BIOCHEMICAL PHARMACOLOGY 76 (2008) 91–97

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Carnosic acid and carnosol potently inhibit human 5-lipoxygenase and suppress pro-inflammatory responses of stimulated human polymorphonuclear leukocytes

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ARTICLE INFO

Article history:
Received 28 March 2008
Accepted 24 April 2008

Keywords:
Carnosol
Carnosic acid
5-Lipoxygenase
Reactive oxygen species
Leukocyte elastase
Inflammation

ABSTRACT

Carnosic acid (CA) and carnosol (CS) are phenolic diterpenes present in several labiate herbs like *Rosmarinus officinalis* (Rosemary) and *Salvia officinalis* (Sage). Extracts of these plants exhibit anti-inflammatory properties, but the underlying mechanisms are largely undefined. Recently, we found that CA and CS activate the peroxisome proliferator-activated receptor gamma, implying an anti-inflammatory potential on the level of gene regulation. Here we address short-term effects of CA and CS on typical functions of human polymorphonuclear leukocytes (PMNL). We found that (I), CA and CS inhibit the formation of pro-inflammatory leukotrienes in intact PMNL (IC_{50} = 15–20 μM [CA] and 7 μM [CS], respectively) as well as purified recombinant 5-lipoxygenase (EC number 1.13.11.34, IC_{50} = 1 μM [CA] and 0.1 μM [CS], respectively), (II) both CA and CS potently antagonise intracellular Ca^{2+} mobilisation induced by a chemotactic stimulus, and (III) CA and CS attenuate formation of reactive oxygen species and the secretion of human leukocyte elastase (EC number 3.4.21.37). Together, our findings provide a pharmacological basis for the anti-inflammatory properties reported for CS- and CA-containing extracts.

1. Introduction

The o-diphenolic diterpene carnosic acid (CA) and its oxidation product carnosol (CS, Fig. 1) are major ingredients of extracts from *Rosmarinus officinalis* (Rosemary) and *Salvia officinalis* [1–4] both of which possess anti-oxidant and anti-inflammatory properties [5–8]. Dried herbs of Rosemary and Sage contain approximately 0.2–1% of CA and of CS, each [9], and in commercially available extracts CA and CS are strongly enriched (e.g. for Rosemary 10.3% CA and 4.4% CS was

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Abbreviations: AA, arachidonic acid; CA, carnosic acid; CS, carnosol; DCF-DA, 2′,7′-dichlorofluorescein diacetate; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HLE, human leukocyte elastase; LO, lipoxygenase; LT, leukotriene; PG buffer, PBS plus 1 mg/ml glucose; PLA_2, phospholipase A_2; PMNL, polymorphonuclear leukocytes; PPAR, peroxisome proliferator-activated receptor; PKC, protein kinase C; PMA, phorbol myristate acetate; ROS, reactive oxygen species.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.04.013

determined [10]). CA and CS have been shown to act as antioxidants, and anti-inflammatory, anti-proliferative, antitumorigenic and neuroprotective effects have been reported [2,11–14] (and references therein). Based on the abundant occurrence of CS and CA as herbal dietary ingredients, the development of natural remedies containing these diterpenes for the treatment of inflammatory diseases appears attractive. In support of this goal, it was shown that orally administered CA is present in the blood and in the brain in pharmacologically relevant concentrations [12].

Recently, we identified the peroxisome proliferator-activated receptor gamma (PPARγ) as a target selectively activated by CA and CS [14]. Activation of PPARγ causes long-term alteration of the expression of inflammatory gene products, implying the modulation of inflammatory cell functions in a comparably slow mode. In contrast, little is known about short-term effects of CS and CA on the molecular level or in pro-inflammatory cells (or animal models) that occur within minutes, distinct from gene expression-related mechanisms. CS has been shown to suppress the formation of pro-inflammatory leukotrienes (LTs) in rat leukocytes [15]. LTs are products of 5-lipoxygenase (5-LO, EC number 1.13.11.34) [16,17]; however, a potential direct interference of CS with 5-LO was not investigated [15]. Furthermore, CS inhibits aggregation of rabbit platelets presumably by blocking collagen-induced Ca^{2+} signals and antagonizing the thromboxane receptor [18]. Despite these findings, it is presently unclear whether the anti-inflammatory properties of CS- and CA-containing extracts are primarily attributable to short-term actions, slow transcriptional events, or a combination of both.

