



## Immunopharmacology and inflammation

## Anti-inflammatory effects of carvacrol: Evidence for a key role of interleukin-10

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## ABSTRACT

Carvacrol, a phenolic monoterpene, has been reported to possess anti-inflammatory properties. However, the mechanisms involved in its pharmacological properties are currently not well understood. In the present study, the contribution of cytokine modulation to the anti-inflammatory effects of carvacrol was investigated in a classical inflammation model: the complete Freund's adjuvant (CFA)-induced paw inflammation in mice. The paw edema was measured using a plethysmometer. Paw tissue was removed 2 h after the inflammatory stimulus to determine the levels of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) by enzyme immunoassay, the levels of interleukin-1 β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-10 (IL-10) by ELISA or the mRNA expression of cyclooxygenase-2 (COX-2), IL-1β, TNF-α, and IL-10 by real-time PCR. Administration of carvacrol produced anti-inflammatory effects against CFA-induced inflammation in mice. Treatment of mice with carvacrol at 50 and 100 mg/kg attenuated the paw edema and reduced the IL-1β and PGE<sub>2</sub>, but not TNF-α, local levels. Similarly, carvacrol (100 mg/kg) reduced the COX-2 and IL-1β mRNA expression. The levels of IL-10, an anti-inflammatory cytokine, and the IL-10 mRNA expression in the inflamed paw were enhanced by carvacrol. In addition, the treatment with carvacrol did not reduce the CFA-induced paw edema in IL-10 knockout mice. The present results suggest that carvacrol causes anti-inflammatory effects by reducing the production of inflammatory mediators, such as IL-1β and prostanoids, possibly through the induction of IL-10 release.

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## 1. Introduction

It is well accepted that cytokines constitute a link between cellular injuries and signs of inflammation, e.g., in cell migration, edema, fever, and hyperalgesia (Conti et al., 2004; Cunha and Ferreira, 2003; Hopkins, 2003). Cytokines are produced and released from various cell types in response to inflammatory stimuli. The release of inflammatory cytokines, such as interleukin-1 β (IL-1β) and tumor necrosis factor-α (TNF-α), is followed by that of anti-inflammatory cytokines, such as interleukin-10 (IL-10) and interleukin-4 (IL-4) (Hopkins, 2003). Inflammatory cytokines induce the production of mediators involved in the induction of inflammatory signs, while anti-inflammatory cytokines inhibit the production

of inflammatory cytokines (Verri et al., 2006). In line with this idea, the balance between pro- and anti-inflammatory cytokine production modulates the intensity of inflammation.

It has been demonstrated that natural products are a potential source of cytokine production inhibitors (Lima et al., 2011; Nonato et al., 2011; Oliveira et al., 2011). In contrast with non-steroidal anti-inflammatory drugs, widely used medications to treat inflammatory conditions, inhibitors of cytokine production can exhibit disease-modifying activities, thus representing an alternative therapeutic strategy for the control of inflammatory diseases. Carvacrol (5-isopropyl-2-methylphenol) is a phenolic monoterpene presented in the essential oil of the family Lamiaceae, which includes the genera *Origanum* and *Thymus* (Baser, 2008). Previous studies have demonstrated that carvacrol has anti-inflammatory properties (Botelho et al., 2009; Guimarães et al., 2012; Hotta et al., 2010; Landa et al., 2009; Wagner et al., 1986); however, the mechanisms involved in its pharmacological activities are currently not well understood. Considering the key role of cytokines during inflammation, the present study was

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undertaken to evaluate the contribution of cytokine modulation to the anti-inflammatory effects of carvacrol.

## 2. Materials and methods

### 2.1. Animals

Experiments were performed using male Swiss mice (22–28 g) raised and maintained in the animal facilities at Gonçalo Moniz Research Center, FIOCRUZ/BA. IL-10 knockout experiments were performed using male C57BL/6 wild-type (WT) and IL-10-deficient (IL-10 KO) mice. Animals were housed in temperature-controlled rooms (22–25 °C), under a 12:12 h light-dark cycle, with access to water and food ad libitum until use. All behavioral tests were performed between 8:00 a.m. and 5:00 p.m., and animals were only used once. Animal care and handling procedures were in accordance with the Institutional Animal Care and Use Committee of the Gonçalo Moniz Research Center (FIOCRUZ L-029/2009). All efforts were made to minimize the number of animals used and their discomfort.

