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2-pentadecyl-2-oxazoline: Identification in coffee, synthesis and activity in a rat model of carrageenan-induced hindpaw inflammation

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ABSTRACT

N-acylethanolamines (NAEs) comprise a family of bioactive lipid molecules present in animal and plant tissues, with *N*-palmitoylethanolamine (PEA) having received much attention owing to its anti-inflammatory, analgesic and neuroprotective activities.

2-Pentadecyl-2-oxazoline (PEA-OXA), the oxazoline of PEA, reportedly modulates activity of *N*-acylethanolamine-hydrolyzing acid amidase (NAAA), which catabolizes PEA. Because PEA is produced on demand and exerts pleiotropic effects on non-neuronal cells implicated in neuroinflammation, modulating the specific amidases for NAEs (NAAA in particular) could be a way to preserve PEA role in maintaining cellular homeostasis through its rapid on-demand synthesis and equally rapid degradation. This study provides the first description of PEA-OXA in both green and roasted coffee beans and Moka infusions, and its synthesis. In an established model of carrageenan (CAR)-induced rat paw inflammation, PEA-OXA was orally active in limiting histological damage and thermal hyperalgesia 6 h after CAR intraplantar injection in the right hindpaw and the accumulation of infiltrating inflammatory cells. PEA-OXA appeared to be more potent compared to ultramicronized PEA given orally at the same dose (10 mg/kg). PEA-OXA markedly reduced also the increase in hindpaw myeloperoxidase activity, an index of polymorphonuclear cell accumulation in inflammatory tissues. NAAA modulators like PEA-OXA may serve to maximize availability of NAEs (e.g. PEA) while providing for recycling of the NAE components for further resynthesis.

1. Introduction

Inflammation is widely recognized as a key element across a spectrum of neuropathological conditions, including chronic pain and neuropathic pain, neurodegenerative diseases, stroke, spinal cord injury, and neuropsychiatric disorders [1–5]. Few would dispute that non-resolving inflammation is one of the principal contributors to the medical burden in industrialized societies. It is thus not surprising that a great deal of effort is being directed to identifying effective pharmacological strategies to deal with neuroinflammation [6,7].

Tissue damage, stress and their associated inflammatory response may trigger an endogenous program of resolution that encompasses the production of lipid mediators with the capacity to switch off inflammation and restore a homeostatic balance [8,9]. A number of molecules have been identified which take part in these protective mechanisms. Among these are the *N*-acylethanolamines (NAEs), a class of naturally occurring lipid signaling molecules composed of a fatty acid and ethanolamine—the so-called fatty acid ethanolamides (FAEs). The main family members are the endocannabinoid *N*-arachidonoylethanolamine (anandamide), together with its congeners *N*-stearoylethanolamine, *N*-oleoylethanolamine, and *N*-palmitoylethanolamine (PEA) (chemical name: *N*-(2-hydroxyethyl)hexadecanamide). That PEA plays a role in maintaining cellular homeostasis by acting as mediator of resolution of inflammatory processes draws support from a number of studies [10,11]. These past years have witnessed a continually growing number of studies confirming the anti-neuroinflammatory and neuroprotective actions of PEA [12–15]. Modulating responses induced by inflammatory stimuli can also be achieved by

Abbreviations: CAR, carrageenan; NAAAN, -acylethanolamine-hydrolysing acid amidase; NAE, N-acylethanolamines; PEA, *N*-palmitoylethanolamine; PEA-OXA, pentadecyl-2-oxazoline.

