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α-Phellandrene, a cyclic monoterpene, attenuates inflammatory response through neutrophil migration inhibition and mast cell degranulation

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ABSTRACT

Aims: We aimed to investigate the modulating effect of α-phellandrene on neutrophil migration and mast cell degranulation processes.
Main methods: Male Wistar rats or Swiss mice were treated p.o. with vehicle (3% Tween 80, p.o.), α-phellandrene (50, 100, or 200 mg/kg, p.o.), or dexamethasone (0.5 mg/kg, p.o.) 1 h before carrageenan injection. Then, the neutrophil migration in 6-day-old air pouches or peritoneal cavities. The leukocyte rolling and adhesion were measured in real time and assessed by intravital microscopy. ELISA was used to detect TNF-α and IL-6 in peritoneal lavage. Compound 48/80–induced mast cell degranulation was assessed in mesenteric rat tissues.
Key findings: In all the tested doses, α-phellandrene prevented carrageenan-induced neutrophil accumulation ($P < 0.05$). As detected by intravital microscopy, α-phellandrene also inhibited leukocyte rolling and adhesion, as well as significantly inhibited the production of the pro-inflammatory cytokines TNF-α and IL-6. Moreover, the degranulation of compound 48/80–induced mast cells was also inhibited by α-phellandrene ($P < 0.001$).
Significance: These results suggest that α-phellandrene plays an important role as an anti-inflammatory agent through neutrophil migration modulation and mast cell stabilization.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids are among the most commonly prescribed medications worldwide for several acute and chronic pains and inflammatory conditions [1]. However, the long-term use of these drugs is broadly related to serious adverse side effects, including gastric ulceration and perforation, bleeding, liver and kidney toxicities, hypertension, myocardial infarction, opportunistic infections, hyperglycemia, and several other toxicities, depending on the drugs and doses used [2].

In this context, the introduction of more efficacious and safe drugs is highly demanded. Over the past decades, natural products have been an important source of complex mixtures and new lead molecules with anti-inflammatory properties [3–5]. Essential oils (EOs) are complex volatile natural product mixtures produced as secondary metabolites in aromatic plants [6]. These volatile compound mixtures exert different biological actions on humans, animals, and other plants [6] and are rich in terpenoid constituents, such as mono- and sesquiterpenes with anti-inflammatory potential [7].

α-Phellandrene (5-isopropyl-2-methyl-1,3-cyclohexadiene) is a cyclic monoterpene found in the essential oils of several plants, such as *Schinus terebinthifolius* Raddi (15.7%) [8], *Solanum erianthum* D. Don (17.5%) [9], *Thymus kotschyanus* Boiss and Hohen (10.8%) [10], *Cupressus atlantica* Gaussen (5.5%) [11], *Anethum graveolens* L. (32%) [12], and *Myrica gale* L. (8%) [13]. Furthermore, the anti-inflammatory and antinociceptive properties of some plants, for instance, *Schinus polygamus* (Cav.) Cabr. [14] and *Zingiber officinale* Roscoe [15], have been associated with the presence of α-phellandrene. Accordingly, the antinociceptive effect of α-phellandrene has been confirmed more recently [16]. In addition, a low systemic toxicity for α-phellandrene (5.7 g/kg in rats) has been reported in the literature [17].

The mechanisms involved in the suggested anti-inflammatory activity of α-phellandrene [16] are poorly understood however. Therefore,

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we aimed to investigate the modulating effect of α-phellandrene on neutrophil migration and mast cell degranulation processes.

2. Material and methods

2.1. Animals

Male Wistar rats (180–220 g) and Swiss mice (25–35 g) were obtained from the Local Animal Facility of the Federal University of Piauí or the Federal University of Ceará and divided into experimental groups ($n = 6$). The animals were kept in a temperature-controlled room under dark/light cycles with free access to food and water; they were deprived of food 18 h before the experiments. After the experimental procedures, the animals were euthanized by sodium thiopental overdose (100 mg/kg, i.p.). The local Ethics Committee for Animal Experiments approved the whole protocol, which strictly followed the NIH guidelines for the care and use of laboratory animals (CEEA-PI 008/12).

2.2. Chemicals

α-Phellandrene, cyproheptadine, λ-carrageenan, compound 48/80, toluidine blue, and ketotifen were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tween 80 (Merck AG, Darmstadt, Germany). Dexamethasone (Decadron®; São Paulo, SP, Brazil) was purchased from Aché (São Paulo, SP, Brazil). Ketamine chloride and xylazine chloride were purchased from Syntec (Cotia, São Paulo, Brazil). The chemicals and other solutions used were all of analytical grade. α-Phellandrene was dissolved in 3% Tween 80 and diluted in a saline solution (NaCl 0.9%) just before use. The concentrations of α-phellandrene and other drugs were adjusted for treatment to yield a volume of 10 mL/kg.

2.3. In vivo neutrophil migration induced by carrageenan in air pouch cavities

Six-day-old rat skin air pouches were produced as described by Ribeiro et al. [18]. The rat backs were shaved and injected subcutaneously with 20-mL sterile air. Three days later, 10-mL sterile air was again injected to maintain the pouch patency. Six days after the initial air injection, the pouches were used. Sterile PBS or 1% carrageenan (100 μL/pouch) was injected into 6-day-old rat air pouches previously treated with vehicle (3% Tween 80, 10 mL/kg, p.o.), α-phellandrene (50, 100, or 200 mg/kg, p.o.), or dexamethasone (0.5 mg/kg, p.o.). 4 h after the carrageenan injection into the air pouches, the animals were killed, and the air pouches were washed by injecting 10-mL PBS containing 5 U/mL heparin. Cell counts were carried out as described by Ribeiro et al. [18]. The results were reported as the number of leukocytes/mm^3 of exudate.

