Limonene Suppresses Lipopolysaccharide-Induced Production of Nitric Oxide, Prostaglandin E₂, and Pro-inflammatory Cytokines in RAW 264.7 Macrophages

Weon-Jong Yoon¹, Nam Ho Lee²,³ and Chang-Gu Hyun¹,²*  

¹ Jeju Biodiversity Research Institute (Namwon, Seogwipo-si, Jeju 699-943, KOREA)  
² Research Group for Beauty and Cosmetics, Cheju National University (Ara-1-Dong, Jeju, 690-756, KOREA)  
³ Department of Chemistry, Cheju National University (Ara-1-Dong, Jeju, 690-756, KOREA)

Abstract: The monoterpene D-limonene and its metabolites have been shown to exert chemopreventive and chemotherapeutic effects against different tumours in animal models and clinical trials. However, it is unknown whether these compounds modulate the inflammatory response in RAW 264.7 macrophage cells. The present study was therefore designed to elucidate the pharmacological and biological effects of D-limonene on the production of pro-inflammatory cytokines and inflammatory mediators in macrophages. The results indicate that D-limonene is an effective inhibitor of lipopolysaccharide (LPS)-induced NO and prostaglandin E₂ production in RAW 264.7 cells. These inhibitory effects of D-limonene included dose-dependent decreases in the expression of iNOS and COX-2 proteins. To evaluate the inhibitory effects of D-limonene on other cytokines, we also measured TNF-α, IL-1β, and IL-6 levels in the cell supernatants of LPS-stimulated RAW 264.7 macrophages by enzyme-linked immunosorbent assay. In these assays, D-limonene decreased the expression of TNF-α, IL-1β, and IL-6 in a dose-dependent manner. To assess the suitability of D-limonene for cosmetic applications, we also performed 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assays on HaCaT keratinocytes. D-limonene did not display any cytotoxicity in these assays. From these results, we suggest that D-limonene may be considered a potential anti-inflammatory candidate.

Key words: cytokine, inflammation, iNOS, D-limonene, PGE₂

1 INTRODUCTION

D-limonene (1-methyl-4-(1-methylethenyl)cyclohexene) is one of the most common terpenes in nature, having a lemon-like sweet smell and being a major constituent in several citrus oils such as orange, lemon, mandarin, lime, and grapefruit. Owing to its pleasant citrus fragrance, D-limonene is widely used as a flavour and fragrance additive in perfumes, soaps, foods, chewing gum, and beverages⁴⁻⁷. D-limonene may also be used as an inert ingredient in pesticides and as a natural replacement for petroleum-based solvents in paints and cleaning products. It is considered to be an ingredient with relatively low toxicity. Upon oral administration, D-limonene is rapidly and almost completely absorbed from the gastrointestinal tract of both humans and animals⁸⁻¹⁰. Other studies have shown that D-limonene does not pose a mutagenic, carcinogenic, or nephrotoxic risk to humans. At present, D-limonene is clinically used to dissolve cholesterol-containing gallstones and to relieve heartburn. It also has well-established chemopreventive activity against many types of cancers¹,²,⁵⁻⁷. However, the anti-inflammatory effects of D-limonene are as yet unknown. Therefore, we conducted a detailed study to investigate the anti-inflammatory effects of D-limonene in RAW 264.7 cells.

Inflammation is a complex process mediated by the activation of various immune cells. Macrophages play a central role in mediating many different immunopathological phenomena during inflammation, including the overproduction of pro-inflammatory cytokines and inflammatory mediators, such as interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)-α, and nitric oxide (NO) synthesized by inducible NO synthase (iNOS) and prostaglandin (PG)E₂ synthesized by cyclooxygenase (COX)-2¹¹⁻¹³. In murine macrophage RAW 264.7 cells, lipopolysaccharide (LPS) stimulation alone
can induce the production of pro-inflammatory cytokines and inflammatory mediators. Therefore, this cell system provides an excellent model for drug screening and evaluation of potential inhibitors of the inflammatory response. In this study, we examine the effect of d-limonene on LPS-stimulated production of pro-inflammatory cytokines and inflammatory mediators by RAW 264.7 cells.

