



Spain

Original Article

Anti-inflammatory activity of β -sitosterol in a model of oxazolone-induced contact-delayed-type hypersensitivity

Actividad antiinflamatoria del β -sitosterol en un modelo de hipersensibilidad retardada inducida por oxazolona.

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The referees of this article were: Damaris Silveira, Universidad de Brasilia, Brasil and Jorge Alonso, Sociedad Argentina de Fitomedicina, Buenos Aires, Argentina.

Received September 22nd, 2005. Accepted December 19th, 2005.

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Resumen

Hemos comprobado el efecto *in vivo* del β -sitosterol en un modelo de dermatitis de contacto por hipersensibilidad retardada (DTH). Este compuesto fue igualmente ensayado en modelos de liberación de eicosanoides en leucocitos polimorfonucleares de rata y plaquetas humanas estimuladas con ionóforo A23817 en incubaciones de 15 min. El compuesto reduce de manera significativa el edema inducido por oxazolona a las 24 horas, sin mostrar efecto alguno sobre las enzimas de la cascada del ácido araquidónico implicadas en la iniciación del fenómeno inflamatorio, en las condiciones descritas. Los resultados indican que este compuesto puede modular el edema mediado por respuesta celular sin ningún efecto a corto plazo en la cascada del ácido araquidónico. La ubicuidad del β -sitosterol puede explicar, y predecir, el efecto de muchos extractos vegetales en este modelo *in vivo*, y por tanto este dato puede ser de ayuda en procesos de "de-replicación".

Palabras clave: β -Sitosterol; hipersensibilidad retardada; dermatitis de contacto; eicosanoides

Abstract

The *in vivo* effect of β -sitosterol in a model of delayed-type hypersensitivity (DTH) contact dermatitis was tested. The compound was also tested in intact, A23817 stimulated rat-peritoneal polymorphonuclear leukocytes and human platelets for the inhibition of eicosanoids release in 15 min incubations. The compound reduced in a significant manner the oedema induced by oxazolone only at 24 h, without any effect on the enzymes of the arachidonate pathway involved in the onset of the inflammatory process in the conditions above described. The results indicate that this compound can modulate a cell-mediated oedema without any short term *in vitro* effect on the arachidonate pathway of intact cells. The ubiquity of this compound can explain, and predict, the effect of many plant extracts in this *in vivo* model, and so this data could be of help in dereplication processes.

Key words β -Sitosterol; delayed-type hypersensitivity; contact dermatitis; eicosanoids.

Introduction

β -Sitosterol is one of the most ubiquitous substances in plant extracts. In the last 10 years the biological role of phytosterols in human and animal health has been established, with an emphasis in their *in vitro* and *in vivo* immune modulatory activity (1, 2). There are references of the immunomodulatory properties of β -sitosterol and its glucosides (3, 4). It also has been reported that it has *in vivo* topical anti-inflammatory properties in the acute TPA-induced ear oedema in mice but not in the chronic one (5). However it inhibits leukocyte infiltration in both models. Little more is known about the biochemical mechanism of this anti-inflammatory activity. Mat Ali and Houghton (6) showed that this compound inhibits soy 5-lipoxygenase *in vitro*. Keratinocytes also respond to skin irritation and injury by means of a rapid, but transient activation of arachidonic acid (AA) metabolism through the lipoxygenase (LOX) and cyclooxygenase (COX) pathways (7). Topical inflammation involves some chemotactic and chemokinetic agents produced from arachidonic acid by LOX activity, like 12-hydroxy-6,8,11,14-eicosatetraenoic acid (12(S)-HETE) (8) from platelets and 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5(S)-HETE) and leukotriene B₄ (LTB₄) from polymorphonuclear leukocytes (PMNL). They contribute, together with the prostaglandins (PGs) and thromboxanes (TXs) produced by the COX activity, to the inflammatory response of the skin. After this initial phase, LTB₄ is the responsible for the long-term maintenance of the inflammation, and for this reason, in the last years there was an increasing interest on the LTB₄ role and now it seems that it surpass the idea of a chemotactic agent (9). Therefore, an understanding of the effect of this compound on the enzymatic pathways involved in the eicosanoids biosynthesis is important. Thus we decided to explore its anti-inflammatory effects on *in vitro* models of LTB₄ (from 5-lipoxygenase), 12-HHTrE (from cyclooxygenase-1), and 12-HETE (from 12-lipoxygenase) release by mammalian intact cells, as well as on an *in vivo* model of contact delayed hypersensitivity to check its ability to modulate immune mediated topical inflammatory processes.