### 2.2. Inflammatory pain model

Mice were slightly anesthetized with halothane and received 10 µl of complete Freund's adjuvant (CFA; 1 mg/ml of heat-killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monoleate; Sigma, St. Louis, MO, USA) subcutaneously in the plantar region of the right hind paw, according to a method reported previously (Kassuya et al., 2003). Paw edema and cytokines production were measured as described below.

### 2.3. Paw edema measure

The volume of the mouse paws was measured with a plethysmometer (Ugo Basile, Comerio, Italy) before (Vo) and after (VT) the intraplantar injection of CFA, as described previously (Winter et al., 1962). The amount of paw swelling was determined for each mouse and the difference between VT and Vo was taken as the edema value (mm<sup>3</sup>/paw).

### 2.4. Cytokine measurement by ELISA

Skin tissues were removed from the paws 2, 4, and 24 h after the intraplantar injection of CFA (10 µl/paw), in mice terminally anesthetized from each experimental group. Tissue proteins were extracted from 100 mg tissue/ml PBS to which 0.4 M NaCl, 0.05% Tween 20, and protease inhibitors (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KIU aprotinin A/100 ml) were added. The samples were centrifuged for 10 min at 3000g and the supernatant was frozen at –70 °C for later quantification (Santiago et al., 2006). IL-1β, TNF-α and IL-10 levels were estimated using commercially available immunoassay ELISA kits for mice (R&D System, Minneapolis, MN, USA), according to the manufacturer's instructions. The results are expressed as picograms of cytokine per milliliter of protein solution. As controls, the concentrations of these cytokines in saline and vehicle-treated mice were determined.

### 2.5. Determination of PGE<sub>2</sub> levels

The methodology used was similar to that previously described (Pinheiro and Calixto, 2002). Two hours after the intraplantar injection of CFA (10 µl/paw), mice were sacrificed using deep CO<sub>2</sub> anesthesia, and paw skin tissue samples were collected and soaked in 2 ml of PBS containing heparin (5 IU/ml). Indomethacin (50 µg/ml)

was added to prevent further production of PGE<sub>2</sub>. After homogenization, samples were centrifuged at 1300g for 10 min at 4 °C, and the supernatants collected and rapidly frozen and stored at –70 °C until analysis. Levels of PGE<sub>2</sub> were determined using commercially available enzyme immunoassay (EIA) kit according to the manufacturer's recommendations (Cayman Chemical, Ann Arbor, MI, USA).

### 2.6. Real-time PCR

The transcription of cyclooxygenase-2 (COX-2), IL-1β, TNF-α and IL-10 genes was evaluated by real-time quantitative polymerase chain reaction (qRT-PCR) in mice sacrificed 2 h after the CFA injection. Total RNA was isolated from the paw tissue with TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA), and the concentration was determined by photometric measurement. A High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to synthesize cDNA from 1 µg of RNA following the manufacturer's recommendations. The qRT-PCR assays were performed to detect the expression levels of COX-2, TNF-α, IL-1β, and IL-10 genes. Amplification mixtures for qRT-PCR contained 20 ng template cDNA, 10 µl Taqman Master Mix (Applied Biosystems) and probes in a final volume of 20 µl. All reactions were run in duplicate on an ABI7500 Sequence Detection System (Applied Biosystems) under standard thermal cycling conditions. Experiments with coefficients of variation greater than 5% were excluded. A no-template control (NTC) and no-reverse transcription controls (No-RT) were also included. The results are presented as the fold-increase of expression of the individual mRNAs, with the target internal control GADPH using the cycle threshold method.

### 2.7. Test compounds and stimuli

Complete Freund's adjuvant, carvacrol (98% purity), and dexamethasone were purchased from Sigma (St. Louis, MO, USA). Drugs were administrated by intraperitoneal (i.p.) route. Carvacrol was dissolved in 5% Tween 20 plus saline, and dexamethasone was dissolved in ethanol (10% in saline solution).

