Fitoterapia 82 (2011) 414–421

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Suppression of COX-2, IL-1β and TNF-α expression and leukocyte infiltration in inflamed skin by bioactive compounds from *Rosmarinus officinalis* L.

Eleonora S. Mengoni [a], Gabriel Vichera [a], Luciano A. Rigano [a], Marcelo L. Rodriguez-Puebla [b], Silvia R. Galliano [c], Eduardo E. Cafferata [d], Omar H. Pivetta [e], Sivia Moreno [d], Adrián A. Vojnov [a,*]

[a] *Instituto de Ciencia y Tecnología Dr. César Milstein, Fundación Pablo Cassará and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Saladillo 2468, C1440FFX Buenos Aires, Argentina*
[b] *Department of Molecular Biomedical Sciences, North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606, USA*
[c] *Instituto Universitario CEMIC and Universidad Maimónides, Buenos Aires, Argentina*
[d] *Fundación Instituto Leloir, CONICET, Av. Patricias Argentinas 435, 1035, Buenos Aires, Argentina*
[e] *Universidad Austral, Universidad de Ciencias Médicas, J.D. Perón 1500, Pilar (B1629AHJ) Buenos Aires, Argentina*

ARTICLE INFO

Article history:
Received 22 July 2010
Accepted in revised form 24 November 2010
Available online 1 December 2010

Keywords:
Skin inflammation
Carnosic acid
Carnosol
Cyclooxygenase
Interleukin
Tumor necrosis factor

ABSTRACT

In the present study, we evaluated the effects of extracts and purified compounds from fresh leaves of *Rosmarinus officinalis* L. Pretreatment with the major anti-inflammatory compounds, carnosic acid (CA) and carnosol (CS), inhibited phorbol 12-myristate 13-acetate (PMA)-induced ear inflammation in mice with an EC_{50} of 10.20 μg/cm^2 and 10.70 μg/cm^2, respectively. To further understand the anti-inflammatory mechanism of these compounds, we analyzed the *in vivo* expression of several inflammation-associated genes in mouse skin by reverse transcriptase-polymerase chain reaction (RT-PCR). Our data showed that CA and CS reduced the expression of IL-1β and TNF-α but had less effect on fibronectin and ICAM-1 expression. Interestingly, both compounds selectively inhibited COX-2 but not COX-1. Histopathological analysis of hematoxylin and eosin (H&E)-stained tissue revealed a marked reduction in leukocyte infiltration and epidermal ulceration of PMA-treated ears when ears were pretreated with ethanolic extracts or pure CA. *In vitro*, we showed that ethanolic extract, carnosic acid and carnosol significantly inhibited the overproduction of nitric oxide (NO) in a dose-dependent manner in the RAW 264.7 murine macrophage cell line. For the first time *in vivo*, we showed that CA and CS differentially regulate the expression of inflammation-associated genes, thus demonstrating the pharmacological basis for the anti-inflammatory properties reported for CA and CS.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Inflammation is a rapid response of tissue to injury and is characterized in the acute phase by increased blood flow and vascular permeability along with the accumulation of fluid, leukocytes, and inflammatory mediators, such as cytokines [10]. During the inflammatory process, a variety of soluble factors promote leukocyte recruitment through increased expression of cellular adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1). Several cytokines play key roles in mediating acute inflammatory responses, such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α), which are extremely potent inflammatory molecules [19]. IL-1β induces fever and shock-related hypotension in part by inducing the production of prostanoids through increased synthesis of cycloxygenase-2 (COX-2), a process that can occur in fibroblasts, endothelial cells and human monocytes [9]. The COX-2 protein catalyzes the biosynthesis of prostaglandins from arachidonic acid [39]. Unlike the constitutively expressed cycloxygenase-1 (COX-1), which is important for maintaining normal physiological functions, COX-2 is

* Corresponding author. Instituto de Ciencia y Tecnología "Dr. Cesar Milstein", Fundación Pablo Cassará, CONICET, Saladillo 2468, C1440FFX Buenos Aires, Argentina. Tel./fax: +54 116872542.
E-mail address: avojnov@fundacioncassara.org.ar (A.A. Vojnov).

0367-326X/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.fitote.2010.11.023

undetectable in tissue under basal conditions and is induced transiently by pro-inflammatory cytokines, growth factors, oncogenes and tumor promoters, such as phorbol 12-myristate 13-acetate (PMA) [6]. Nitric oxide (NO) is another inflammatory mediator secreted by monocytes and macrophages. Inducible nitric oxide synthase (iNOS) is one of three enzymes that generate NO and mediate many physiological events [21]. Vasodilatation in response to endothelium-derived relaxing factor has also been attributed to NO [26], and NO has been reported to play an important role in inflammatory responses, such as edema in mouse skin [1,37].

Plant-derived phytochemicals are an important and promising group of potential anti-inflammatory agents because of their low toxicity and apparent benefit in acute and chronic diseases [6]. Several rosemary extracts (RE) from dried leaves have recently been characterized as having topical anti-inflammatory activity. The RE extracts, ursolic acid, oleanolic acid, and micromeric acid, were found to have the most effective anti-inflammatory [2,27] and anti-tumor activities [27]. Carnosic acid (CA) and carnosol (CS) (Fig. 1) are the most abundant diterpenes in rosemary leaves [23,24]. *In vitro* studies in human polymorphonuclear leukocytes (PMNL), U937 cells and human mammary epithelial cells have shown that CA and CS act as strong anti-inflammatory compounds [28,34,43]. However, to date, the expression of pro-inflammatory genes and the histopathological analysis in intact or inflamed skin tissue have not been analyzed.