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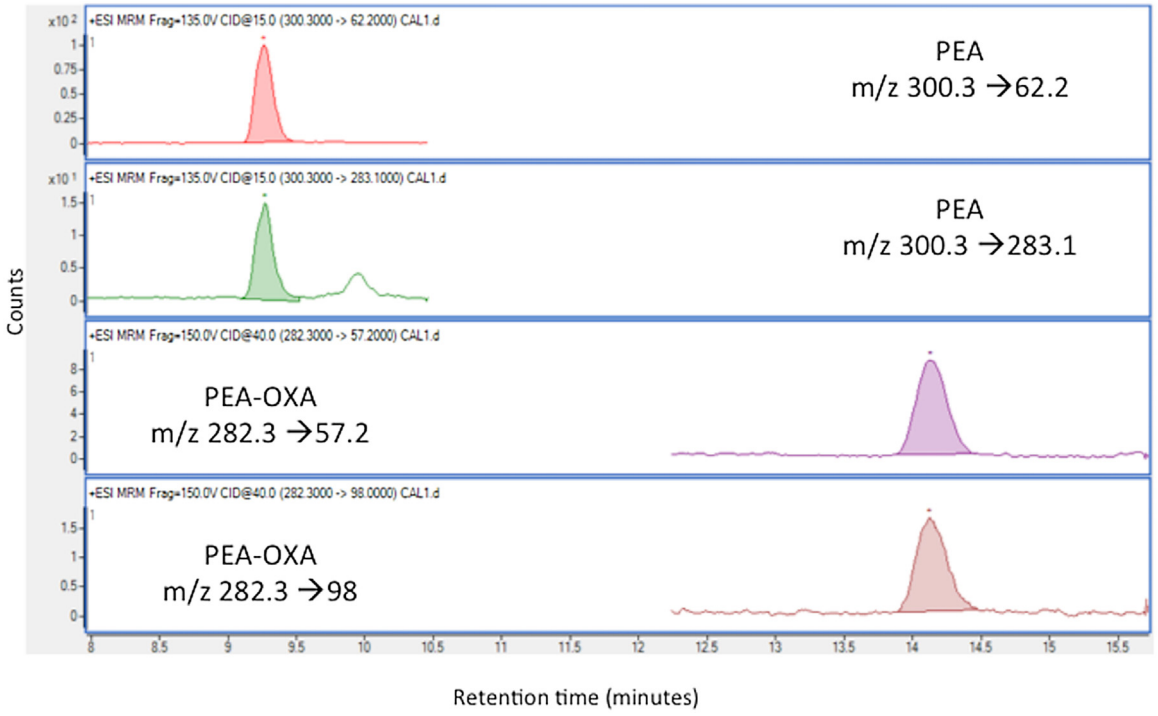

Fig. 1. Chromatographic separation of PEA and PEA-OXA (0.001 μg/ml each) and Multiple Reaction Monitoring. See Section 2 for further details.

increasing endogenous PEA levels through inhibition of its degradation, targeting either fatty acid amide hydrolase [16] or its principal catabolic enzyme, *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) [16–20]. Paradoxically, genetic or pharmacological manipulation of PEA catabolism may result in some instances lead to undesirable effects [21–24].

Because PEA is produced on demand rather than being constitutive, its ability to exert a regulatory effect on non-neuronal cells (e.g. glia and mast cells) implicated in neuroinflammation [25,26] would necessitate that PEA pleiotropic effects [11] be tightly controlled by a mechanism allowing for inactivation. One might thus suppose that NAE catabolic enzymes (NAAA in particular) are intended to modulate substrate availability. It has been proposed that pharmacologically modulating—and not blocking—the specific amidases for *N*-acylamides (in particular NAAA) could be a way to preserve PEA role in maintaining cellular homeostasis through its rapid on-demand synthesis and equally rapid degradation [27]. Recent studies described the pharmacological modulation of NAAA with the oxazoline of PEA (2-pentadecyl-2-oxazoline or PEA-OXA) [28]. The present study was designed to identify natural plant sources of PEA-OXA, describe its synthesis, and therapeutic application in a model of carrageenan (CAR)-induced inflammation in the rat paw, which represents a classical paradigm of edema formation and hyperalgesia [29] that has been extensively used in the development of anti-inflammatory drugs.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, U.K.). All other chemicals were of the highest commercial grade available. Ultramicronized PEA was kindly provided by Epitech SpA, Saccolongo, Italy. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, Biogenerica srl, Catania, Italy).

2.2. Identification of PEA and PEA-OXA in green and roasted coffee beans and moka infusions by LC/MS/MS

Coffee samples were obtained from DIEMME SpA (Albignasego, Italy). Roasted seeds were obtained by heating at 180 °C for 20 min. A common coffee grinder was employed to turn seeds in powder. Powders obtained from green and roasted coffee were extracted with dichloromethane while Moka infusion solutions were extracted with ethylacetate. Solvent extracts were then evaporated to dryness. The sample residues were re-suspended in 1 ml of acetonitrile and analysed without further purification. The present chromatographic method was adapted from Gouveia-Figueira and Nording [30], while maintaining the same mobile phases but modifying the gradient. Phase A, water; phase B, methanol added to 10 mM ammonium acetate. The gradient was: 0–5 min, 75% B; 5–6.5 min, 6.5–19 90% B; 19–19.1 min, 75% B, flow rate, 0.25 ml/min. The acquisition of MS/MS occurred prior optimization of collision energies for each analyte with infusion technique. The best source conditions [ESI+] model Jet Stream were: gas temp 140 °C, gas flow 8 l/min, nebulizer 20 psi, sheath gas temperature 375 °C, sheath gas flow 12 l/min.