Polymorphonuclear leukocytes (PMNL) are important early effectors of the innate immune response and play a crucial role in the development of an inflammatory phenotype [19]. They are highly responsive to chemotactic agonists such as the bacterial tripeptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) [20,21]. Activation of the G protein-coupled receptor for fMLP causes elevations of cytosolic [Ca^{2+}] [22]. Ca^{2+} acts as second messenger and induces a number of distal signalling events, resulting in the elicitation of functional PMNL responses [23,24] including the release of reactive oxygen species (ROS), predominantly generated by NADPH oxidase [25,26]. Ca^{2+} also promotes the exocytosis of stored vesicles containing proteolytic enzymes like human leukocyte elastase (HLE, EC number 3.4.21.37) or cathepsin G [27]. Elevated ROS levels and enhanced protease release are typical early events in inflammatory reactions [28–30].

Here, we address the ability of CA and CS to directly interfere with typical short-term pro-inflammatory functions of human PMNL including LT formation, generation of ROS, and secretion of HLE. Moreover we show that the suppression of these PMNL functions correlates with reduced mobilisation of intracellular Ca^{2+}, and we present 5-LO as a molecular target of CA and CS.

2. Materials and methods

2.1. Reagents

CA, CS, fMLP, and Fura-2/AM were obtained from Axxora (Lörrach, Germany). 2′,7′-Dichlorofluorescein diacetate (DCF-DA) was obtained from Molecular Probes (Leiden, Netherlands); Nycoprep was from PAA Laboratories (Pasching, Austria); dextran was from Fluka (Seelze, Germany); cytochalasin B, HLE substrate MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide and all other chemicals were obtained from Sigma (Munich, Germany).

2.2. Cells

Human PMNL were freshly isolated from leukocyte concentrates obtained at St. Markus Hospital (Frankfurt, Germany) or at the Institute for Clinical and Experimental Transfusion Medicine, University Medical Center Tuebingen, Germany. In brief, venous blood was taken from healthy adult donors and subjected to centrifugation for preparation of leukocyte concentrates. PMNL were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions, and hypotonic lysis of erythrocytes as described [31]. PMNL were finally resuspended in PBS plus 1 mg/ml glucose (PG buffer).

2.3. Cell viability assessment

Potential acute cytotoxic effects of CA and CS on human PMNL were assessed by trypan blue exclusion. Cells were exposed to CA, CS (30 μM, each) or DMSO (0.3%), respectively, for 45 min at 37 °C, and the number of viable and dead cells was counted under a light microscope. No reduction in cellular viability of CS- or CA-exposed PMNL versus DMSO-treated cells was detectable ($n = 6$).

2.4. Determination of 5- and 12-lipoxygenase products

5×10^6 freshly isolated human PMNL were resuspended in PG buffer plus 1 mM $CaCl_2$ and pre-incubated with the test compounds for 15 min at room temperature (RT). 5-LO and 12-LO product formation was started by the addition of 2.5 μM Ca^{2+} ionophore A23187 plus 20 μM arachidonic acid (AA) as indicated. After 10 min at 37 °C, the reaction was stopped with 1 ml of methanol and 30 μl of 1N HCl, 200 ng prostaglandin B_1 and 500 μl of PBS were added. Formed 5-LO and 12-LO metabolites were extracted and analyzed by HPLC as described [31,32]. 5-LO products include LTB_4 and its all-trans isomers, 5(S),12(S)-di-hydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid (5(S),12(S)-DiHETE), and 5(S)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-H(p)ETE). Cysteinyl LTs (LTC_4, D_4 and E_4) were not detected and oxidation products of LTB_4 were not determined. 12-LO products, generated presumably by contaminating platelets, include 12(S)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (12-H(P)ETE). 12-HETE plus 12-HPETE elute as one major peak.

