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Anti-inflammatory activity of abietic acid, a diterpene isolated from *Pimenta racemosa* var. grissea

M. A. Fernández, M. P. Tornos, M. D. García, B. de las Heras, A. M. Villar and M. T. Sáenz

Abstract

The anti-inflammatory activity of abietic acid, a diterpene isolated from *Pimenta racemosa* var. grissea (Myrtaceae), was evaluated in-vivo and in-vitro. This compound significantly inhibited rat paw oedema induced by carrageenan in a time- and dose-dependent manner, and mouse ear oedema induced by 12-*0*-tetradecanoylphorbol acetate, after oral or topical administration. The inhibition of myeloperoxidase enzyme showed that its topical activity was influenced by neutrophil infiltration into the inflamed tissues (ears). In addition, the effect of abietic acid on some macrophage functions was analysed in-vitro. Non-toxic concentrations of abietic acid inhibited prostaglandin E_2 (PGE_2) production in lipopolysaccharide-treated macrophages, whereas nitrite, tumour necrosis factor α and interleukin-1β production were only weakly affected by this diterpene. PGE_2 production from A23187-stimulated macrophages was only inhibited at high doses (100 μM) and it failed to modify leukotriene C_4 production. These results indicate that abietic acid exerts in-vivo anti-inflammatory activity after oral or topical administration and has partial ability to prevent the production of some inflammatory mediators.

Introduction

The genus *Pimenta* (Myrtaceae) is widely represented in the Caribbean region and includes a large number of species which are traditionally used in local folk medicine. Decoctions of leaves from *Pimenta racemosa* var. grissea, commonly known as 'ozua' (Germosen Robineau 1995) are used for their anti-inflammatory and analgesic properties (Duke 1986; Robineau 1991). We have been investigating the phytochemistry of this plant species and the diterpene abietic acid was isolated from the methanolic extract obtained from the leaves (Tornos 2000; Figure 1). Diterpenoids display a wide range of pharmacological activity (Alcaraz & Rios 1991), including anti-inflammatory activity (Chen et al 1988; Wu et al 1988). Abietic acid had also been isolated from *Pygeum africanum*, a species used in phytotherapy to treat prostatic hypertrophy related-disturbances (Capasso & Grandolini 1996), but there are to our knowledge no reports on its anti-inflammatory activity.

Macrophages play a crucial role in modulating the initiation and perpetuation of the inflammatory response. Activation of these cells promotes the synthesis and release of eicosanoids such as prostaglandins, leukotrienes and nitric oxide (NO), mediators involved in the inflammatory onset (DeWitt 1991; Moncada et al 1991;

Department of Pharmacology, Faculty of Pharmacy, C/Professor García Gonzalez s/n, 41012-Sevilla, Spain

M. A. Fernández, M. P.Tornos, M. D. García, M. T. Sáenz

Department of Pharmacology, Faculty of Pharmacy, UCM, Avda Complutense s/n, 28040 Madrid, Spain

B. de las Heras, A. M. Villar

Correspondence: M. D. García, Department of Pharmacology, Faculty of Pharmacy, University of Seville, C/Professor García Gonzalez s/n, 41012-Seville, Spain. E-mail: gimenez@fafar.us.es

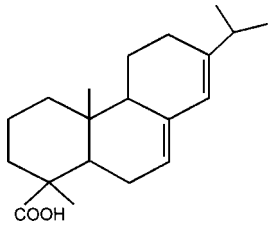

Figure 1 Chemical structure of abietic acid.

Dugas et al 1995; MacMicking et al 1997; Albina & Reichner 1998), and proinflammatory cytokines, including tumour necrosis factor α (TNF-α) and interleukin-1β (IL-1β) (Moilanen & Vapaatalo 1995; Vane et al 1998).

The purpose of the present study was to investigate the anti-inflammatory activity of abietic acid on different experimental models of inflammation in-vivo, and its ability to influence the generation of some mediators and cytokines related to the inflammatory process to determine the mechanisms underlying its anti-inflammatory activity.

Materials and Methods

Materials

Abietic acid was isolated and identified from the methanolic extract of leaves from *P. racemosa* var. grissea (Tornos 2000). Cell culture reagents were purchased from Life Technologies (Barcelona, Spain). Enzyme-linked immunosorbent assay (ELISA) kits for determination of prostaglandin E_2 (PGE_2) and leukotriene C_4 (LTC_4) were provided by Cayman Chemical Co (USA). TNF-α and IL-1β ELISA kits were from Amersham Iberica (Madrid, Spain). All other reagents were purchased from Sigma Chemical Co. (USA).