In the present study, we examined the anti-inflammatory properties of organic extracts and their bioactive compounds, CA and CS, obtained from fresh rosemary leaves. Using a formaldehyde-induced model of paw edema in mice, we first established that topical application had the strongest anti-inflammatory effects. We then used a PMA-induced model of ear edema to analyze the effect of CA and CS on the expression of COX-1, COX-2, IL-1β, ICAM-1, TNF-α and fibronectin. Our results showed that inflamed tissue treated with CA and CS exhibited a decreased expression of IL-1β and TNF-α, a selective inhibition of COX-2 and a reduction in leukocyte migration.

2. Materials and methods

2.1. Chemicals and reagents

Water and ethanolic rosemary extracts were prepared as previously described [23]. The chemicals used in the study were phorbol 12-myristate 13-acetate (PMA) (Sigma, St. Louis, USA), formaldehyde (Merck Química Argentina S.A.I.C, Argentina), carnosic acid (CA) and carnosol (CS), (Alexis Biochemicals, Lausen, Switzerland) and indomethacin (Montepellier, Argentina).

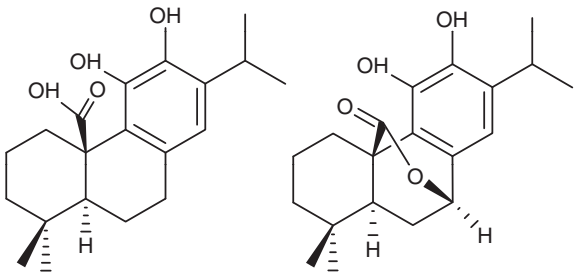

Fig. 1. Chemical structures of carnosic acid (1) and carnosol (2).

2.2. Animals

Male Balb/c mice weighing 20–25 g were obtained from the Laboratorio Pablo Cassará. Animals were housed in steel cages under standard conditions and allowed free access to food and water. Prior to use, animals were given two weeks to acclimate to the new environment. All animal experiments were in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals (Pub No. 85-23, revised 1985).

2.3. Formaldehyde-induced plantar edema

Preliminary studies of the effect of RE on the acute phase of inflammation were performed. The Ref. [35] model was used with some modifications. Acute inflammation was induced by injection of 20 μl of 3% formaldehyde in phosphate buffered saline (PBS) into the sub-plantar region of both hind paws. The control group received an equivalent volume of the vehicle (PBS). Two hours later, the left hind paws were treated with 10 μl of RE (12 mg/ml). The mice were divided into groups of six mice each to test different treatment protocols with RE. The first group received topical application of RE, the second group was injected with RE and the third group received both modes of treatment (injected and topical application). After 24 h, the mice were sacrificed by cervical dislocation, and the diameter of edema was measured with a caliber. The degree of edema in each treatment group was determined using the following equation:

Edema reduction (%) = 100 − ((EP/A) 100), where EP (edema production) is the difference between (right leg–left leg) diameters from animals given the same treatment and (right leg–left leg) diameters from control animals, and A is the difference in the inflammation group.

2.4. Anti-inflammatory activity in the ear edema model

The ear edema model was used as previously described [7]. Anti-inflammatory activity was evaluated as the inhibition of PMA-induced ear edema in mice. Cutaneous inflammation in ears of Balb/c mice (n = 10 for each group) was induced by topical application of PMA (25 ng/cm^2), and this condition was considered to represent 100% inflammatory activity. Doses of 10, 20, 100, 250, 500, or 1000 μg/cm^2 of RE or 2, 10 or 20 μg/cm^2 of CA were topically applied to the inner surface of the right ear of mice 30 min before PMA application. The left ear remained untreated. We used the non-steroidal anti-inflammatory drug (NSAID), indomethacin as a reference. Control animals received only PMA. Four hours after treatment, the mice were sacrificed by cervical dislocation, and a plug (6 mm Ø) was removed from both treated and untreated ears for analysis. The anti-inflammatory activity (AI) was measured as follows: AI = ((Wcontrol–Wtrial)/Wcontrol) × 100% where Wcontrol = right ear disk weight − left ear disk weight from control animals; Wtrial = right ear disk weight − left ear disk weight from animals given the same concentration of extract. The anti-

inflammatory activity was expressed as a percentage of the edema reduction in treated mice compared to the control mice. The activity was quantitatively evaluated by determining the 50% effective concentration (EC_{50}) using nonlinear regression with a sigmoidal dose–response curve fit.

2.5. Histology

The tissue specimens were fixed overnight in 4% buffered formaldehyde, processed by standard methods, and stained for hematoxylin and eosin (H&E). Semi-quantitative analysis of tissues was performed by a blinded observer.

2.6. Cell lines

The murine monocyte macrophage cell line, RAW 264.7, was kindly provided by Dr. Fernando Goldbaum (Fundación Instituto Leloir, Buenos Aires, Argentina) and cultured at 37 °C in 5% CO_2 with 95% air in Dulbecco's modified Eagle's medium (DMEM) containing heat inactivated 10% (vol/vol) fetal bovine serum (FBS, Bioser, Argentina), 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin.

2.7. Measurement of nitrite concentration in culture medium

RAW 264.7 cells were cultured in 96-well plates and grown to confluence. The medium was then replaced with fresh medium, and cells were stimulated with 1 μg/ml of *Pseudomonas aeruginosa* LPS (Sigma, USA). Two hours prior to challenge with LPS, 12.5, 25 or 50 μg/ml of RE or 3.125, 6.25, 12.5 or 25 μg/ml of CA or CS was added to cultures. Cell viability was assessed by trypan blue exclusion.