2.5. Expression, purification and activity studies of human recombinant 5-lipoxygenase

Expression of 5-LO, performed in *Escherichia coli* BL 21 cells transfected with pT3-5LO, and partial purification of 5-LO by ATP affinity chromatography (Sigma A2767) was performed as described previously [33]. The partially purified 5-LO (0.5 μg in 5 μl) was added to 1 ml of a 5-LO reaction mix (PBS, pH 7.4, 1 mM EDTA, and 1 mM ATP). Test compounds or vehicle

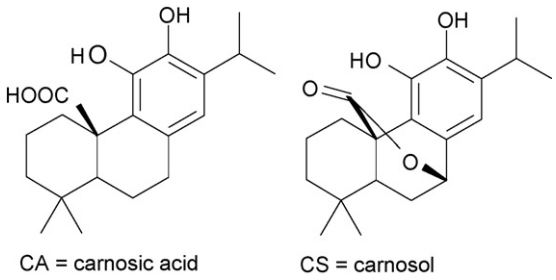

Fig. 1 – Chemical structures of carnosic acid (CA) and carnosol (CS).

(DMSO) were added and after 5–10 min at 4 °C, samples were prewarmed for 30 s at 37 °C. Then, 2 mM $CaCl_2$ and 20 μM AA were added to start 5-LO product formation. The reaction was stopped after 10 min at 37 °C by addition of 1 ml ice-cold methanol and the formed metabolites were analyzed as described above for PMNL.

2.6. Measurement of intracellular Ca^{2+} mobilisation

Freshly isolated PMNL (1×10^7) were resuspended in 1 ml PG buffer and incubated with 2 μM Fura-2/AM for 30 min at 37 °C in the dark. After washing, cells were finally resuspended in 1 ml PG buffer, pre-incubated with the indicated compounds for 15 min, supplemented with 1 mM $CaCl_2$, and transferred into a thermally controlled (37 °C) fluorimeter cuvette in a spectrofluorometer (Aminco-Bowman Series 2, Thermo Fisher, Dreieich, Germany) with continuous stirring. Release of $[Ca^{2+}]_i$ was initiated by addition of fMLP (0.1 μM). The fluorescence emission at 510 nm was measured after excitation at 340 and 380 nm, respectively. Intracellular Ca^{2+} levels were calculated according to the method of Grynkiewicz et al. [34]. F_{max} (maximal fluorescence) was obtained by lysing the cells with 0.5% Triton-X 100 and F_{min} by chelating Ca^{2+} with 10 mM EDTA.

2.7. Formation of reactive oxygen species

PMNL (1×10^7 in 1 ml PG buffer) were preincubated with the test compounds for 15 min at RT. Then, the peroxide-sensitive fluorescence dye DCF-DA (1 μg/ml) and $CaCl_2$ (1 mM) were added 2 min prior addition of fMLP (1 μM) or phorbol myristate acetate (PMA, 0.1 μM). The fluorescence emission at 530 nm was measured after excitation at 485 nm in a thermally controlled (37 °C) cuvette with continuous stirring in a spectrofluorometer (Aminco-Bowman Series 2). The mean fluorescence data determined before and 210 s after stimulus addition are expressed as percentage of control.

2.8. Determination of leukocyte elastase release

PMNL (3×10^6), resuspended in 100 μl PG buffer plus 1 mM $CaCl_2$, were pre-incubated with the test compounds for 15 min. Then, cells were subsequently stimulated with cytochalasin B (cytB, 10 μM) and fMLP (2.5 μM) for 5 min (each) at 37 °C, and stopped on ice. After centrifugation ($1000 \times g$, 5 min, 4 °C), supernatants containing released HLE were incubated with MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide (200 μM) for 15 min at 37 °C. The extent of *p*-nitrophenol release was measured at 405 nm using a Victor^3 plate reader (PerkinElmer, Rodgau, Germany).

