression of iNOS protein and mRNA expression and (ii) to increase PGE_2 production in LPS- and IFNγ-stimulated RAW 264.7 cells. Future work will employ other cell lines as well as animal models to characterize the effect of CDP on immune system reactions.

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