Nitrite accumulation in RAW 264.7 culture supernatants was measured using the Griess method as described previously [32]. Briefly, 50 μl of 1% sulphanilamide in 5% phosphoric acid and 50 μl of 0.1% N-(1-naphtyl)-ethylenediamine in water were added to 50 μl of culture medium. After 10 min of incubation at room temperature, the absorbance at 570 nm was read with a microplate spectrophotometer. Micromolar concentrations of nitrite were calculated from a standard curve made with sodium nitrite as the reference compound.

2.8. RT-PCR analysis

After cutting into small pieces, ear tissue was homogenized in TRIZOL (Invitrogen) containing 1% beta-mercaptoethanol for 30 s using a Polytron homogenizer. Total RNA was extracted using the Total RNA kit (Bioamerica, Inc.) according to the manufacturer's protocol. The concentration of RNA was determined by measuring the absorbance at 260 and 280 nm, and samples were stored at −70 °C until RT-PCR analysis. Complimentary DNA was synthesized using an RT reaction at 42 °C for 60 min in a T-18 thermal cycler (IVEMA). Primers were designed based on the mouse cDNA sequences for COX-1, COX-2, IL-1β, ICAM-1, TNF-α, fibronectin, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The primer sequences used for PCR were as follows: COX-1: sense, 5′-TGC ATG TGG CTG TGG ATG TCA TCA A-3′, antisense, 5′-CAC TAA GAC AGA CCC GTC ATC TCC A-3′, 450 bp; COX-2: sense, 5′-TTC AAA AGA AGT GCT GGA AAA GGT-3′, antisense, 5′-GAT CAT CTC TAC CTG AGT GTC TTT-3′, 583 bp; IL-1β: sense, 5′-TGC AGA GTT CCC CAA CTG GTA CAT C-3′, antisense, 5′-GTG CTG CCT AAT GTC CCC TTG AAT C-3′, 387 bp; ICAM-1: sense, 5′-TCG GAG GAT CAC AAA CGA AGC-3′, antisense, 5′-AAC ATA AGA GGC TGC CAT CAC G-3′, 471 bp; TNF-α: sense, 5′-ACA AGC CTG TAG CCC ACG-3′, antisense, 5′-TCC AAA GTA GAC CTG CCC-3′, 428 bp; fibronectin: sense, 5′-GCA ACG TGT TAT GAC GAT GG-3′, antisense, 5′-CTA ACG GCA TGA AGC ACT CA-3′, 253 bp; G3PDH: sense, 5′-TGA AGGGTA GGC CAT GAG GTC CAC CAC-3′, 983 bp. The amplification was performed using 25 μl of the reaction mixture at 94 °C for 15–60 s, 50–68 °C for 30–60 s, and 72 °C for 45–90 s with 28 cycles for IL-1β and 35 cycles for the other genes. After amplification, 10 μl of reaction mixture was analyzed by 1.5% agarose gel electrophoresis. The bands were visualized by GelRed™ solution (Biotium). The signal intensities were normalized by comparing tested gene expression with G3PDH and represented as relative ratios.

2.9. Statistical analysis

Results are expressed as the mean ± SEM of n experiments. Analyses were performed using a one-way ANOVA test. Differences were considered statistically significant at $p<0.05$.

3. Results

3.1. Anti-inflammatory effects of ethanolic rosemary extracts

Preliminary studies were done to test the anti-inflammatory properties of rosemary extracts using the formaldehyde-induced plantar edema mouse model. Formaldehyde was injected into the plantar tissue of mice, and 2 h later, water and ethanolic REs were applied to the inflamed area either topically or by injection, or both. Whereas treatment with water RE showed no anti-inflammatory properties (data not shown), ethanolic RE treatment resulted in a strong reduction of edema (Fig. 2D and Table 1).

Injection of ethanolic RE reduced inflammation by 22% when compared with the PBS control injection. However, when the ethanolic RE was topically administered, inflammation was reduced by 80% (Fig. 2D and Table 1).

In agreement with these results, histopathological analysis of formaldehyde-induced plantar edema showed that topical treatment with ethanolic RE strongly diminished the number of leukocytes (a marker of the inflammatory process) at the site of inflammation (Fig. 2C) compared with the control samples (Fig. 2B). Based on these data, we used topical application in further experiments and a PMA-induced model of ear inflammation because it is a more effective system to evaluate the major bioactive compounds of rosemary extract.

3.2. CA and CS are the major anti-inflammatory bioactive compounds

CA and CS have been reported to be the most abundant compounds with antioxidant activity in ethanolic extracts of rosemary leaves [23]. To assess the anti-inflammatory properties of these compounds, we administered ethanolic RE or pure CA or CS to animals prior to PMA-induced ear inflammation. In agreement with our previous data, the ethanolic RE administered at the indicated doses had a potent

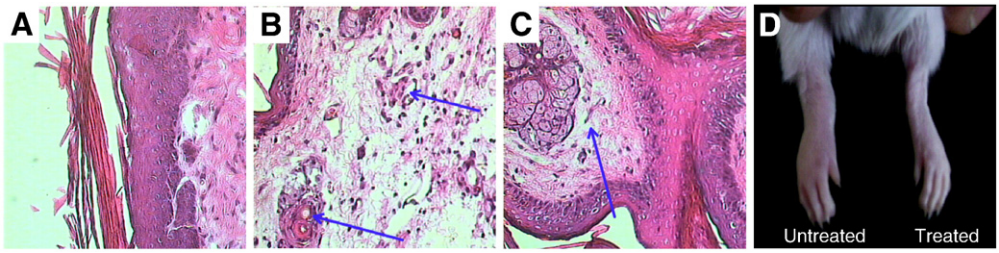

Fig. 2. Reduction of paw edema in Balb/c mice treated topically with RE. Acute inflammation was induced by injection of 20 μl of 3% formaldehyde in PBS into the sub-plantar side of both paws. Two hours later, mice were treated topically with 10 μl of RE (12 mg/ml). Samples were fixed overnight in 4% buffered formaldehyde and stained with H&E. (A) Vehicle-treated control. (B) Formaldehyde (3%)-induced paw edema showing intense dermal edema and inflammatory cell infiltration with presence of mononuclear and polymorphonuclear cells. (C) Topical RE treatment after formaldehyde injection showing reduced dermal edema and inflammatory cell infiltration. (D) Image of paw showing edema reduction upon topical administration of RE.