2.9. Statistics

IC_{50} values are approximations determined by graphical analysis (linear interpolation between the points between 50% activity). Statistical evaluation of the original data (determined in at least three independent experiments) prior to normalization was performed by Student's *t*-test for paired observations or one-way analysis of variance (ANOVA) for independent or correlated samples, followed by Tukey HSD post hoc tests. A p value < 0.05 (*) was considered significant (**$p < 0.01$).

3. Results

3.1. Inhibition of lipoxygenases by carnosic acid and carnosol

We investigated the inhibitory potential of CA and CS on 5-LO product formation in intact human PMNL stimulated with Ca^{2+} ionophore A23187 in the presence of exogenous AA. CS inhibited the formation of 5-LO products with an $IC_{50} = 7$ μM (Fig. 2A), which is in the range of the value (2 μM) determined by others in rat leukocytes [15]. The IC_{50} of CA was determined at 15–20 μM (Fig. 2A). Interestingly, both compounds also inhibited purified human recombinant 5-LO with increased potency. The IC_{50} values were determined at 0.1 μM (CS) and 1 μM (CA), respectively (Fig. 2C). In parallel, we studied the effects on 12-LO. Whereas CA did not exert any inhibition of cellular 12-LO product synthesis, CS inhibited 12-LO activity with an IC_{50} of approximately 13 μM (Fig. 2B). Thus, CS is a potent inhibitor of both 5- and 12-LO product formation, whereas CA is less efficient but may exhibit some selectivity for 5-LO.

3.2. Influence of carnosic acid and carnosol on Ca^{2+} homeostasis

Following pre-treatment with CA or CS, fMLP was used to evoke an increase in intracellular Ca^{2+} in PMNL. We observed a concentration-dependent inhibitory effect of CA on the fMLP-activated Ca^{2+} signal with a maximum of ~80% inhibition at 30 μM. CS also inhibited Ca^{2+} elevations although with slightly reduced efficiency (Fig. 3). Together, both CA and CS markedly antagonise intracellular Ca^{2+} mobilisation evoked by fMLP.

3.3. Effects of carnosic acid and carnosol on the formation of reactive oxygen species

Next, we addressed whether CA and CS interfere with the formation of ROS in PMNL. CA concentration-dependently inhibited the formation of ROS in fMLP-stimulated PMNL ($IC_{50} = 5$ μM, Fig. 4). The increase in background fluorescence of unstimulated, DMSO-treated cells (29% of stimulated control) is indicated by a dotted line. In accordance with the

