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Original article

2-Phthalimidethanol and 2-phthalimidethyl nitrate inhibit mechanical allodynia, neutrophil recruitment and cytokine and chemokine production in a murine model of articular inflammation

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ABSTRACT

Background: Phthalimide analogs have been shown to exhibit anti-inflammatory, analgesic and immunomodulatory activities in different preclinical assays. This study aimed to investigate the potential role of 2-phthalimidethanol (PTD-OH) and 2-phthalimidethyl nitrate (PTD-NO) in a murine model of antigen-induced articular inflammation.
Methods: Articular inflammation was induced by intra-articular injection of methylated bovine serum albumin (mBSA) in the knee joint of immunized male C57BL/6J mice. The animals were pre-treated with PTD-OH or PTD-NO (500 mg/kg, *per os*, – 1 h). Nociceptive threshold was measured using an electronic von Frey apparatus. The total number of leukocytes in the synovial cavity was determined. Concentrations of tumor necrosis factor (TNF)-α and CXCL-1 and myeloperoxidase (MPO) activity were determined in periarticular tissue.
Results: Both PTD-OH and PTD-NO inhibited at similar extent the mechanical allodynia, neutrophil recruitment to the synovial cavity and periarticular tissue and TNF-α and CXCL-1 production induced by intra-articular challenge with mBSA in immunized mice.
Conclusions: PTD-OH and PTD-NO exhibit a marked activity in a murine model of antigen-induced articular inflammation in immunized animals. These results reinforce the interest in the investigation of phthalimide analogs devoid of the glutarimide ring as candidates to analgesic and anti-inflammatory drugs.

Introduction

Many preclinical assays have shown that phthalimide analogs exhibit anti-inflammatory, analgesic, immunomodulatory, anti-angiogenic and antineoplastic activities [1–4]. These activities have been associated with reduced production of inflammatory cytokines, inhibition of cyclooxygenases and nitric oxide (NO) synthases, among other effects [5–17].

We have recently demonstrated that two phthalimide analogs, 2-phthalimidethanol (PTD-OH) and 2-phthalimidethyl nitrate (PTD-NO), exhibit activities in models of acute pain and inflammation induced by carrageenan and formaldehyde [18,19]. These two phthalimide analogs markedly differ from the prototypical phthalmide analog, thalidomide, as they are devoid of the glutarimide ring (Fig. 1). There is evidence that the glutarimide ring may contribute to the teratogenic activity of some phthalimide analogs, thus justifying the increasing interest in the evaluation of the activities of phthalimide analogs devoid of this functional group [20,21].

In the present study, aiming to further characterize the pharmacological profile of the two phthalimide analogs, PTD-OH and PTD-NO, we evaluated their activities in a murine model of antigen-induced articular inflammation. The administration of antigen into the knee joint of immunized mice induces a marked inflammatory response characterized by accumulation of leukocytes and the release of inflammatory cytokines [22,23]. The

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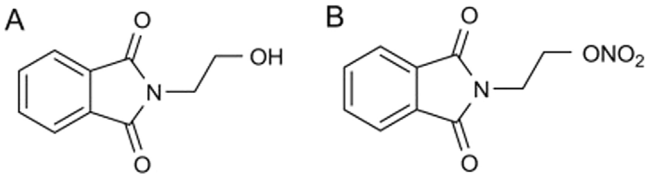

Fig. 1. Chemical structures of (a) 2-phthalimidethanol (PTD-OH) and (b) 2-phthalimidethyl nitrate (PTD-NO).

evaluation of the activities of the two phthalimide analogs in this experimental model may provide information about their potential as candidates in the treatment of chronic inflammatory diseases that are driven primarily by immune processes and cause extensive bone and cartilage erosion such as rheumatoid arthritis.

Materials and methods

Animals

Eight-week-old male C57BL/6J mice were used. The animals had free access to food and water and were maintained in a room with a 12 h light-dark cycle for at least three days before the experiment to allow acclimatization. The experiments were carried out at a room temperature of 27 °C. This temperature was used as the thermoneutral zone for mice and rats ranges between 26 and 34 °C, a temperature range that markedly differs from that of standard laboratory environments which could be stressful and affect many aspects of physiology and behavior of the rodents [24,25]. All experiments were conducted according to the ethical guidelines for investigation of experimental pain in conscious animals and approved by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (CETEA – UFMG, Protocol 48/2011) [26].