anti-inflammatory effect. As shown in Table 2, ethanolic RE exerted a dose-dependent topical anti-inflammatory activity in PMA-induced oedema, where an EC_{50} (dose giving 50% oedema inhibition) was 128.3 μg/cm^2.

Pure CA and CS were evaluated in the same model for anti-inflammatory activity. Both compounds CA showed an EC_{50} of 10.20 μg/cm^2 and 10.70 μg/cm^2, respectively (Table 2). Ethanolic RE, CA and CS were compared to indomethacin, a nonsteroidal anti-inflammatory drug used to relieve the symptoms caused by different inflammatory processes [12,25,40,41]. The ethanolic RE showed an anti-inflammatory effect similar to that observed with indomethacin, whereas CA and CS exhibited a significantly higher activity than this drug (Table 2). Thus, CA and CS appear to be responsible for the anti-inflammatory activity observed in rosemary extracts. Notably, a lower concentration of CA and CS than rosemary extract and indomethacin was required to produce the same effect.

3.3. Histopathological analysis of mouse ears treated with ethanolic RE and carnosic acid

Histopathological analysis of hematoxylin and eosin (H&E)-stained tissue revealed a marked infiltration of leukocytes and epidermal ulceration in PMA-treated ears (Fig. 3B and Table 2), which is characteristic of acute infection, but not in untreated ears (Fig. 3A). Pretreatment with ethanolic RE strongly reduced the accumulation of leukocytes. In addition, we observed vasodilatation similar to that observed with indomethacin, thus confirming the protective effect of ethanolic RE (Fig. 3C and D). The effect

Table 1
Effect of the *Rosmarinus officinalis* extract on formaldehyde-induced paw edema.

Treatment	No. of mice	Edema (cm)	EP = [Treatment − control]	Edema reduction (%)
Control	6	0.280 ± 0.010	0	100
Control inflammation	6	0.330 ± 0.016	0.050	–
Injected application	6	0.319 ± 0.010	0.039	22
Topical application	6	0.290* ± 0.004	0.010	80
Injected application + topical application	6	0.318* ± 0.010	0.038	24

EP, edema production. Experimental groups were compared with control; $^*p<0.05$.

Table 2
Anti-inflammatory effect of RE and CA on PMA-induced edema in mouse ears.

Compound	Dose (μg/cm^2)	No. of mice	Edema (mg)	Edema reduction (%)	EC_{50} (μg/cm^2)
	10	10	3.26 ± 1.48	3.9	
	20	10	3.13 ± 1.27	7.8	
RE	100	10	2.10* ± 0.50	38.2	128.3
	250	10	0.71* ± 0.14	78.9	
	500	10	0.50* ± 0.14	84.3	
	1000	10	0.05* ± 0.08	98.6	
	2	10	2.03* ± 0.51	40.2	
CA	10	10	1.73* ± 0.24	49.3	10.2
	20	10	1.22* ± 0.26	64.1	
	2	10	2.10* ± 0.80	38.2	
CS	10	10	2.03* ± 0.40	40.2	10.7
	20	10	1.36* ± 0.40	59.8	
Indomethacin	500	10	0.27 ± 0.17	92.2	
PMA	4	10	3.40 ± 0.15		

EC_{50}, dose inhibiting the edematous response by 50%. Experimental groups were compared with control; $^*p<0.05$.

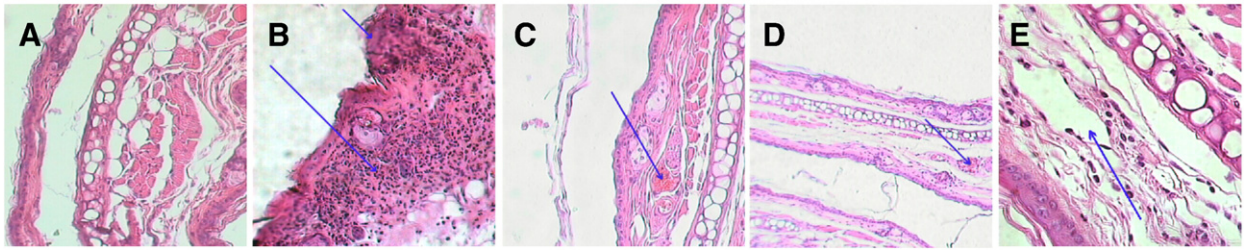

Fig. 3. Suppression of PMA-induced ear edema in Balb/c mice upon treatment with RE and CA. RE or CA was topically applied to the inner surface of the right ear of mice 30 min prior to PMA (25 ng/cm^2) administration. After 4 h, samples were fixed overnight in 4% buffered formaldehyde and H&E stained as described in Materials and Methods. Control (A). PMA (B). Ethanolic RE (1000 μg/cm^2) (C). Indomethacin (500 μg/cm^2) (D). CA (20 μg/cm^2) (E). Arrows indicate leukocyte infiltration (B), low vasodilatation (C and D), and reduction in leukocyte accumulation (E).

of one of the major bioactive compounds, CA, was tested, and reduced numbers of leukocytes were also observed when this compound was topically applied (Fig. 3E).