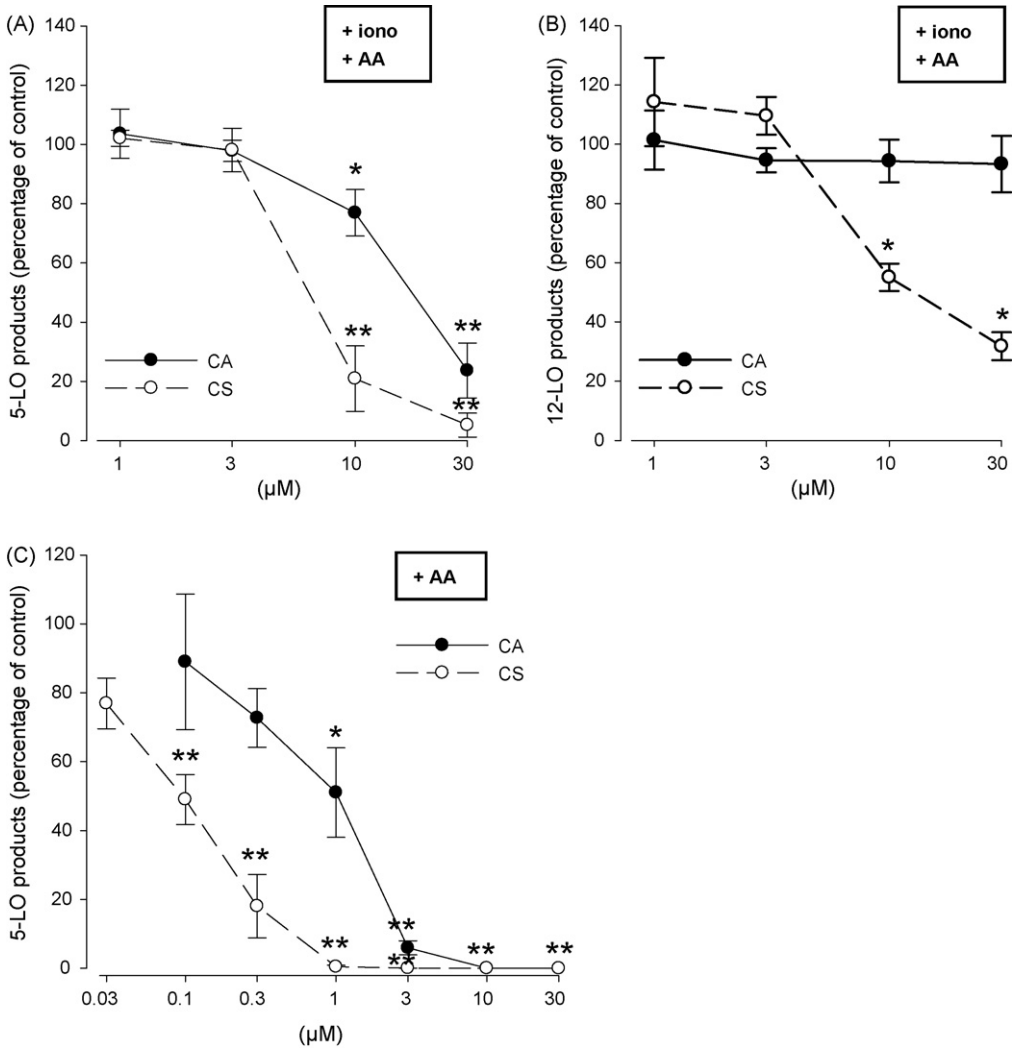

Fig. 2 – Inhibition of 5-lipoxygenase and 12-lipoxygenase. Intact PMNL were pre-incubated with the indicated concentrations of CA and CS for 15 min. 5-LO (A) and 12-LO (B) product formation was initiated by addition of 2.5 μM Ca^{2+} ionophore (iono) plus 20 μM AA. Averaged data were normalised to control cells pre-incubated with solvent (DMSO, 0.1%). (C) Purified recombinant 5-LO was supplemented with CA or CS and 1 mM ATP, and product formation was started with the addition of 2 mM $CaCl_2$ and 20 μM AA. Formed products within 10 min at 37 °C were analysed as described. In the absence of test compounds (100%, control), cellular 5-LO product formation was 1816 ± 153 ng, cellular 12-LO product synthesis was 161 ± 41 ng, and product formation of purified 5-LO was 1345 ± 621 ng. Values are given as mean ± S.E., $n = 3$; data were analysed by ANOVA followed by Tukey-HSD post hoc test: $^{*}p < 0.05$, $^{**}p < 0.01$ vs. vehicle control (w/o).

anti-oxidant properties of CS reported in the literature [8], CS almost completely inhibited ROS formation at 10 μM. Since the signals of both CS and CA were already close to basal levels at 10 μM, no higher concentrations were tested.

In order to assess whether the inhibitory action of CA and CS was due to unspecific antioxidant properties, the effects of CA on ROS generation were determined in PMNL challenged with PMA. In PMNL, ROS are mainly generated by NADPH oxidase [26], a target of protein kinase C, which is activated by PMA [35]. The PMA-induced ROS formation was significantly reduced by CS at 10 μM, reflecting its known radical-scavenging properties [36]. In contrast, CA did not interfere with PMA-activated ROS generation up to 10 μM (Fig. 4), excluding unspecific anti-oxidant properties. Instead, a selective interference within the fMLP-induced signalling cascade is likely.