Carrageenan-induced rat paw oedema

Swelling was induced by the technique of Winter et al (1962). Male Wistar rats (200–250 g) were divided into groups of six. Drugs and vehicle (saline solution) were administered orally 1 h before inflammation was induced on the right hind paw by the subplantar injection of carrageenan (0.1 mL, 1% w/v). Paw volume (mL) was measured with a plethysmometer (LI-7500, Letica) before carrageenan injection and 1, 3 and 5 h later. The oedema was reported as the difference between the initial and the final paw volume. The anti-inflammatory effect was expressed as the percentage inhibition compared with vehicle-treated animal. A reference group was treated with indometacin (25 mg kg^{-1}).

12-*O*-Tetradecanoylphorbol acetate (TPA)-induced mouse ear oedema

Oedema was induced by topical application of 2.5 μg TPA in 20 μL acetone to the right ear of male Swiss mice (25 ± 5 g). The left ear (control) received only acetone. Abietic acid was applied topically in acetone (0.25, 0.5 and 1 mg/ear) before TPA administration. A reference group was treated with indometacin (0.5 mg/ear). After 4 h, mice were killed by cervical dislocation and a 6-mm biopsy was obtained from both ears and weighed. The increase in the weight of the right ear punch over that of the left indicated the oedema (Carlson et al 1989). Ear sections were homogenized in 750 μL saline, and after centrifugation at 10 000 *g* for 15 min at 4°C, myeloperoxidase (MPO) activity was measured in supernatants as described previously by De Young (1989).

Production of nitrite, PGE_2, TNF-α and IL-1β in lipopolysaccharide (LPS)-activated macrophages

Swiss male mice were injected intraperitoneally with 1 mL thioglycolate broth 4 days before use (Lopez-Collazo et al 1998). Peritoneal macrophages were prepared as follows: light ether-anaesthetized mice ($n = 4$–6) were killed by cervical dislocation and injected intraperitoneally with 5 mL sterile RPMI 1640 medium. The peritoneal fluid was carefully aspirated to avoid haemorrhage and kept at 4°C to prevent the adhesion of macrophages to the plastic. After centrifugation at 200 *g* for 10 min at 4°C, the cell pellet was washed twice with 45 mL ice-cold phosphate-buffered saline (PBS). Cells were seeded at 1×10^6 mL^{-1} in RPMI 1640 supplemented with 10% fetal calf serum (FCS). After incubation for 2 h at 37°C in 5% CO_2, non-adherent cells were removed by extensive washing with PBS. Cells were incubated with *Escherichia coli* LPS (0.5 μg mL^{-1}) with or without test compounds at 37°C for 24 h. Culture supernatants were used for measurements of NO, PGE_2, TNF-α and IL-1β. NO released was assessed spectrophotometrically as the stable end product nitrite in the culture supernatant with the Griess reagent (Green et al 1982). PGE_2, TNF-α and IL-1β were measured by ELISA kits according to the manufacturer's instructions.

Production of PGE_2 and LTC_4 in calcium ionophore A23187-stimulated macrophages

Macrophages from Swiss male mice were collected after peritoneal lavage with PBS, as previously described by De las Heras & Hoult (1994). Briefly, cells were suspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS and seeded into 24-well plates at a concentration of 0.5×10^6 cells mL^{-1}. After adhering to plates (2 h at 37°C in an atmosphere of 5% CO_2/95% air), non-adherent cells were washed off and then cultured in DMEM without FCS. Cells were pretreated for 30 min at 37°C with test compounds or vehicle and then stimulated for a further 2 h by adding calcium ionophore A23187 (final concn 1 μM). The medium was withdrawn from each well and levels of PGE_2 and LTC_4 quantified using ELISA kits.

Cytotoxicity assays

Lactate dehydrogenase (LDH) was determined by measuring the rate of oxidation of nicotinamide adenine dinucleotide (NADH) (Bergmeyer & Bernt 1974). Cells treated with Triton X-100 (0.5%) were used to measure the total cellular content of LDH. Cells in 96-well plates were incubated at 37°C with 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT; 0.2 mg mL^{-1}) for 60 min. The medium was removed by aspiration and the cells were treated with dimethylsulfoxide (DMSO) (100 μL). The absorbance at 550 nm was read in a microtiter plate reader (Pang 1996).

Data analysis

The results are presented as mean ± s.e.m. (analysis of variance followed by Dunnet's test). Differences were considered significant when $P < 0.05$.