3.4. Reduction of nitric oxide production by rosemary and its bioactive compounds

Compounds able to reduce NO production by iNOS are effective anti-inflammatory agents. For this reason, the effect of ethanolic RE, CA and CS on iNOS activity was determined [22]. Stimulation of RAW 264.7 cells with LPS has been shown to promote NO generation and nitrite (NO_2^-) accumulation in the media [16]. In our studies, RAW 264.7 cells were pre-incubated with 12, 25 or 50 μg/ml of ethanolic RE or 3.125, 6.25, 12.50 or 50 μg/ml of CA or CS and then stimulated with 1 μg/ml of LPS for 24 h. As shown in Fig. 4, ethanolic RE, CA and CS significantly inhibited the production of NO in a dose-dependent manner. The cytotoxic effects of the ethanolic RE and CA were evaluated in the presence or absence of LPS. Both RE and CA did not affect the viability of RAW 264.7 cells at the concentrations used (data not shown). These results indicate that the ethanolic RE and CA have a potent inhibitory effect on NO synthesis.

3.5. CA and CS reduce mRNA expression of pro-inflammatory molecules

RT-PCR was used to measure expression levels of several inflammation-associated genes in PMA-induced skin injury upon pretreatment with CA or CS.

Analysis of tissue without PMA treatment showed constitutive expression of genes, such as COX-1, fibronectin and G3PDH, as expected. The expression of inducible pro-inflammatory genes was not (or was only slightly) detected (Fig. 5).

In contrast, PMA treatment significantly induced IL-1β, TNF-α and COX-2 expression. Skin pretreated with 20 μg/cm^2 of CA or CS suppressed the expression of IL-1β with a reduction of 67.64% and 65.06%, respectively. Moreover,

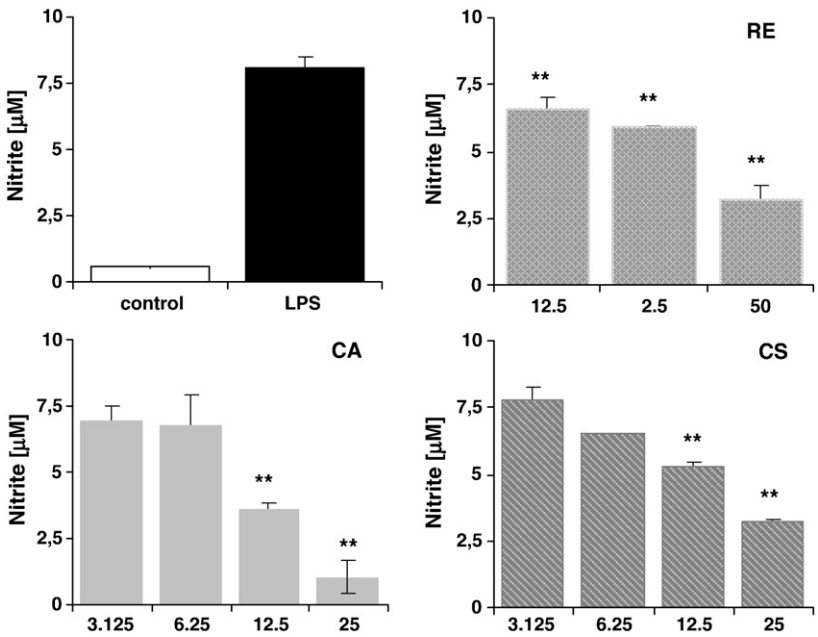

Fig. 4. Effect of RE, CA and CS on nitrite accumulation in culture medium of RAW 264.7 macrophages. RAW 264.7 cells were treated with increasing concentrations of RE, CA or CS and stimulated with LPS (1 μg/ml) 2 h later. After incubation for 24 h, nitrite concentration in the culture medium was measured as a marker of NO production. Data are representative of three independent experiments and show the mean ± SD from triplicate samples. $^{**}p<0.05$, compared with cells treated with LPS only.