3.4. *Effects of carnosic acid and carnosol on the secretion of elastase*

Upon PMNL activation, HLE may be released from intracellular granules [37]. This degranulation process is triggered by intracellular Ca^{2+} elevations and can be evoked by PMNL stimulation with cytochalasin B (cytB) and fLMP [38]. CA and CS concentration-dependently inhibited the secretion of HLE, with CA being slightly superior to CS (Fig. 5). Conclusively, CA and CS potently prevent the secretion of elastase from fMLP-challenged PMNL.

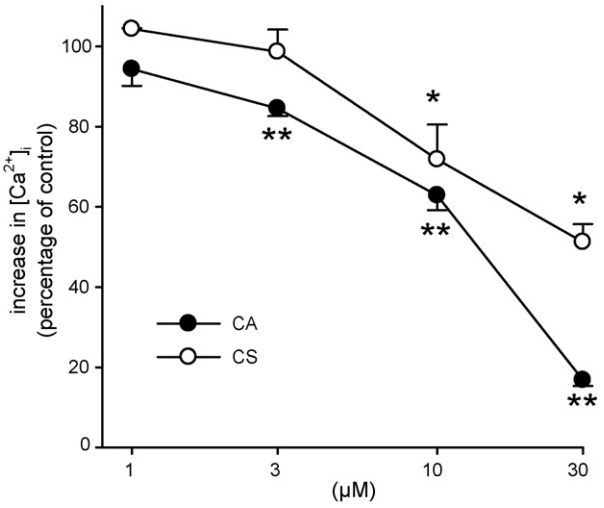

Fig. 3 – Inhibition of Ca^{2+} mobilisation. Fura-2-labeled PMNL were pre-incubated with the indicated compounds for 15 min, supplemented with 1 mM $CaCl_2$, and stimulated with fMLP (0.1 μM). Concentration–response curves for CA and CS display the increases in $[Ca^{2+}]_i$ (mean ± S.E.; $n \geq 3$) at maximum amplitude. Data are expressed as percentage of control cells, pre-incubated with vehicle (DMSO). In the absence of test compounds (100%, control), the increase in $[Ca^{2+}]_i$ was 86 ± 11 nM. Student's *t*-test for paired observations: $^{*}p < 0.05$; $^{**}p < 0.01$.

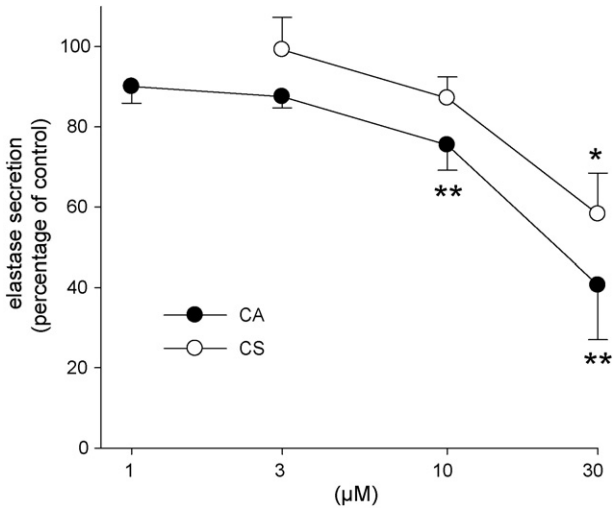

Fig. 5 – Inhibition of human leukocyte elastase secretion. PMNL were pre-incubated for 15 min with the indicated compounds and stimulated with cytochalasin B (10 μM) plus fMLP (2.5 μM) for 5 min at 37 °C. The amount of released HLE was compared by the levels of substrate conversion after 15 min at 37 °C. Concentration–response curves of CA and CS are shown. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control cells (background-corrected basal absorbance increase 0.42 ± 0.05 units), pre-incubated with vehicle (DMSO). Student's *t*-test for paired observations: $^{*}p < 0.05$; $^{**}p < 0.01$.