Material and Methods

Animals

Female Wistar rats (180-200 g) and female Swiss mice (25-30 g) were provided by the animal facility of the Faculty of Pharmacy (University of Valencia). They were housed in

standard environmental conditions. The institutional Ethical Committee of the Faculty of Pharmacy, University of Valencia (Spain) approved all *in vivo* experiments, which were performed according to the guidelines established by the European Union on Animal Care (CEE Council 86/609).

Cells

Human platelets were obtained by diluting human buffy-coats (obtained at the Centre de Transfusions de la Generalitat Valenciana, València, Spain) with PBS (1:3) and centrifuging twice (300 × g, 10 min), discarding the pellets and keeping the platelet rich supernatants. After centrifugation, the resulting pellet was washed twice (1000 × g, 10 min) and finally resuspended in Hank's Balanced Salt Solution (HBSS) with Ca²⁺ (1 mM) and Mg²⁺ (0.5 mM). The differential counting was done by Coulter Counter (Sysmex D-800). Semi-quantitative estimation of the platelets' viability was performed before each experiment by fluorescence microscopy (Nikon®, Japan) staining with acridine orange/ethidium bromide solution.

Rat peritoneal polymorphonuclear leukocytes (PMNLs) were harvested by intraperitoneal injection of glycogen (1 mg/g body weight in 10 mL of PBS, 37 °C) and washed by centrifugation (10 min, 300 × g, room temperature, twice).

The cell viability of the elicited rat peritoneal PMNLs and human leukocytes was assessed before each experiment using the Trypan blue exclusion test. Only harvested cells with viability greater than 95% were used.

Chemicals

Boswellia serrata standardised resin was obtained from H15 tablets (Gufic Chemicals®, India). β -sitosterol, isolated from *Ranunculus sceleratus* L. as described previously (10). Dexamethasone and oxazolone were purchased from Sigma® (St. Louis, USA). All other chemicals were of the highest available analytical grade and purchased from Sigma® (St. Louis, USA) and Merck® (Darmstadt, Germany). Solvents (HPLC grade) were provided by JT Baker® (Deventer, Holland).

Analytical high-performance liquid chromatography (HPLC)-diode array detector (DAD)

HPLC-DAD analysis was performed on a Merck-Hitachi® system equipped with a Pump L-6200, L-7455 Diode Array Detector and Auto Sampler L-7200, injection valve (Reodyne®), loop of 100 μ L, precolumn Lichrospher® C18 (4 × 4 mm, 5 μ m, Merck®), and column Lichrospher® C18

(250 × 4 mm, 5 μ m, Merck). The data were collected and processed with the software HSM-7000 (Merck-Hitachi®).

HPLC-DAD for eicosanoids analysis was carried out using an isocratic elution program with methanol/H₂O (74:26) + trifluoroacetic acid (0.007%) as eluent at a flow rate 1 mL/min (11).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The assay described by Mosmann (12) was used as a criterion of cytotoxicity for human leukocytes. Briefly, 10⁶ cells were preincubated at 37 °C for 30 min with Dulbeccos' PBS, pH 7.4, containing the extracts at concentrations up to 200 μ g/mL. Controls received vehicle and correspond to 100% viability. The yellow water-soluble substrate MTT is converted by living cells into a dark blue formazan product that is insoluble in water. The coloured metabolite was dissolved in DMSO in an ultrasonic bath and measured using a Labsystem Multiskan MCC/340 plate reader, at 490 nm.

Determination of 5-lipoxygenase (5-LOX) activity

We followed the protocol established by Safayhi et al. (11). Rat peritoneal PMNLs (5 × 10⁶) were resuspended in 1 ml PBS (with glucose, 1 g/l) and pre-incubated for 5 min with the extracts or reference compounds at 37 °C. Then, the reaction was started by addition of ionophore A23187 and Ca²⁺ (1.9 μ M and 1.8 mM final concentrations, respectively). After 5 min at 37 °C, the reaction was stopped with 1 mL of cold methanol / 1N-HCl (97:3), and 500 pmol of prostaglandin B₂ (PGB₂) were added as an internal standard. After centrifugation (10000 × *g*, 5 min, 0 °C), the samples were applied to C18 SPE columns (100 mg), pre-conditioned with 1 mL of methanol and 1 mL of water. The columns were washed with 1 mL of water and 1 mL of 25% methanol. The 5-LOX metabolites 5(S)-HETE, LTB₄, 6-*trans*-leukotriene B₄ and 6-*trans*-12-*epi*-leukotriene B₄ (LTB₄ all-*trans*-isomers) were extracted with 300 μ L of methanol and analysed by high performance liquid chromatography (HPLC-DAD).