Results

Carrageenan-induced oedema

The effect of abietic acid on carrageenan-induced oedema is shown in Figure 2. Oral administration of this diterpene (50 and 100 mg kg^{-1}) significantly and dose-dependently inhibited oedema formation, achieving the greatest inhibitory effect (39 and 45% inhibition at doses of 50 and 100 mg kg^{-1}, respectively) 3 h after carrageenan injection. The reference drug, indometacin, had a similar effect, with a maximum response (59% inhibition) at 3 h.

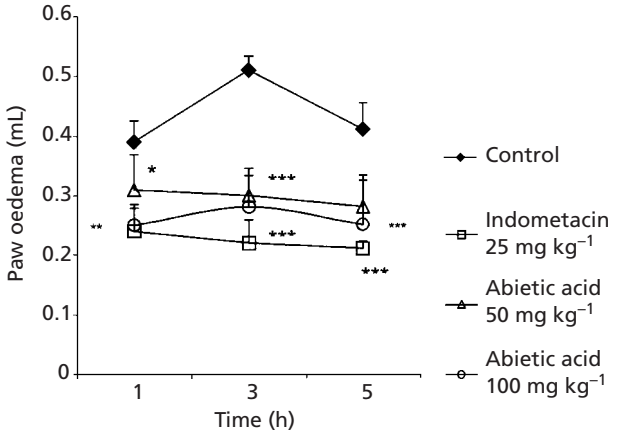

Figure 2 Effects of abietic acid and indometacin on carrageenan-induced paw oedema. Values represent means ± s.e.m. from six rats. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (analysis of variance followed Dunnet's test).

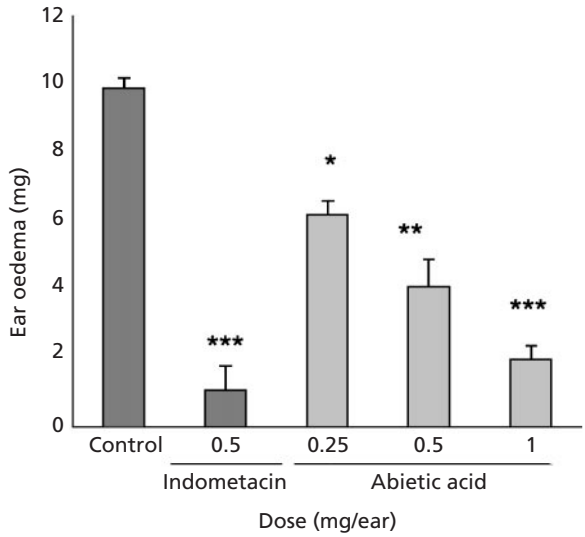

Figure 3 Effects of abietic acid and indometacin on TPA-induced mouse ear oedema. Compounds were administered topically at the time of TPA application (2.5 μg mL^{-1}). Values are expressed as mean ± s.e.m. from six mice. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (analysis of variance followed Dunnet's test).

TPA-induced oedema

Topical application of this compound significantly inhibited the development of swelling in a dose-dependent manner (46.4, 58.6 and 74.8% inhibition at doses of 0.25, 0.5 and 1 mg/ear, respectively, compared with control animals; Figure 3). The observed topical anti-inflammatory activity was confirmed by quantifying levels of the neutrophil specific marker MPO, which was

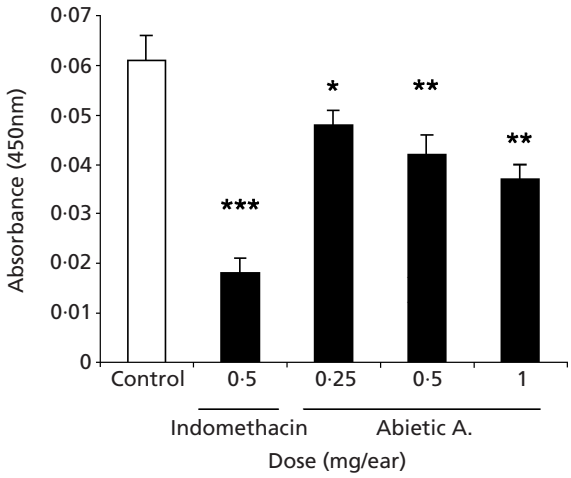

Figure 4 Effects of abietic acid and indometacin on myeloperoxidase activity (increase in absorbance at 450 nm). Values are expressed as means ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (analysis of variance followed Dunnet's test).

extracted from ear biopsies. These studies revealed that abietic acid significantly inhibited MPO activity by 21.3, 31.1 and 39.3% at the doses tested (0.25, 0.5 and 1 mg/ear; Figure 4). Indometacin effectively inhibited oedema formation and MPO activity, as expected.