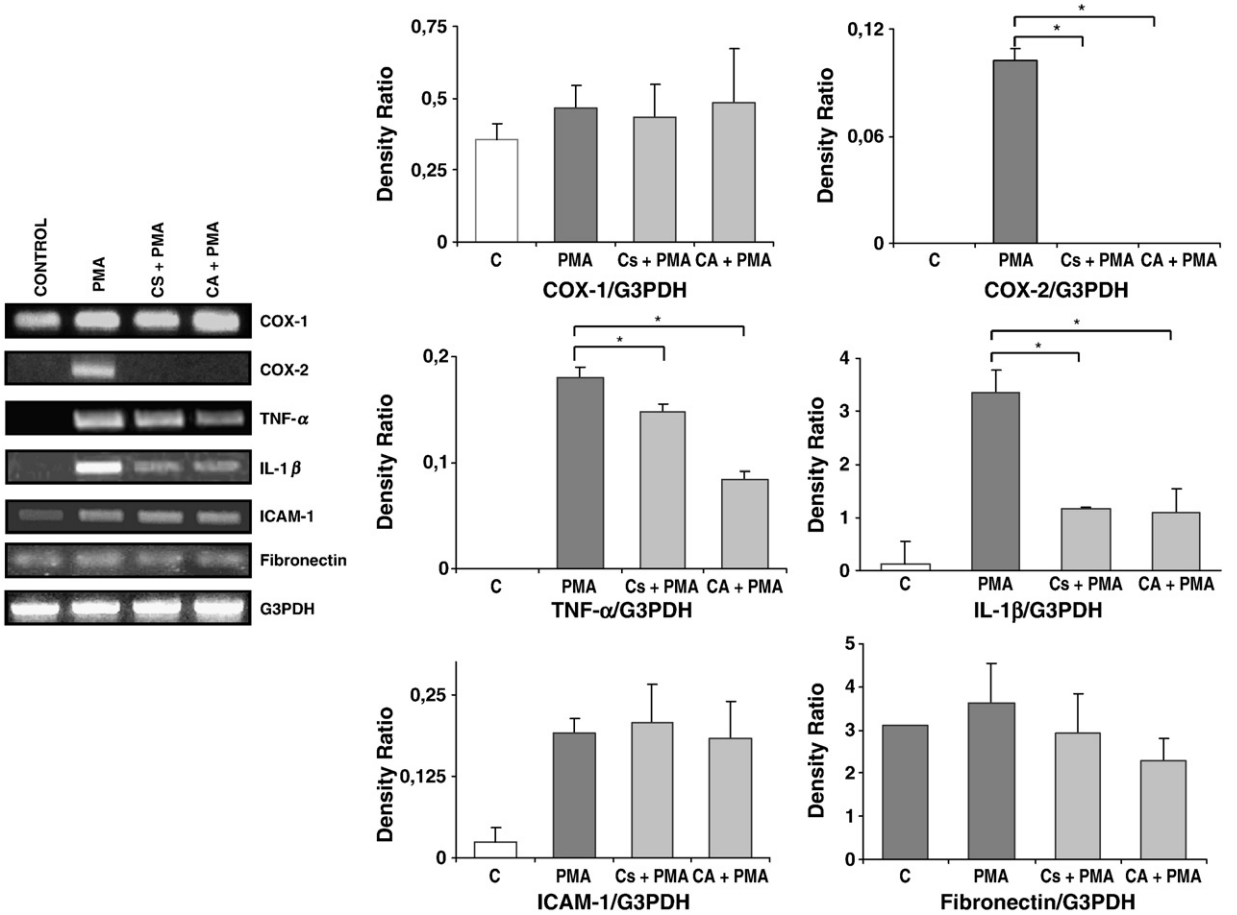

Fig 5. Effect of CA and CS on PMA-induced ear edema. PMA and test compounds/vehicle were topically applied to the skin of ears. Ear edema was measured 4 h after PMA treatment. Mice were then immediately sacrificed, and ears were removed for RT-PCR analysis. One representative PCR result from three analyses from each group is shown. The ratios of band density were compared to G3PDH.

pretreatment with CA reduced TNF-α expression to a greater extent than pretreatment with CS (53% compared with 18.2%, respectively), and both compounds completely inhibited COX-2. The expression levels of COX-1, ICAM-1 and fibronectin were not significantly reduced by topical application of CA or CS compared to the PMA-treated group (Fig. 5).

4. Discussion

Currently, a major strategy in the alleviation of inflammation is to seek new agents with anti-inflammatory and antioxidant properties [18]. The association between antioxidants and inflammation initially originated from studies that found that free radicals are produced during the inflammatory process by macrophages [38]. Based on this finding, several synthetic and natural antioxidants have been evaluated for their analgesic and anti-inflammatory activities [4].

Previous studies showed that the rosemary extracts, carnosic acid and its derivative, carnosol, and rosmarinic acid possess potent antioxidant activity [23]. In the current study, we used *in vivo* and *in vitro* models to evaluate the anti-inflammatory properties of rosemary extracts and the most abundant bioactive compounds from the extracts.

Using two *in vivo* inflammation models, we evaluated both water and ethanolic fresh rosemary leaf extracts as well as pure CA and Cs, the most abundant compounds in ethanolic RE. We determined that ethanolic but not water extracts reduced inflammation (Tables 1 and 2), and pure CA and CS isolated from ethanolic RE showed important anti-inflammatory activity. The EC_{50} was 128.3 μg/cm^2 for the ethanolic extract; this extract was about 45% less active than indomethacin, which has been reported to have an EC_{50} of 93 μg/cm^2 [2]. Interestingly, pure CA and CS showed an activity about nine-fold higher than indomethacin (10.2 μg/cm^2 and 10.70 μg/cm^2, respectively) (Table 2).

Supporting the potential use of CA and CS for inflammatory disorders, these compounds had the highest topical anti-inflammatory activity in mouse models compared to other compounds already reported from rosemary plants. The potency of CA (0.030 μmol/cm^2) and CS (0.032 μmol/cm^2) was higher than ursolic acid (0.12 μmol/cm^2), oleanolic acid (0.29 μmol/cm^2) and micromeric acid (0.22 μmol/cm^2), which were all obtained from dried rosemary leaves [2].

The ethanolic RE and CA effects were confirmed by histopathological analysis of tissue from mouse ears and paws in edema models of inflammation (Figs. 2 and 3). The event initiating the inflammatory process is the infiltration of

neutrophils into the wound site to prevent infection through phagocytosis [3]. Our results suggested that ethanolic RE and CA interfered with the recruitment of PMNL into the blood in response to the acute inflammation induced by formaldehyde (Fig. 2) or PMA (Fig. 3). Moreover, our results demonstrated for the first time *in vivo* that CA and CS significantly decreased the expression of IL-1β and TNF-α and completely inhibited COX-2 expression (Fig. 5). Interestingly, both compounds were highly selective in inhibiting COX-2 but not COX-1. Therefore, we demonstrate here that the selective inhibition of COX-2, which has been recently reported for rosemary extracts [42], is due to the bioactive compounds, CA and CS, from rosemary extracts. Our results suggest that these compounds may be useful as anti-inflammatory agents with fewer side effects than regular non-steroidal drugs.