### 2.8. Statistical analyses

Data are presented as means ± standard error of the mean (S.E.M.) of measurements made on 6 animals in each group. Comparisons between three or more treatments were made using one-way ANOVA with Tukey's post-hoc test or, repeated measures of two-way ANOVA with Bonferroni's post-hoc test, as appropriate. All data were analyzed using Prism 5 Computer Software (GraphPad, San Diego, CA, USA). Statistical differences were considered to be significant at  $P < 0.05$ .

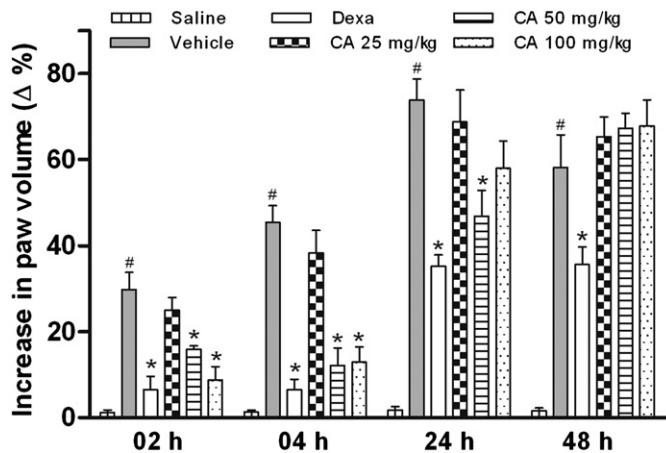
## 3. Results

Administration of carvacrol (50 and 100 mg/kg, i.p.), 40 min before CFA, reduced significantly ( $P < 0.01$ ) the CFA-induced paw edema up to 24 h after the stimuli (Fig. 1). The results obtained with control groups supported the effects observed with carvacrol administration, since vehicle (5% Tween 20 in saline) had no activity, whereas dexamethasone (2 mg/kg), the reference drug, inhibited the CFA-induced edema ( $P < 0.01$ ).

The modulatory action of carvacrol on cytokine production was next evaluated. As shown by qRT-PCR analysis, pretreatment with carvacrol (100 mg/kg, i.p.) reduced the local expression of IL-1β mRNA ( $P < 0.05$ ), but not TNF-α mRNA, during CFA-induced paw inflammation (Fig. 2B and C, respectively). Confirming these results, the paw levels of IL-1β, but not of TNF-α, were reduced

( $P < 0.01$ ) by carvacrol at 100 mg/kg (Fig. 3C and A, respectively). The kinetics of IL-1 $\beta$  production showed that the inhibitory effects of carvacrol persisted until 24 h after CFA challenge (Fig. 3D). As expected, the pretreatment with dexamethasone (2 mg/kg, i.p.) reduced the production of TNF- $\alpha$  and IL-1 $\beta$  induced by CFA (Fig. 3;  $P < 0.05$ ).

Aiming to evaluate a possible inhibition of prostanoid production by carvacrol, the levels of PGE<sub>2</sub> and the expression of COX-2 mRNA were evaluated. Mice pretreated with carvacrol (50 and 100 mg/kg) or dexamethasone (0.5 mg/kg) had a significant ( $P < 0.01$ ) decrease



**Fig. 1.** Effects of carvacrol on complete Freund's adjuvant (CFA)-induced paw inflammation. Paw edema measured at 2, 4, 24, and 48 h after the CFA injection. Mice were injected with carvacrol (CA; 25, 50 and 100 mg/kg), vehicle (5% Tween 20 in saline; control group) or dexamethasone (Dexa; 2 mg/kg; reference drug) by the intraperitoneal route 40 min before CFA (injected at time zero). The saline group received intraplantar injection of saline solution (10  $\mu$ l/paw) instead of CFA. Data are expressed as means  $\pm$  S.E.M.;  $n=6$  mice per group. \* Significantly different from the control group ( $P < 0.01$ ); #significantly different from the saline group ( $P < 0.001$ ). Two-way ANOVA followed by Bonferroni's test.