Production of nitrite, PGE_2, TNF-α and Il-1β in LPS-stimulated macrophages

Abietic acid did not affect mitochondrial reduction of MTT after 24 h challenge to formazan or LDH release at the concentrations tested (data not shown).

As can be seen in Table 1, incubation of macrophages with LPS (0.5 μg mL^{-1}) greatly increased the production of NO, PGE_2 and cytokines. Abietic acid was a weak inhibitor of NO release as a significant reduction of nitrite production was only observed at the highest concentration tested (100 μM, 54% inhibition). However, when macrophages were stimulated with the same amount of LPS in presence of abietic acid, a significant concentration-dependent inhibition of PGE_2 production was observed. Cytokine production (TNF-α, IL-1β) was significantly reduced by 100 μM abietic acid. Dexamethasone, used as reference compound, potently inhibited the four parameters assayed.

Table 2 Effects of abietic acid and reference drugs on prostaglandin E_2 (PGE_2) and leukotriene C_4 (LTC_4) release (ng mL^{-1}) from calcium ionophore A23187-stimulated peritoneal macrophages.

Treatment	PGE_2	LTC_4
Control	3.2 ± 0.8	3.0 ± 0.3
Cells alone	0.3 ± 0.01	0.2 ± 0.1
Abietic acid (1 μM)	2.5 ± 0.3	3.1 ± 0.2
Abietic acid (10 μM)	2.0 ± 0.1	3.2 ± 0.1
Abietic acid (100 μM)	0.9 ± 002**	2.9 ± 0.3
Indometacin (10 μM)	0.4 ± 0.01***	ND
Nordihidroguayaretic acid (10 μM)	ND	1.2 ± 1.0**

Results are expressed as mean ± s.e.m., n = 8, from two separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs A23187 (analysis of variance followed Dunnet's test). ND, not determined.

Production of PGE_2 and LTC_4 release in A23187-stimulated macrophages

Addition of calcium ionophore A23187 to mouse peritoneal macrophages caused the generation of nanogram amounts of eicosanoids via both cycloxygenase-1 (COX-1) and 5- lipoxygenase (5-LOX) pathways, measured in

Table 1 Effect of abietic acid and dexamethasone on nitrite, prostaglandin E_2 (PGE_2), tumour necrosis factor α (TNF-α) and interleukin-1β (IL-1β) production by lipopolysaccharide (LPS)-stimulated peritoneal macrophages.

Treatment	Nitrite μM	PGE_2 (ng mL^{-1})	TNF-α (ng mL^{-1})	IL-1β (pg mL^{-1})
Cells alone	4.6 ± 0.5	0.07 ± 0.1	3.4 ± 0.3	22.0 ± 0.001
LPS	43.6 ± 2.6	3.74 ± 0.3	16.2 ± 1.4	200.0 ± 2.3
Abietic acid (1 μM)	41.4 ± 2.5	2.45 ± 0.9	19.3 ± 1.8	180.1 ± 2.1
Abietic acid (10 μM)	37.2 ± 1.9	1.65 ± 0.7*	15.9 ± 1.7	170.0 ± 2.2
Abietic acid (100 μM)	20.1 ± 0.7**	1.25 ± 0.3**	8.9 ± 1.1**	110.0 ± 3.3*
Dexamethasone (1 μM)	19.0 ± 0.5**	0.8 ± 0.1**	2.0 ± 0.4***	30.0 ± 3.1***

Results are expressed as mean ± s.e.m., n = 8, from two separate experiments. Abietic acid was added to cells 30 min before LPS and incubation continued for 24 h. $^{*}P < 0.05$, $^{**}P < 0.001$ vs LPS (analysis of variance followed Dunnet's test).

terms of immunoassayable PGE_2 and LTC_4, respectively. Validation of this system for the identification of inhibitors of the two divergent pathways of arachidonate metabolism was obtained using indometacin, a well-characterized COX-1 inhibitor, and nordihidroguayaretic acid, a known inhibitor of 5-LOX, as potently reduced LTC_4 synthesis. Pre-treatment of cells with abietic acid resulted in a decrease of PGE_2 levels at the highest dose 100 μM. As expected, indometacin strongly reduced this parameter. Abietic acid did not show any inhibitory effect on LTC_4 release (Table 2).

Discussion

Previous studies have demonstrated that diterpenoids display a wide range of pharmacological activity, including antineoplasic, antimicrobial and anti-inflammatory activity (Alcaraz & Rios 1991; Singh 1999). The present study focused on the anti-inflammatory activity of abietic acid on two experimental models of acute inflammation in-vivo (carrageenan-induced paw oedema and TPA-induced ear oedema), as well as its influence on the release of some inflammatory mediators.