It has been suggested that high levels of NO induced by iNOS stimulate neutrophil accumulation [11]. The anti-inflammatory effects of compounds of diverse origins have been attributed to the inhibition of events involved in inflammation, such as histamine release, cyclooxygenase-2,5-lypoxigenase activity, elastase activity, complement activity, and nitric oxide production [8,13–15,20,29,30,33,36]. In addition, NO produced by iNOS has been implicated in pathological conditions of the skin. Dysregulated expression of iNOS has been demonstrated in skin biopsies from patients with psoriasis and patients with inflammatory arthritides [17,31]. More recently, it has been demonstrated that NO mediates inflammatory responses in human keratinocytes [5]. Based on these reports and the fact that NO is increased significantly during the process of edema in guinea pig skin [37], we assessed the inhibitory activity of the ethanolic RE, CA and CS on NO production *in vitro* by LPS-activated macrophages. We demonstrated that ethanolic RE, CA and CS inhibit NO production (Fig. 4) and that ethanolic RE suppresses NF-κB activation using EMSA (data not shown), suggesting that the anti-inflammatory activity of RE, CA and CS may be due to their inhibition of pathways involving both NO and NF-κB.

As far as we know, this study is the first to demonstrate *in vivo* the anti-inflammatory effects of CA and CS with a selective inhibition of COX-2.

Acknowledgements

This work was supported in part by grants from Fundacion Roemmers. AAV is a member of the Career Investigator of CONICET, Argentina. We are grateful to Fundación para el Bienestar del Niño: Programa de Asistencia a la Fibrosis Quística, Córdoba-Argentina, for awarding the present work. We thank Fernando J. Dyzenchauz, Professional Associate of the CONICET and Gastón Dominguez for technical assistance, and Paloma Dyzenchauz for image analysis.

References

[1] Ahmad N, Srivastava RC, Agarwal R, Mukhtar H. Nitric oxide synthase and skin tumor promotion. Biochem Biophys Res Commun 1997;232:328–31.
[2] Altinier G, Sosa S, Aquino RP, Mencherini T, Della Loggia R, Tubaro A. Characterization of topical anti-inflammatory compounds in *Rosmarinus officinalis* L. J Agric Food Chem 2007;55:1718–23.
[3] Barbosa PS, Martins AM, Havt A, Toyama DO, Evangelista JS, Ferreira DP, et al. Renal and antibacterial effects induced by myotoxin I and II isolated from *Bothrops jararacussu* venom. Toxicon 2005;46:376–86.
[4] Calixto JB, Santos AR, Cechinel Filho V, Yunes RA. A review of the plants of the genus *Phyllanthus*: their chemistry, pharmacology, and therapeutic potential. Med Res Rev 1998;18:225–58.
[5] Chang HR, Tsao DA, Wang SR, Yu HS. Expression of nitric oxide synthases in keratinocytes after UVB irradiation. Arch Dermatol Res 2003;295:293–6.
[6] Chung WY, Park JH, Kim MJ, Kim HO, Hwang JK, Lee SK, et al. Xanthorrhizol inhibits 12-O-tetradecanoylphorbol-13-acetate-induced acute inflammation and two-stage mouse skin carcinogenesis by blocking the expression of ornithine decarboxylase, cyclooxygenase-2 and inducible nitric oxide synthase through mitogen-activated protein kinases and/or the nuclear factor-kappa B. Carcinogenesis 2007;28:1224–31.
[7] Della Loggia R, Tubazo A, Sosa S, Becker H, Saar S, Isaac O. The role of triterpenoids in the topical antiinflammatory activity of *Calendula officinalis* flowers. Planta Med 1994;60:516–20.
[8] Diaz AM, Abad MJ, Fernandez L, Recuero C, Villaescusa L, Silvan AM, et al. *In vitro* anti-inflammatory activity of iridoids and triterpenoid compounds isolated from *Phillyrea latifolia* L. Biol Pharm Bull 2000;23:1307–13.
[9] Duque J, Diaz-Munoz MD, Fresno M, Iniguez MA. Up-regulation of cyclooxygenase-2 by interleukin-1beta in colon carcinoma cells. Cell Signal 2006;18:1262–9.
[10] Feghali CA, Wright TM. Cytokines in acute and chronic inflammation. Front Biosci 1997;2:d12–26.
[11] Greenacre SA, Rocha FA, Rawlingson A, Meinerikandathevan S, Poston RN, Ruiz E, et al. Protein nitration in cutaneous inflammation in the rat: essential role of inducible nitric oxide synthase and polymorphonuclear leukocytes. Br J Pharmacol 2002;136:985–94.
[12] Hellewell PG, Jose PJ, Williams TJ. Inflammatory mechanisms in the passive cutaneous anaphylactic reaction in the rabbit: evidence that novel mediators are involved. Br J Pharmacol 1992;107:1163–72.
[13] Honda T, Gribble GW, Suh N, Finlay HJ, Rounds BV, Bore L, et al. Novel synthetic oleanane and ursane triterpenoids with various enone functionalities in ring A as inhibitors of nitric oxide production in mouse macrophages. J Med Chem 2000;43:1866–77.
[14] Huss U, Ringbom T, Perera P, Bohlin L, Vasange M. Screening of ubiquitous plant constituents for COX-2 inhibition with a scintillation proximity based assay. J Nat Prod 2002;65:1517–21.
[15] Kapil A, Sharma S. Effect of oleanolic acid on complement in adjuvant- and carrageenan-induced inflammation in rats. J Pharm Pharmacol 1995;47:585–7.
[16] Kleinert H, Pautz A, Linker K, Schwarz PM. Regulation of the expression of inducible nitric oxide synthase. Eur J Pharmacol 2004;500:255–66.
[17] Kolb-Bachofen V, Fehsel K, Michel G, Ruzicka T. Epidermal keratinocyte expression of inducible nitric oxide synthase in skin lesions of psoriasis vulgaris. Lancet 1994;344:139.
[18] Kourounakis AP, Galanakis D, Tsiakitzis K, Rekka AE. Synthesis and pharmacological evaluation of novel derivatives of anti-inflammatory drugs with increased antioxidant and anti-inflammatory activities. Drug Dev Res 1999;47:9–16.
[19] Lantz RC, Chen GJ, Solyom AM, Jolad SD, Timmermann BN. The effect of turmeric extracts on inflammatory mediator production. Phytomedicine 2005;12:445–52.
[20] Liu J. Pharmacology of oleanolic acid and ursolic acid. J Ethnopharmacol 1995;49:57–68.
[21] MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. Annu Rev Immunol 1997;15:323–50.
[22] Maiuri MC, De Stefano D, Di Meglio P, Irace C, Savarese M, Sacchi R, et al. Hydroxytyrosol, a phenolic compound from virgin olive oil, prevents macrophage activation. Naunyn-Schmiedebergs Arch Pharmacol 2005;371:457–65.
[23] Moreno S, Scheyer T, Romano CS, Vojnov AA. Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. Free Radic Res 2006;40:223–31.
[24] Munne-Bosch S, Alegre L. Subcellular compartmentation of the diterpene carnosic acid and its derivatives in the leaves of rosemary. Plant Physiol 2001;125:1094–102.
[25] Needham L, Hellewell PG, Williams TJ, Gordon JL. Endothelial functional responses and increased vascular permeability induced by polycations. Lab Investig 1988;59:538–48.
[26] Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987;327:524–6.
[27] Peng CH, Su JD, Chyau CC, Sung TY, Ho SS, Peng CC, et al. Supercritical fluid extracts of rosemary leaves exhibit potent anti-inflammation and anti-tumor effects. Biosci Biotechnol Biochem 2007;71:2223–32.
[28] Poeckel D, Greiner C, Verhoff M, Rau O, Tausch L, Hornig C, et al. Carnosic acid and carnosol potently inhibit human 5-lipoxygenase and