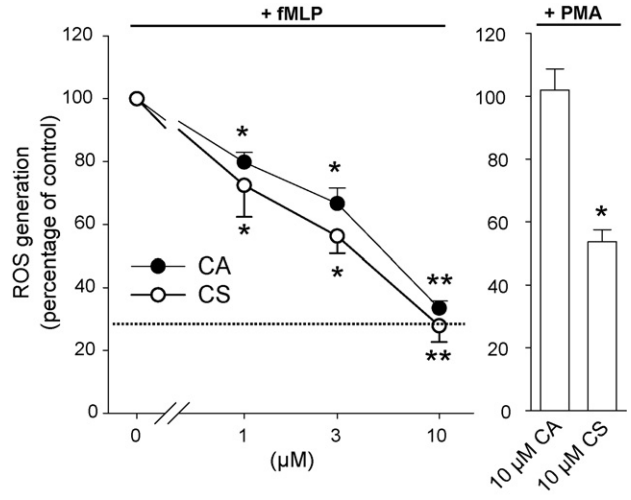

Fig. 4 – Inhibition of ROS formation. PMNL were pre-incubated with the compounds for 15 min at the indicated concentrations, loaded with the fluorescent dye DCF-DA, supplemented with 1 mM $CaCl_2$, and stimulated with fMLP (1 μM) or PMA (0.1 μM) as indicated. The relative increase in ROS formation was determined after 210 s at 37 °C. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control cells pre-incubated with vehicle (DMSO). The increase in background fluorescence of DMSO-treated cells is indicated by a dotted line (29% of control). Student's *t*-test for paired observations: $^{*}p < 0.05$; $^{**}p < 0.01$.

4. Discussion

Anti-oxidant and anti-inflammatory effects of extracts from Rosemary and Sage have been demonstrated in animal models [5–7]. Similarly, anti-inflammatory and anti-tumorigenic properties of CA and CS were reported in various *in vitro* studies (Ref. [14] and references therein). Transcriptional activation of the anti-inflammatory PPARγ was proposed as the underlying mechanism [14], but this model does not apply to the immediate effects of the compounds that have been observed by others [15,18,39]. In the present report we explored the possibility of short-term anti-inflammatory effects of CA and CS by assessing their effectiveness on the functionality of human PMNL. Three distinct features of activated PMNL were addressed: (1) the capacity to generate 5-LO and 12-LO products; (2) rapid cellular activation reflected by intracellular Ca^{2+} mobilisation; (3) distal cellular functions represented by the formation of ROS and the secretion of HLE.

First, both CA and CS inhibit the biosynthesis of pro-inflammatory LTs in intact PMNL in the low micromolar range. This effect is not conferred by the abrogation of substrate supply for 5-LO (AA provided by phospholipase (PL)A_2), as supplementation of exogenous substrate circumvented the need for liberation of endogenous AA by PLA_2s. Also, purified human recombinant 5-LO was directly inhibited by both CS and CA (IC_{50} = 0.1 and 1 μM, respectively). Thus, inhibition of 5-LO most likely accounts for the suppression of LT formation in intact PMNL. Differences in the IC_{50} values between the

recombinant 5-LO enzyme and intact cells are frequently apparent for 5-LO inhibitors [16] and might be due to limited availability of the inhibitor in intact cells, potential inhibitor degradation, or enrichment of the inhibitor in subcellular compartments different from those where 5-LO resides. From structural considerations, the *o*-diphenolic diterpenes CA and CS belong to the category of redox-active 5-LO inhibitors which encompass lipophilic reducing compounds such as flavonoids and other polyphenols, hydroquinones, or coumarines [16]. These agents often act non-specifically on iron-containing enzymes, which might explain the LO inhibitory action of CS, since it also inhibits the related 12-LO. However, the select inhibitory effect of CA on 5-LO may imply a different mode of action for this molecule which will require further assessment. Although CA was somewhat superior over CS to suppress Ca^{2+} mobilisation, ROS formation and HLE release, CS was more potent than CA regarding inhibition of 5-LO (and 12-LO). This could be related to the higher hydrophobicity of CS (a lactone) versus the anionic structure of CA (a carboxylate), since the 5-LO inhibitory potency of *o*-diphenolic compounds appears to correlate to their lipophilicity [16].