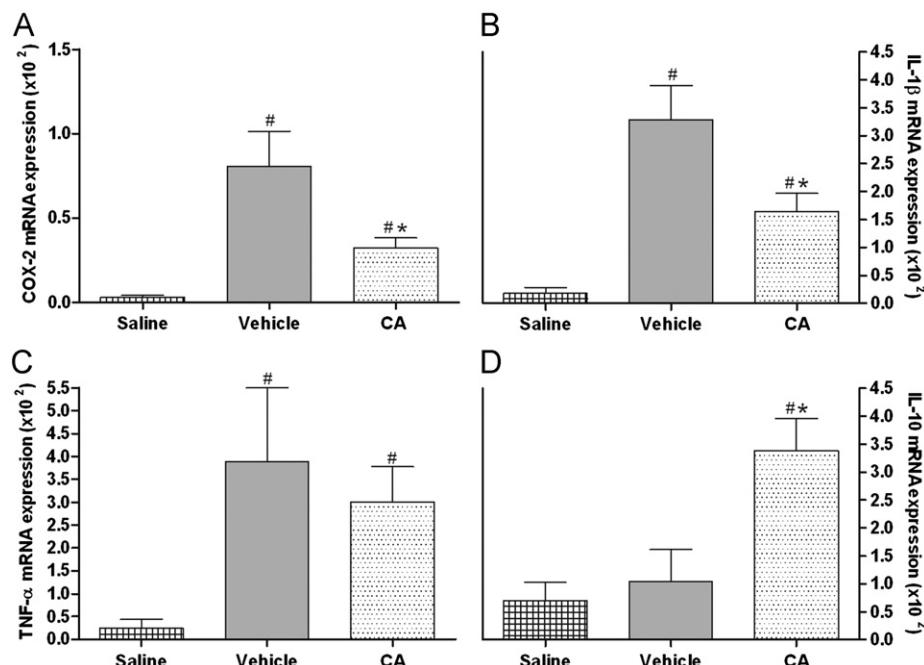
in PGE<sub>2</sub> levels in the paw after CFA injection (Fig. 4). Similarly, a reduction of COX-2 mRNA expression was observed in the paws of carvacrol-treated mice (Fig. 2A;  $P < 0.05$ ).

Next we evaluated the contribution of IL-10, an anti-inflammatory cytokine, to the carvacrol-induced anti-inflammatory effect. Pretreatment with carvacrol (100 mg/kg) induced a significant increase ( $P < 0.01$ ) of the paw expression of IL-10 mRNA (Fig. 2D). Similarly, the local production of IL-10 (Fig. 3E) was enhanced by carvacrol at 50 mg/kg ( $P < 0.01$ ) and 100 mg/kg ( $P < 0.001$ ). The effects of carvacrol on IL-10 production lasted for 24 h (Fig. 3F). Moreover, pretreatment with carvacrol, at a similar range of doses, did not reduce the CFA-induced paw edema in IL-10 knockout mice (Fig. 5).