2 EXPERIMENTAL

2.1 Reagents

Foetal bovine serum (FBS) and Dulbecco’s Modified Eagle Medium (DMEM) were obtained from Gibco (Grand Island, NY). iNOS and COX-2 antibodies were purchased from Calbiochem (San Diego, CA, USA) and BD Biosciences (San Diego, CA, USA). Recombinant mouse TNF-α, IL-1β, IL-6, and PGE₂ were purchased from R&D Systems Inc. (St. Louis, MO). All other chemicals were from Sigma.

2.2 Cell culture

The murine macrophage cell line, RAW264.7, was purchased from ATCC (Rockville, MD), maintained in DMEM supplemented with 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 10% FBS. Cells were grown at 37°C in humidified air containing 5% CO₂ and were sub-cultured every 3 days.

2.3 Measurement of nitrite (NO₂⁻) production using the Griess reaction

RAW264.7 cells (1.5 × 10⁶ cells/mL) were pre-incubated for 18 h before treatment with LPS (1 µg/mL) and various concentrations of d-limonene for a further 24 h. Nitrite in culture supernatants was measured by adding 100 µL of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 µL samples of medium. All measurements were performed in triplicate. The concentration of NO₂⁻ was determined using a standard curve generated with NaNO₂.

2.4 Western blot analysis

RAW 264.7 cells were pre-incubated for 18 h before co-treatment with LPS (1 µg/mL) and test reagents for 24 h. After incubation, the cells were collected and washed twice with cold PBS. The cells were lysed in a lysis buffer [50 mM Tris- HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethylene glycol-bis(β-amino-ethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM NaVO₃, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 µg/mL leupeptin] and kept on ice for 30 min. Cell lysates were centrifuged at 12,000 × g at 4°C for 15 min, and supernatants stored at −70°C until required for analysis. Protein concentrations were measured using a protein assay kit (Bio-Rad, HC, U.S.A.). Aliquots of the lysates (30–40 µg of protein) were separated on a 8–12% sodium dodecyl sulphate (SDS) -polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, HC, U.S.A.) using a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)]. After blocking nonspecific sites with 5% non-fat milk powder solution, membranes were incubated with a specific primary antibody (mouse monoclonal anti-rabbit iNOS or anti-mouse COX-2) overnight at 4°C. Membranes were further incubated for 1 h with a peroxidase-conjugated secondary antibody (goat IgG, 1:5000; Santa Cruz, CA, U.S.A.). The immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) western blotting detection kit (Amersham Pharmacia Biotech, NY, U.S.A.).

2.5 Measurement of PGE₂ production by enzyme immunoassay

RAW 264.7 cells (1.5 × 10⁶ cells/mL) were pre-incubated for 18 h before co-treatment with LPS (1 µg/mL) and d-limonene at various concentrations for 24 h. Culture medium was centrifuged at 12,000 rpm for 3 min at 4°C to pellet cells. Culture supernatants were harvested for PGE₂ measurement using a mouse ELISA kit.

2.6 Measurement of cell viability (MTT assay)

Cell viability was measured by conventional MTT assay. HaCaT cells (1.0 × 10⁶ cells/mL) were seeded on 96-well plates and cultured as described above for 18 h. At 4 h prior to culture termination, 10 µL of MTT solution (10 mg/mL in phosphate buffered-saline, pH 7.4) was added and the cells were continuously cultured until termination. The incubation was stopped by addition of 15% sodium dodecyl sulfate (SDS) into each well for solubilization of formazan and the optical density (OD) at 570 nm (OD₅₇₀₋₆₃₀) was measured by a microplate reader.