The present study showed that abietic acid inhibited experimental acute inflammation after topical or oral administration. Carrageenan-induced oedema in the rat hind paw is one of the most commonly used models of inflammation. It is highly sensitive to non-steroidal anti-inflammatory drugs and it has long been accepted as a useful phlogistic tool for investigating new anti-inflammatory drugs. The initial phase of carrageenan paw oedema is mediated by histamine and serotonin, and the mediators in the later phase are suspected to be arachidonate metabolites producing oedema after mobilization of neutrophils. It seems that the primary effect of carrageenan as an inflammatory agent is the activation of phospholipase A_2 (PLA_2) though its cytotoxic effect may initiate further inflammatory action. Inhibitors of arachidonate cycloxygenase are much more effective than those of arachidonate lipoxygenase in inhibiting carrageenan-induced inflammation (Lo et al 1987). Abietic acid significantly inhibited this oedematous response over time at all doses assayed. However, the greatest inhibitory effect was obtained 3 h after carrageenan injection and this was also observed with the reference drug, indometacin.

Topical application of the phorbol ester TPA provides a skin inflammation model suitable for evaluation of anti-inflammatory drugs. The majority of its activity appears to be involved with or dependent on arachidonate release and metabolism, which may occur simultaneously with the interaction of TPA with a receptor site on protein kinase C (Young & De Young 1989). The inflammation induced by this agent is related to phospholipase A_2 activation and cycloxogenase inhibitors are very effective in this test, indicating a role for prostaglandins (Carlson et al 1989). Abietic acid can act as a topical anti-inflammatory agent as it significantly and dose-dependently inhibited this oedematous response.

MPO, a haemoprotein located in the azurophil granules of neutrophils has been used as a biochemical marker for neutrophil infiltration into tissues. A high level of MPO was noted on TPA-treated ears 4 h after induction of inflammation. Abietic acid significantly reduced this parameter in ear homogenates which suggests that a moderate control on leukocyte migration is involved in the observed topical anti-inflammatory activity.

It is interesting in the context of anti-inflammatory drug design to investigate the possible mechanisms underlying the potentially beneficial activity of natural products to establish their mode of action. To this end, abietic acid was further tested on some macrophage functions.

Activated macrophages secrete eicosanoids, NO and cytokines. Identification of selective inhibitors of inflammatory mediator production by these cells may lead to advances in the control of inflammation. The induction of the enzymes inducible nitric oxide synthase (iNOS) and COX-2 results in a great increase in the synthesis of NO and PGE_2, which contribute to the pathophysiology of different inflammatory processes. An NO-mediated increase in the production of pro-inflammatory prostaglandins in macrophages, that may result in an exacerbated inflammatory response, has been described. Thus, NOS inhibitors could modulate inflammatory processes by the inhibition of NO biosynthesis and related PG generation (Vane et al 1998; Salvemini et al 1995). We focused on the synthesis of NO and PGE_2 after cellular challenge with LPS and we have shown that abietic acid can prevent PGE_2 release into the culture medium, but only reduces nitrite levels at higher doses.

Activated macrophages also secrete proinflammatory cytokines, including TNF-α and IL-1β. Proinflammatory cytokines induce the expression of enzymes such as COX-2 and iNOS in macrophages and other cells, which results in the release of inflammatory mediators. Inflammatory cytokines induce the enzymes responsible for arachidonic acid release and metabolism, thus leading

to increased levels of eicosanoids, and they are involved in the perpetuation of the inflammatory response.

Of the anti-inflammatory drugs in use, non-steroidal anti-inflammatory drugs cannot prevent the progression of chronic inflammation. The inhibition of PG synthesis can potentiate the expression of TNF-α induced by LPS (Bondeson & Sundler 1996). Abietic acid only reduced TNF-α and IL-1β release at the highest dose (100 μM). This effect is similar to that of COX and 5-LOX inhibitors.

We evaluated the effect of abietic acid on A23187-stimulated macrophages, a cellular system generating COX-1 and LOX metabolites. Abietic acid did not show any significant inhibitory effect on PGE_2 and LTC_4 production, which strongly suggests that this diterpene does not affect these arachidonate pathways.

In conclusion, the present study indicates that abietic acid is an effective in-vivo anti-inflammatory agent in acute inflammation models after oral or topical administration, with partial involvement of the inhibitory action on some macrophage functions. Further investigation on its mechanism of action is necessary.

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