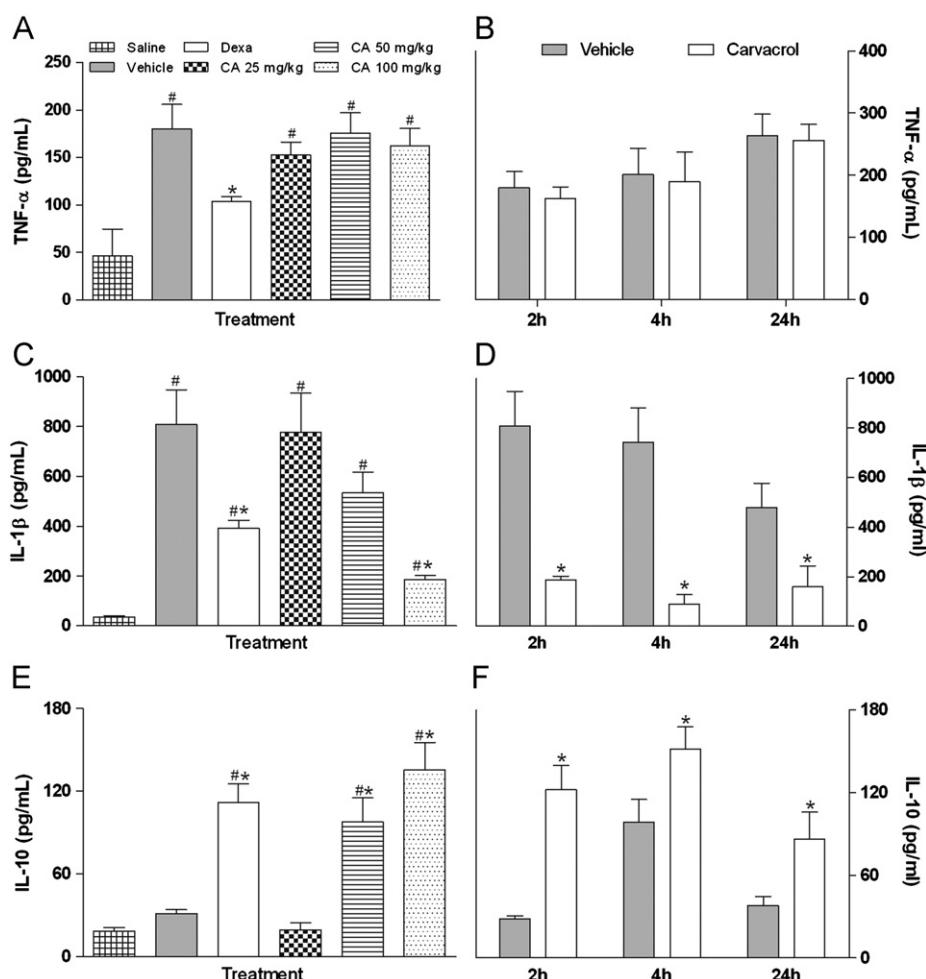
#### 4. Discussion

In the present study, aiming to understand the mechanisms by which carvacrol induces its anti-inflammatory effects, the production of important mediators of inflammation was evaluated. Treatment of mice with carvacrol, at a similar range of doses, attenuated the paw edema and inhibited key events related to inflammation, namely, the local increase of IL-1 $\beta$  and PGE<sub>2</sub> levels and the induction of COX-2 expression. In addition, carvacrol enhanced the local IL-10 release, while was unable to reduce the inflammation in IL-10 knockout mice. Therefore, the present data suggest that carvacrol induces anti-inflammatory effects by reducing the production of IL-1 $\beta$  and PGE<sub>2</sub>, important inflammatory mediators, possible through a mechanism dependent on IL-10 production.

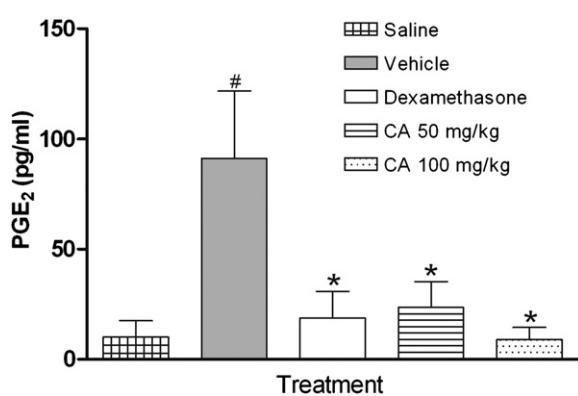
Using an experimental protocol for screening of new anti-inflammatory drugs, the CFA-induced paw inflammation model, the mechanisms involved with the anti-inflammatory action of carvacrol were investigated. Administration of carvacrol reduced the CFA-induced paw edema, confirming the data from Guimarães et al. (2012) showing that carvacrol has anti-edematogenic properties in



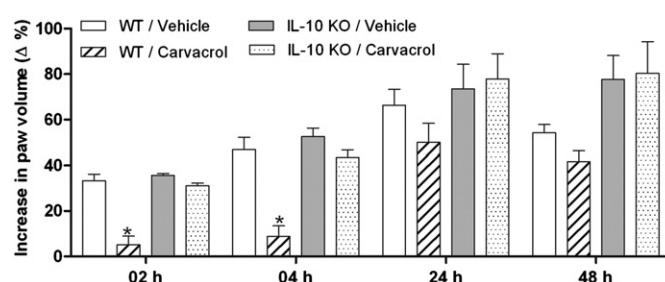
**Fig. 2.** Effects of carvacrol on COX-2 mRNA (A), IL-1 $\beta$  mRNA (B), TNF- $\alpha$  mRNA (C), and IL-10 mRNA (D) paw levels. Mice were injected with carvacrol (CA; 100 mg/kg) or vehicle (5% Tween 20 in saline; control group) by the intraperitoneal route 40 min before the intraplantar injection of complete Freund's adjuvant (CFA). The paw levels of COX-2 mRNA, IL-1 $\beta$  mRNA, TNF- $\alpha$  mRNA, and IL-10 mRNA were measured by qRT-PCR 2 h after the CFA injection. The saline group received intraplantar injection of saline (10  $\mu$ l/paw) instead of CFA. Data are reported as means  $\pm$  S.E.M.;  $n=6$  mice per group. \* Significantly different when compared to the control group ( $P < 0.05$ ); #significantly different when compared to the saline group ( $P < 0.01$ ). One-way ANOVA followed by Tukey's test.