2.7 Statistical analysis.

The Student’s t-test was used to determine the statistical significance of differences between values for a variety of experimental and control groups. Data are expressed as the mean ± standard error of the mean (SEM) of at least three independent experiments performed in triplicate. P values of 0.05 or less were considered statistically significant.

3 RESULTS

3.1 d-Limonene inhibits LPS-mediated production of nitrite

In order to evaluate the capacity of d-limonene to inhibit iNOS, nitrite accumulation was examined in the culture
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medium of LPS-stimulated RAW 264.7 macrophages. 

3.2 D-Limonene decreases LPS-induced PGE2 production in RAW 264.7 macrophages

PGE2 was measured in the supernatant of cultured RAW 264.7 macrophages stimulated with LPS (1 μg/mL) for 18 h in the presence of D-limonene. While PGE2 production by unstimulated cells was low (data not shown), LPS stimulated an increase in PGE2 production (Fig. 1B). D-limonene and dexamethasone (20 μM) both inhibited LPS-stimulated PGE2 production in a concentration-dependent manner (Fig. 1B).

3.3 D-Limonene inhibits LPS-stimulated iNOS protein expression

In order to elucidate the mechanism by which D-limonene inhibits LPS-stimulated nitrite production, we investigated its effect on iNOS protein expression. While barely detected in unstimulated RAW 264.7 macrophages by western blot analysis, robust expression of iNOS protein was observed after stimulation with LPS for 18 h (Fig. 2). The presence of D-limonene in LPS-stimulated cell cultures markedly inhibited the induction of iNOS expression in a concentration-dependent manner (Fig. 2). These data demonstrate that the inhibition of NO2 production by D-limonene, observed in Fig. 1, could have resulted from the inhibition of LPS-induced iNOS protein expression in RAW 264.7 macrophages.

3.4 D-Limonene inhibits COX-2 protein expression

To assess whether PGE2 repression is a result of the inhibition of COX-2 by D-limonene, RAW 264.7 macrophages treated with D-limonene together with LPS for 18 h were subjected to western blot analysis. Figure 2 shows that D-limonene inhibits LPS-induced COX-2 protein expression in a concentration-dependent manner. This corroborates the above finding that D-limonene decreases LPS-stimulat-
ed PGE₂ production.

3.5 D-Limonene inhibits macrophage-cytokine production.

To assess the effects of D-limonene on TNF-α, IL-1β, and IL-6 production by activated macrophages, LPS-stimulated RAW 264.7 cells were incubated in the presence of increasing concentrations of D-limonene and cytokine concentrations in culture supernatants were measured by ELISA. D-Limonene suppressed LPS-stimulated TNF-α, IL-1β, and IL-6 production in a dose-dependent manner (Fig. 3). A housekeeping protein, β-actin, was constitutively expressed and was unaffected by D-limonene treatment. Interestingly, of the two positive controls used, 2-amino-4-methyl pyridine did not affect production of the proinflammatory cytokines but dexamethasone (DEX; 20 μM) significantly reduced them. Thus, D-limonene modulates the LPS-induced production of TNF-α, IL-1β, and IL-6 in RAW 267.4 macrophages.

3.6 Cytotoxicity of D-limonene on human keratinocytes HaCaT cells

Finally, we examined the cytotoxic effects of D-limonene in HaCaT human keratinocyte cells to assess the suitability of this terpene for use as a therapeutic agent on the human skin. As shown in Fig. 4, cell viability remained at almost 100% following treatment with D-limonene at concentrations up to the effective dose of 0.08%. These data suggest that D-limonene has low cytotoxicity against mammalian cell lines.