suppress pro-inflammatory responses of stimulated human polymorphonuclear leukocytes. Biochem Pharmacol 2008;76:91–7.
[29] Ringbom T, Segura L, Noreen Y, Perera P, Bohlin L. Ursolic acid from *Plantago major*, a selective inhibitor of cyclooxygenase-2 catalyzed prostaglandin biosynthesis. J Nat Prod 1998;61:1212–5.
[30] Ryu SY, Oak MH, Yoon SK, Cho DI, Yoo GS, Kim TS, et al. Anti-allergic and anti-inflammatory triterpenes from the herb of Prunella vulgaris. Planta Med 2000;66:358–60.
[31] Sakurai H, Kohsaka H, Liu MF, Higashiyama H, Hirata Y, Kanno K, et al. Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. J Clin Invest 1995;96:2357–63.
[32] Yoon SB, Lee YJ, Park SK, Kim HC, Bae H, Kim HM, et al. Anti-inflammatory effects of *Scutellaria baicalensis* water extract on LPS-activated RAW 264.7 macrophages. Ethnopharmacology 2009;125: 286–90.
[33] Sheng D, Liu R, Xu Z, Singh P, Shen B, Hua Y. Dual negative regulatory mechanisms of RecX on RecA functions in radiation resistance, DNA recombination and consequent genome instability in *Deinococcus radiodurans*. DNA Repair (Amst) 2005;4:671–8.
[34] Subbaramaiah K, Cole PA, Dannenberg AJ. Retinoids and carnosol suppress cyclooxygenase-2 transcription by CREB-binding protein/p300-dependent and -independent mechanisms. Cancer Res 2002;62: 2522–30.
[35] Suleyman H, Yildirim D, Aslan A, Gocer F, Gepdiremen A, Guvenalp Z. An investigation of the anti-inflammatory effects of an extract from *Cladonia rangiformis* HOFFM. Biol Pharm Bull 2002;25:10–3.
[36] Tamura H, Yamashita S, Kusano N, Suzuki C, Yamaguchi Y, Tanigawa K, et al. Fulminant hepatitis complicated by small intestine infection and massive hemorrhage. J Gastroenterol 1998;33:412–8.
[37] Teixeira MM, Williams TJ, Hellewell PG. Role of prostaglandins and nitric oxide in acute inflammatory reactions in guinea-pig skin. Br J Pharmacol 1993;110:1515–21.
[38] Ushikubi F, Hirata M, Narumiya S. Molecular biology of prostanoid receptors; an overview. J Lipid Mediat Cell Signal 1995;12:343–59.
[39] Vane SJ. Differential inhibition of cyclooxygenase isoforms: an explanation of the action of NSAIDs. J Clin Rheumatol 1998;4:s3–s10.
[40] Williams TJ, Hellewell PG, Jose PJ. Inflammatory mechanisms in the Arthus reaction. Agents Actions 1986;19:66–72.
[41] Williams TJ, Peck MJ. Role of prostaglandin-mediated vasodilatation in inflammation. Nature 1977;270:530–2.
[42] Yi W, Wetzstein HY. Biochemical, biological and histological evaluation of some culinary and medicinal herbs grown under greenhouse and field conditions. J Sci Food Agric 2010;90:1063–70.
[43] Yu YM, Lin CH, Chan HC, Tsai HD. Carnosic acid reduces cytokine-induced adhesion molecules expression and monocyte adhesion to endothelial cells. Eur J Nutr 2009;48:101–6.