**Fig. 3.** Effects of carvacrol on TNF- $\alpha$  (A and B), IL-1 $\beta$  (C and D), and IL-10 (E and F) paw levels. Mice were injected with carvacrol (CA; 25–100 mg/kg), vehicle (5% Tween 20 plus saline; control group) or dexamethasone (Dexa; 2 mg/kg; reference drug) by the intraperitoneal route 40 min before the intraplantar injection of complete Freund's adjuvant (CFA). The saline group received intraplantar injection of saline (10  $\mu$ L/paw) instead of CFA. The paw levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-10 were measured by ELISA 2 h (A, C and E) or 2, 4, and 24 h (B, D and F) after the CFA injection. Data are reported as means  $\pm$  S.E.M.;  $n=6$  mice per group. \* Significantly different when compared to the control group ( $P < 0.01$ ); # significantly different from the saline group ( $P < 0.01$ ). One-way ANOVA followed by Tukey's test.



**Fig. 4.** Effect of carvacrol on PGE $_2$  paw levels. Mice were injected with carvacrol (CA; 50 and 100 mg/kg), vehicle (5% Tween 20 plus saline; control group) or dexamethasone (2 mg/kg; reference drug) by the intraperitoneal route 40 min before the intraplantar injection of complete Freund's adjuvant (CFA). The saline group received intraplantar injection of saline (10  $\mu$ L/paw) instead of CFA. The paw levels PGE $_2$  were measured by EIA 2 h after the CFA injection. Data are reported as means  $\pm$  S.E.M.;  $n=6$  mice per group. \* Significantly different when compared to the control group ( $P < 0.05$ ); #significantly different when compared to the saline group ( $P < 0.01$ ). One-way ANOVA followed by Tukey's test.



**Fig. 5.** Effects of carvacrol on complete Freund's adjuvant (CFA)-induced paw inflammation in wild-type (WT) and IL-10-deficient (IL-10 KO) mice. Paw edema measured at 2, 4, 24, and 48 h after the CFA injection. Mice were injected with carvacrol (100 mg/kg) or vehicle (5% Tween 20 in saline; control group) by the intraperitoneal route 40 min before CFA (injected at time zero). Data are expressed as means  $\pm$  S.E.M.;  $n=6$  mice per group. \* Significantly different when compared to the WT/Vehicle group ( $P < 0.01$ ). Two-way ANOVA followed by Bonferroni's test.

mice. In fact, the anti-inflammatory activity of carvacrol has been demonstrated by in vitro and in vivo assays (Botelho et al., 2009; Hotta et al., 2010; Landa et al., 2009; Wagner et al., 1986).

CFA, which consists of heat-killed mycobacteria suspended in a mineral oil vehicle, produces a chronic inflammatory condition when injected into rodents. It is well recognized that the injection of CFA produces inflammation initiated by a local release of mediators, such as cytokines and prostanoids, which are involved in the inflammatory signs (Conti et al., 2004; Cunha and Ferreira, 2003; Dinarello, 2000; Fehrenbacher et al., 2012; Woolf et al., 1997). Therefore, to investigate the mechanisms involved in the anti-inflammatory action of carvacrol, its possible modulatory action on cytokine production was evaluated. Data obtained by qRT-PCR and ELISA analyses show that carvacrol reduced the local production of IL-1 $\beta$ , but not TNF- $\alpha$ , during paw inflammation. In contrast, a recent work, Guimarães et al. (2012) demonstrated that carvacrol decreased TNF- $\alpha$  concentrations in pleural lavage fluid in a mouse pleurisy model induced by carrageenan. The difference on the effects in TNF- $\alpha$  production by carvacrol may depend on the characteristics of the inflammation models (pleurisy vs. paw inflammation) or on the inflammatory stimuli (carrageenan vs. CFA) used in these studies. In fact, the cytokine response to inflammation is extremely complex and depends on the inflammatory stimuli (Fehrenbacher et al., 2012).