4 DISCUSSION

D-Limonene, a natural monoterpene occurring in citrus peels and other plants, is present in foods and in many essential oils. It is extensively used as a food additive to provide a citrus flavour; as a fragrance in perfumes, air fresheners, and personal care products; and as a natural replacement for petroleum-based solvents in paints and cleaning products. D-limonene may also be used as an inert ingredient in pesticide formulations. Previously, we demonstrated that citrus essential oils, which contain D-limonene as a major component, are able to alleviate inflammation. In order to validate the use of D-limonene as an anti-inflammatory agent in the cosmetic industry, we have, in this study, investigated the effects of D-limonene on the production of pro-inflammatory cytokines and inflammatory mediators by LPS-activated RAW 264.7 macrophages.

Macrophages play an important role in the initiation and amplification of a variety of inflammatory diseases. Therefore, the development of methods to reduce the number of activated macrophages, to inhibit the activation signals and/or their specific macrophage receptors, or to selectively counteract the macrophage products that act as disease amplifiers has been suggested as a promising therapeutic approach against various inflammatory diseases.

Pro-inflammatory cytokines and inflammatory mediators such as COX-2, NO, IL-1β, IL-6, and TNF-α play important roles in the inflammatory process. NO is derived from arginine after the activation of iNOS, and is an important effector molecule involved in immune regulation and defence. COX-2 catalyzes the production of prostaglandins, which represents an important step in the inflammatory process. PGE₂ production in LPS-treated macrophages is primarily due to the transcriptional activation of the COX-2 gene. Thus, NO and COX-2 inhibitors are considered potential anti-inflammatory agents. Therefore, in this study, we investigated the effects of D-limonene on the production of pro-inflammatory cytokines as well as NO and COX-2 in RAW 264.7 macrophages. After exposure to LPS, RAW 264.7 cells displayed elevated production of pro-inflammatory cytokines and inflammatory mediators. D-Limonene, at concentrations not producing evident cytotoxicity, dramatically prevented the production of NO, PGE₂, IL-1β, IL-6,
and TNF-α, as well as the expression of iNOS and COX-2 proteins in LPS-treated RAW 264.7 cells, suggesting that it down-regulated pro-inflammatory mediators and cytokines. However, although the anti-inflammatory effects of d-limonene were identified, the mechanisms of action underlying these effects should be evaluated in further studies. It is generally accepted that many intracellular signal pathways participate in the LPS-induced activation of macrophages and resultant production of pro-inflammatory cytokines/mediators, and that nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) pathways play key roles. NF-κB is one of the most ubiquitous transcription factors and coordinates the expression of pro-inflammatory enzymes and cytokines, making it a popular target for the development of potent inhibitors as novel anti-inflammatory drugs. Given the role of NF-κB in modulating the expression of inflammatory mediators, it is possible that the inhibitory effects of d-limonene involve the inhibition of the NF-κB signalling pathway. However, we cannot exclude the involvement of other transcription factors.

Fig. 3 Inhibitory Effects of d-Limonene on TNF-α, IL-1β, and IL-6 Production in RAW 264.7 Cells.
Cells (1.5 × 10⁵ cells/mL) were stimulated by LPS (1 μg/mL) for 24 h in the presence of d-limonene (0.01%, 0.02%, and 0.04%), 2-amino-4-methylpyridine (20 μM), and dexamethasone (DEX; 20 μM). Supernatants were collected, and TNF-α (A), IL-1β (B), and IL-6 (C) concentrations were determined by ELISA. Values are the mean ± SEM of triplicate experiments. *P < 0.05; **P < 0.01
In conclusion, the present study demonstrates the potential cellular anti-inflammatory activity of D-limonene in LPS-activated RAW 264.7 cells. The results indicate that production of NO, PGE2, IL-1β, IL-6, and TNF-α is reduced by D-limonene at the protein level in activated macrophages. Furthermore, assessment of cell viability upon treatment of HaCaT human keratinocytes with D-limonene at effective concentrations revealed no apparent cytotoxicity of the terpene, confirming its suitability for use in cosmetic applications. Thus, D-limonene is a promising candidate anti-inflammatory agent with potential for use in skin care cosmetics or in the treatment of skin diseases such as acne and atopic dermatitis.

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