An increased peroxide tone favours activation of 5-LO by facilitating the enzyme to enter the catalytic cycle [16,17]. CA and CS have reducing properties [13,15] (overview given in Ref. [14]), and our data suggest that CS indeed acts as an anti-oxidant by blocking the PMA-induced ROS formation. Hence, besides direct interference with the active site iron, deprivation of peroxides could also contribute to the inhibitory action of CS on 5-LO. In a previous report about inhibition of 5-LO product formation by CS in rat leukocytes, no AA was supplemented, leaving open the possibility for PLA_2 suppression rather than direct 5-LO inhibition [15]. This altered approach might also explain the approx. threefold lower IC_{50} value determined for CS without exogenous AA (2 μM [15]) versus presence of exogenous substrate (IC_{50} = 7 μM, this study). Species disparities (rat vs. human) could also account for the observed differences. Suppression of 5-LO and 12-LO results in downregulation of the pro-inflammatory activity of leukocytes and platelets [40,41], which may cause a diminished or delayed outcome of the inflammatory reaction. This would be in line with pharmacological evidence for the observed anti-inflammatory effects of CA- and CS-containing extracts [5–7].

In addition to the potent inhibition of 5-LO, we demonstrate a prominent reduction of the fMLP-induced intracellular Ca^{2+} mobilisation by CA and CS. For CS, this is consistent with a recent report [18], where CS antagonised Ca^{2+} transients in rabbit platelets evoked by collagen. Interference with Ca^{2+} homeostasis by CA is shown for the first time here. CA also exhibits a specific interference with the signalling pathway of fMLP leading to the formation of ROS. At 10 μM, the effect of fMLP is completely blunted by CA, indicating a potent blockade of this pro-inflammatory event. Finally, the release of HLE as a distal functional response of activated leukocytes was used as experimental model. Secretion of elastase-containing granules is a Ca^{2+}-dependent process [27,28], and it appeared reasonable that CA and CS interfere at this stage as well. Increased HLE activity is correlated to the break down of matrix proteins at sites of inflammation [29] and raises the risk of a chronic inflammatory state [30]. Thus, the presence of CA and CS, which antagonise HLE release, would seem beneficial in the prevention of such an inflammatory process.

Conclusively, we provide a detailed analysis of typical immediate anti-inflammatory properties of CA and CS in human PMNL. CA and CS have several common but also distinct effects on PMNL activation and functional characteristics. Both compounds potently suppress 5-LO product synthesis by direct interference with enzyme activity, antagonise Ca^{2+} homeostasis, and reduce ROS production as well as HLE release. The interference with multiple pro-inflammatory processes implies a synergistic and overall high effectiveness of these compounds. Besides direct inhibition of 5-LO, it is, however, unclear which molecular target(s) are affected by CA and CS resulting in the suppressed PMNL functionality. Since CA and CS attenuated all fMLP-evoked responses (i.e. Ca^{2+} mobilisation, ROS formation and HLE release), it is conceivable that these compounds target an element within the fMLP signalling cascade between the fMLP receptor and phospholipase C-mediated Ca^{2+} release. However, this element still remains to be identified which may help to further evaluate the anti-inflammatory potential of these compounds. As oral administration of CA to mice results in serum levels of up to 44 μM [12], it is conceivable that the inhibitory effects presented in our report are of pharmacological relevance. Unfortunately, the bioavailability and metabolism of CA and CS in humans has not been investigated yet. We conclude that CA and CS possess anti-inflammatory qualities beyond simple anti-oxidant action, indicating a potential for future *in vivo* assessment of their efficacy in models of inflammation.

Acknowledgment

The authors would like to acknowledge the financial support by the Deutsche Forschungsgemeinschaft.

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