Cytokines are small regulatory proteins produced by white blood cells and a variety of other cell types. Currently, it is well accepted that cytokines constitute a link between cellular injuries and signs of inflammation (Dinarello, 2000; Faccioli et al., 1990; Verri et al., 2006). IL-1 $\beta$  is a pro-inflammatory cytokine that induce the production of other inflammatory mediators involved with cellular recruitment, fever, acute phase protein release, increase of vascular permeability, and hyperalgesia (Dinarello, 1998). Considering the important role of IL-1 $\beta$  during the inflammatory response, it may be suggested that the anti-inflammatory effects of carvacrol are related to an ability to inhibit the release of this cytokine. It is known that IL-1 $\beta$  release precedes the production of the final inflammatory mediators, such as prostaglandins (Cunha et al., 2005; Verri et al., 2006). IL-1 $\beta$  stimulates the expression of COX-2 and the subsequent production of prostaglandins (Croxford et al., 1994; Zucali et al., 1986). Based on the relevant anti-inflammatory action of non-steroidal anti-inflammatory drugs and its cyclooxygenase inhibitory effect, it is accepted that prostaglandins are important contributors of inflammatory response. Since the inhibition of IL-1 $\beta$  release might lead to the inhibition of prostanoid production, it is possible that carvacrol acts by preventing the production of final mediators of inflammation. Aiming to evaluate this hypothesis, the effect of carvacrol on PGE<sub>2</sub> levels and COX-2 expression were evaluated. In the present study, carvacrol decreased the COX-2 mRNA expression and the PGE<sub>2</sub> levels in the paw after the CFA stimuli. Since the COX-2/PGE<sub>2</sub> pathway is a key event during different inflammatory conditions, the reduction of PGE<sub>2</sub> levels and COX-2 expression can be responsible to the anti-inflammatory effects of carvacrol.

To limit the deleterious consequences of prolonged inflammatory reaction, the release of pro-inflammatory cytokines is followed by the release of anti-inflammatory cytokines, such as IL-4, IL-10, and IL-13, which inhibit the production and action of the pro-inflammatory cytokines (Verri et al., 2006). IL-10, in fact, acts as a potent anti-inflammatory cytokine by suppressing the activation and function of immune cells. Accordingly, a crucial effect of IL-10 is its ability to selectively block the expression of pro-inflammatory cytokines (Moore et al., 2001), including IL-1 $\beta$  (De Waal Malefyt et al., 1991; Karam et al., 2007). Thus, we investigated the possibility that IL-10 production contributes to the anti-inflammatory effect of carvacrol. Pretreatment with carvacrol enhanced the local production of IL-10. Importantly, the edema inhibition, reduction of PGE<sub>2</sub> levels, and enhance of IL-10 production were significant at similar doses of carvacrol,

showing that the anti-inflammatory effect parallels to the IL-10 release. It has been demonstrated that the inhibitory effects of IL-10 on IL-1 $\beta$  and TNF- $\alpha$  production are crucial to its anti-inflammatory activities, because these cytokines often have synergistic activities on inflammatory processes, and amplify these responses by inducing secondary mediators, such as chemokines and prostaglandins (Moore et al., 2001). In accordance, IL-10 inhibits the production of PGE<sub>2</sub>, through down regulation of COX-2 expression (Niiro et al., 1994, 1995). The present data indicates that carvacrol, by stimulating the IL-10 action, produced anti-edematogenic effects, down regulated the COX-2 expression, and reduced the local levels of IL-1 $\beta$  and PGE<sub>2</sub>. The contribution of IL-10 to the anti-inflammatory effect of carvacrol was confirmed in IL-10-deficient mice, in which carvacrol did not produce anti-edematogenic effect.

## 5. Conclusions

In conclusion, it is possible to propose that the anti-inflammatory properties of carvacrol are dependent on its ability to reduce the production of inflammatory mediators, such as IL-1 $\beta$  and prostanoids, possibly through the induction of IL-10 release.

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