Drugs

PTD-OH and PTD-NO were synthesized at the Department of Chemistry, Federal University of Minas Gerais, as described previously [18]. Methylated bovine serum albumin (mBSA) and complete Freund adjuvant (CFA) were purchased from Sigma, USA. Suspensions of PTD-OH and PTD-NO were prepared in 0.5% carboxymethylcellulose (CMC) sodium salt (Sigma, USA) suspension in saline immediately before the experiments. The dose of PTD-OH (406 mg/kg) was equimolar to that of PTD-NO (500 mg/kg). Suspensions were administered *per os (po)* in a volume of 12 ml/kg.

Induction of articular inflammation

The animals were immunized through an intradermal injection at the base of the tail of 500 μg of mBSA in an emulsion of saline (50 μl) and CFA (50 μl) at day 0 [27]. Fifteen days later, mice were challenged with an intra-articular *(ia)* injection of mBSA (10 μg in 10 μl sterile saline) in the knee joint. For the control group, mice received an *ia* injection in the knee joint with 10 μl phosphate-buffered saline (PBS).

Evaluation of mechanical allodynia

Nociceptive threshold was measured by using an electronic von Frey apparatus (electronic pressure-meter, Insight, Brazil), according to the method described by Cunha et al., with minor modifications [28]. The mice were kept individually in acrylic cages (10 × 10 cm with 18 cm-high walls) whose floor was a metal grid. The hand-held force transducer, fitted with a polypropylene tip (0.5 mm^2) filament, was gradually pressed on the plantar surface of the right hind paw. The test consisted of evoking a hind paw flexion reflex. The endpoint was characterized by the removal of the paw usually followed by flinching movements. The pressure that induced the paw withdrawal was automatically recorded. The paw withdrawal threshold was obtained by averaging five measurements. Mice were habituated to the experimental apparatus daily, approximately 60 min a day, for four days before the experiments. On the experimental day, baseline paw withdrawal threshold of each animal was determined. After that, the animals were divided into the experimental groups in such a way that the mean paw withdrawal thresholds of the different groups were similar. mBSA (10 μg, 10 μl) was injected *via ia* route 1 h after *po* administration of PTD-OH (406 mg/kg), PTD-NO (500 mg/kg) or vehicle (CMC 0.5%, 12 ml/kg). Nociceptive threshold of each animal was again measured at 24 h after *ia* injection. The results were expressed as the absolute paw withdrawal threshold (in grams).

Evaluation of leukocytes recruitment

Twenty-four hours after *ia* injection of mBSA, the mice were euthanized. The knee cavity was washed with PBS (2 × 5 μl) and periarticular tissue was removed for evaluation of cytokine and chemokine concentrations and myeloperoxidase (MPO) activity. The total number of leukocytes in the synovial cavity was determined by counting in a Neubauer chamber after staining with Turk's solution. Differential counts were obtained from cytospin (Shandon III, Thermo Shandon, Frankfurt, Germany) preparations by evaluating the percentage of each leukocyte on a slide stained with May–Grünwald–Giemsa. The number of neutrophils in the tissue was indirectly determined by quantifying MPO activity. The pellet samples were frozen and thawed three times in liquid nitrogen. Upon thawing, the samples were centrifuged (10,000 r.p.m.) for 15 min at 4 °C and 25 μl of the supernatant were used for the MPO assay. The enzymatic reaction was assessed by adding 25 μl of 1.6 mM tetramethylbenzidine prepared in dimethylsulfoxide. The mixture was incubated for 5 min at 37 °C. Then, 100 μl of 0.02% hydrogen peroxide were added, followed by incubation for 5 min at 37 °C. After 10 min, the reaction was stopped by adding 100 μl of 1 M sulfuric acid. The absorbance was measured at 450 nm. Previously, a standard curve indicating the range of linear relationship between neutrophils and optical density (O.D.; expressed in relative units) was prepared. Results were expressed as number of neutrophils per 100 mg of tissue.

Evaluation of tumor necrosis factor-α (TNF-α) and chemokine (C-X-C motif) ligand 1 (CXCL-1) concentrations in periarticular tissue

The concentrations of TNF-α and CXCL-1 were measured in periarticular tissues using ELISA assays, following the instructions supplied by manufacturer (DuoSet kits, R&D Systems, Minneapolis, USA). Briefly, the tissue (0.1 g) was homogenized in 1.9 ml PBS containing Tween-20 (0.05%), phenylmethylsulphonyl fluoride (0.1 mM), benzamethonium chloride (0.1 mM), EDTA (10 mM), aprotinin A (2 μg/ml) and BSA (0.5%) and centrifuged (10,000 r.p.m.) for 15 min at 4 °C.

The supernatant and the pellet (resuspended in 0.5% hexadecyltrimethyl ammonium bromide buffer, pH 5.4) were stored separately at −70 °C until further analysis of TNF-α and CXCL-1 concentrations and MPO activity. The supernatant samples were