2.4. Carrageenan-induced peritonitis

The mice were treated with saline (10 mL/kg, p.o.), vehicle (3% Tween 80, 10 mL/kg, p.o.), α-phellandrene (50, 100, or 200 mg/kg, p.o.), or dexamethasone (0.5 mg/kg, p.o.). After 1 h, peritonitis was induced in the mice by intraperitoneal injection of carrageenan diluted in sterile saline (500 μg/500 μL/cavity). The mice were sacrificed 4 h later, and the peritoneal cavity was washed with 3-mL sterile saline containing 5-IU/mL heparin. A sample of fluid (20 μL) recovered from the peritoneal cavity was diluted (1:20) in Turk's solution (380 μL). Then, the total leukocyte count was determined by using a Neubauer chamber, as described by Ribeiro et al. [18]. The differential leukocyte cell count was carried out on cytocentrifuge slides stained with the May-Grunwald–Giemsa solution [19]. The results were expressed as the peritoneal lavage × 10^5/mL neutrophils. In addition, a sample of fluid recovered from the peritoneal cavity was used to measure the pro-inflammatory levels of TNF-α and IL-6 cytokines, as described below.

2.5. Cytokine (TNF-α and IL-6) detection by ELISA

The TNF-α and IL-6 concentrations in peritoneal lavage were measured by using ELISA. Briefly, primary anti-TNF-α or anti–IL-6 antibodies (2 μg/mL) were incubated overnight at 4 °C in 96-well microtiter plates. After the plates were blocked, several sample dilutions and standard curves were added and incubated at 4 °C for 24 h. The plates were washed with buffer, after which secondary anti-TNF-α or anti–IL-6 polyclonal biotinylated antibodies (1:1000 dilution with 1% BSA) were added to the wells. After further incubation at room temperature for 1 h, the plates were washed, and 100-μL streptavidin-HRP at 1:200 dilution was added, followed by 100-μL substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine; R&D Systems, USA). The plates were then incubated in the dark at room temperature for 20 min. The enzyme reaction was stopped with 2N H_2SO_4, and the absorbance was measured at 450 nm. The results were expressed as pg/mL peritoneal exudate and reported as mean ± SE.

2.6. Real-time measurement of leukocyte rolling and adhesion by intravital microscopy

Intravital microscopy was used to determine the extent of leukocyte rolling and adhesion in the mesenteric microcirculation, as previously described [20]. The mice were first treated with vehicle (3% Tween 80, 10 mL/kg, p.o.), α-phellandrene (50 mg/kg, p.o.), or dexamethasone (0.5 mg/kg, p.o.); after 1 h, they received an intraperitoneal injection of carrageenan diluted in saline (500 μg/500 μL/cavity). A saline-treated control group (10 mL/kg, p.o.) was included. 4 h after the carrageenan injection, the mice were anesthetized with ketamine/xylazine (100/10 mg/kg, i.p.; União Química, Brazil). After laparotomy, the mesenteric tissue was exteriorized, placed in a temperature-controlled (37 °C) transilluminated platform, and observed by intravital microscopy. The images were recorded at ×200 magnification. The preparation was kept moist and warm during the whole procedure by irrigation with warmed (37 °C) Ringer Locke's solution (pH 7.2–7.4) containing 1% gelatin. Third-order venules, defined according to their branch-order location within the microvascular network, were selected for the study. These vessels corresponded to post-capillary venules and had diameters of 12–18 μm. Rolling leukocytes were defined as white blood cells that moved at a lower velocity than erythrocytes in the same stream in a 100-μm-long segment in a venular bloodstream, at a sufficiently slow pace to be individually visible [21,22]. The number of rolling leukocytes/100 μm of venule was determined at 10-min intervals. Leukocytes were considered to be adherent to the venular endothelium when they remained stationary for over 30 s. The number of adherent cells/100 μm^2 venule was also determined. The cells were counted in recorded images, with five different fields used for each animal to avoid sampling variability [23]. The data were then averaged for each animal.

2.7. Mast cell degranulation in mesenteric tissue

The rats were given saline (10 mL/kg, p.o.), vehicle (3% Tween 80, 10 mL/kg, p.o.), α-phellandrene (50, 100, or 200 mg/kg, p.o.), or ketotifen (2 mg/kg, p.o.) 2 h before euthanasia. The mesenteric tissues were then carefully collected from the respective groups and placed in petri dishes containing Ringer-Locke's fluid (10 mL). Mast cell degranulation was induced by tissue incubation with compound 48/80 (final concentration, 0.4 μg/mL) for 30 min. A control group was added, in which compound 48/80 was replaced by saline. Hydrated tissue sections were immersed in 0.1% toluidine blue solution (in 0.9% sodium chloride), followed by extensive rinsing in deionized water [24].

The number of mast cells in the inflammatory site is known to have a tendency to decrease as a result of the degranulation phenomenon, whereby chemically active substances (histamine, heparin, and serotonin) that have an affinity for toluidine are released [25]. Therefore, it was considered those granulated mast cells which have extravasation