Determination of cyclooxygenase-1/12-lipoxygenase (COX-1/12-LOX) activity

The assay is based on the protocol proposed by Safayhi et al. (13). Aliquots of 80 × 10⁶ human platelets were pre-incubated for 5 min with extracts or reference compounds at 37°C. Then the reaction was started by addition of ionophore A23187 (1.9 μ M final concentration). After 1 min the reaction was stopped with 1 mL of cold methanol/1N HCl (97:3), and 500 pmol of PGB₂

were added as internal standard. After centrifugation (10000 × *g* for 10 min) the supernatants were pooled, subjected to SPE above and analysed by HPLC-DAD as described in the previous assay. The measured metabolites were 12(S)-hydroxyheptadeca-5Z,8E,10E-trienoic acid (12(S)-HHTrE) from the COX-1 pathway, and 12(S)-HETE from the 12-LOX pathway.

Oxazolone-induced contact-delayed hypersensitivity (DTH) mouse ear edema

The oxazolone-induced DTH test was performed according to Recio et al. (14). Briefly, female mice were sensitised by topical application on the shaven ventral abdomen of 50 μ L of a 2% (w/v) solution of oxazolone in acetone on two consecutive days (days 1 and 2). Challenge was performed on day 6 by application of 30 μ L of 2% oxazolone to both ears. β -sitosterol and dexamethasone were applied (20 μ L) to right ears 6 h after challenge (single application) and 24, 48, 72 and 96 h after challenge (repeated dosage). Ear thickness measurements of treated and control groups were done with a micrometer (Mitutoyo series 293) 24 and 102 h after challenge and just before drug application. The 102 h measurement was performed immediately before sacrifice. Swelling was assessed in terms of mean thickness increase of each ear.

Myeloperoxidase assay

It was performed according to Young and De Young (15). Each biopsy, placed in an Eppendorf tube containing 0.75 mL of 0.5% HTAB in 80 mM sodium phosphate buffer (pH=5.4), was homogenised (45 s). The homogenate was centrifuged and the supernatant (30 μ L) was assayed by mixing it with 20 μ L of TMB 18.4 mM and 15 μ L of H₂O₂ 0.017 % in a 96-well microtiter plate. The mixture was incubated for 3 min at 37 °C. Enzyme activity was determined colorimetrically using a Labsystem Multiskan MCC/340 plate reader set to measure absorbance at 620 nm.

Statistics

Oedemas are expressed as mean \pm S.E.M. and inhibition percentages arise from differences between treated and non-treated tissues, and are referred to the control treated only with the inflammatory agent. Percentages of inhibition of eicosanoids production are shown as mean \pm S.E.M. of three or more independent experiments, and every experiment was performed in duplicate. The inhibition of 5-LOX activity is expressed as percentages with respect

to the control, which includes LTB₄ and 5(S)-HETE. Inhibition of COX-1 and 12-LOX activities is expressed as percentages with respect to the control of 12(S)-HHTrE and 12(S)-HETE

respectively. Statistical evaluation was performed by ANOVA followed by Dunnett's *t*-test for multiple comparisons using Graph-Pad InStat software.

Table 1. Effects of β -sitosterol at 100 μ g/mL on the activity of different enzymes of the AA pathway. 5-LOX activity measured by the release of LTB₄; COX-1 activity measured as 12(S)-HHTrE release; 12-LOX activity measured as 12(S)-HETE release. Values are mean \pm S.E.M. (n = 3); ** *P* < 0.01. (Dunnett's *t*-test). As references we used ethanol extract of gum of *Boswellia serrata* (Bs) (20 μ g/mL) for the 5-LOX activity, Piroxicam (15 μ M) for COX-1 activity and *nor*-dihydroguayaretic acid (NDGA) (10 μ M) for 12-LOX activity.

Compound	LTB ₄	12-HHTrE	12-HETE	N
β -sitosterol	87 \pm 26	134 \pm 36	125 \pm 2	3
<i>B. serrata</i>	44 \pm 9**	N.T.	N.T.	3
Piroxicam	N.T.	39 \pm 2**	204 \pm 18	3
NDGA	N.T.	54 \pm 3**	41 \pm 8**	3

Table 2: Effects of β -sitosterol (0.5 mg/ear) and dexamethasone (0.05 mg/ear) on induced DTH in mice. Values are mean \pm S.E.M. (n = 6); ** *P* < 0.01. (Dunnett's *t*-test) compared with control.

Compound	$\Delta T \pm E$				
	24 h	48 h	72 h	96 h	102 h
Control	232,5 \pm 9,2	176,6 \pm 12,8	227,3 \pm 14,2	228,8 \pm 13,8	226,1 \pm 24,5
β -sitosterol	162,9 \pm 10,9 **	164,6 \pm 12,9	187,6 \pm 8,0	214,3 \pm 16,9	250,2 \pm 13,9
Dexamethasone	80,5 \pm 8,2**	58,5 \pm 9,8**	37,7 \pm 26,9**	57,2 \pm 15,1**	83,7 \pm 15,2**

(ΔT) Thickness variation (mm \times 10⁻³); ** *P* < 0,01 (Dunnett's *t* Test)

Compound	% Inhibition				
	24 h	48 h	72 h	96 h	102 h
β -sitosterol	30	7	17	6	-11
Dexamethasone	65	67	77	75	63

Table 3. Effects of β -sitosterol I and dexamethasone on the activity of MPO in ear biopsies.

Compound	Absorbance	% Activity MPO
Control	1,537 \pm 0,114	100
β -sitosterol	1,376 \pm 0,096	90
Dexamethasone	0,837 \pm 0,167**	46

** *p* < 0,01 (Dunnett's *t* Test)

Results and Discussion

β -sitosterol did not show any cytotoxicity in human PMN at a dose of 200 μ g/mL in the MTT assay (data not shown). The results of the models of eicosanoid release are summarised in Table 1. β -sitosterol did not inhibit LTB₄ production neither the production of 5(S)-HETE. It also failed to inhibit 12(S)-HHTrE release, a valid marker of the COX-1 activity (16). The obtained results in the oxazolone-induced contact dermatitis are showed in Table 2. The extract inhibited the oedema at 24 hours but this effect was not maintained until the end of the assay. It did not inhibit the leukocyte infiltration measured as myeloperoxidase activity in biopsies (Table 3).

In the *in vivo* model of DTH, β -sitosterol has been shown to inhibit the oedema in the oxazolone-induced DTH model in the 24 first hours. The inhibition is of pharmacological significance in accordance with Young and De Young (15), which established that in this model NSAIDs only inhibit in a extend of 30-40% at 24 h. The subjacent mechanisms of DTH models are based on the modification of endogenous proteins by covalent binding of haptens. Oxazolone acts as a hapten that reacts covalently with proteins to lead to an antigen-protein complex that is target of T-lymphocytes. The Langerhan' cells recognize this complex and migrate from epidermis to lymph nodes, thus initiating a specific immune response. After a second contact with the hapten, an inflammatory reaction appears by inducing macrophage cytokine release and PGE₂ synthesis during the respective early vascular and late cellular phases of inflammation (17). It is possible, however, that the response to oxazolone may partly evolve on a different pathway independent of interleukin-4 (18). The results indicate that this compound can participate in the inhibition of a cell-mediated oedema during its earlier stage. If β -sitosterol does not inhibit the COX pathway responsible for PGE₂ synthesis other mechanisms must be considered.

As conclusion, we here establish for first time the *in vivo* anti-inflammatory activity of the β -sitosterol in a model of delayed contact dermatitis. The results indicate that this compound can modulate a cell-mediated oedema without any short term *in vitro* effect in the arachidonate pathway of intact cells. The ubiquity of this compound can explain, and predict, the effect of many plant extracts in this model, and so this data could be of help in dereplication processes.

Acknowledgements

The authors wish to thank the Centre de Transfusions de la Comunitat Valenciana for the kind supply of buffy-coats.

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