The matrix effect was determined using the PEA-corresponding and ^{13}C-labeled compound EPT2110/1 (for dilutions ≥10, matrix effect 100–116%). A dilution of 1:10 in acetonitrile for all experiments was chosen as the best compromise so as not to lose information on the peak of PEA-OXA, which is present in lower concentrations than PEA. Extraction recovery was calculated using the structurally related compound EPT2110/19 (*N*-2-(hydroxyethyl)nonadecanamide) at 2.7 and 0.27 μg/ml. The final product recovery was 72%.

Intraday repeatability for PEA and PEA-OXA was calculated across the concentration range 0.01–0.1–1–10–50–100 μg/ml. The limits of detection were calculated using standard solutions of 0.12 and 0.72 ng/ml for PEA and PEA-OXA, respectively (defined statistically based on 7 repeats and Student's *t*-test, confidence interval 0.01). The extended calibration was tested in the range 0.005–100 μg/ml; this concentration range was reduced the case of

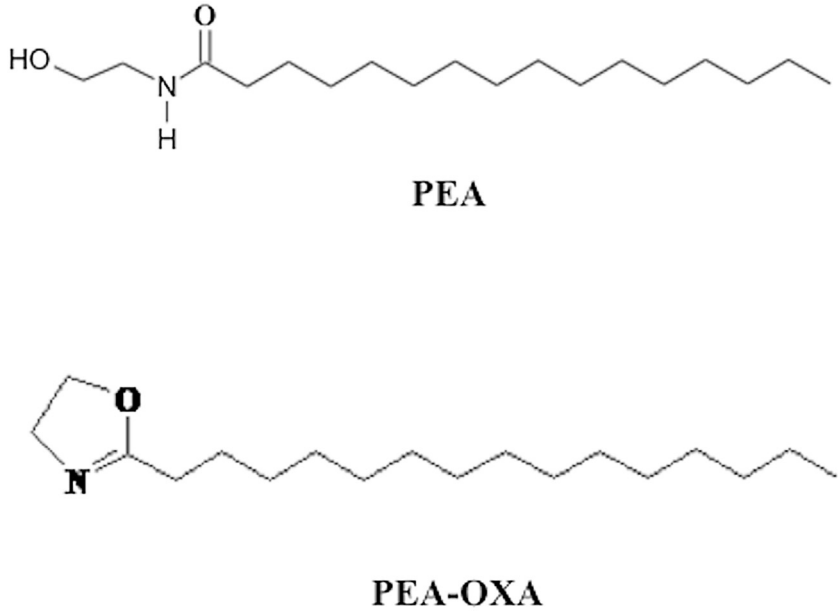

Fig. 2. Chemical structures of *N*-palmitoylethanolamine (PEA) and 2-pentadecyl-2-oxazoline (PEA-OXA). Molecular formula of PEA-OXA: $C_{18}H_{35}NO$, formula weight: 281.47, composition: C(76.81%), H(12.53%), N(4.98%), O(5.68%).

single analyses. The limit of quantification (LOQ) for actual samples was 0.005 μg/ml, taking into account also sample dilution factor. To limit a matrix effect in consideration of the sample quantity extracted (up to 5 g), all samples were quantified by the method of standard addition. Under the above conditions, the peaks for PEA and PEA-OXA eluted at 9.3 and 14.1 min, respectively. The MRM (Multiple Reaction Monitoring) transitions monitored for PEA were 300.3 → 62.2, 300.3. → 283.1, while corresponding values for PEA-OXA were 282.3 → 57.2 and 282.3 → 98. A representative chromatogram for PEA and PEA-OXA prepared in acetonitrile at a concentration of 0.001 μg/ml is shown in Fig. 1. Concentrations of PEA and OXA-PEA in the samples were determined by standard addition methods.

2.3. Synthesis of PEA-OXA

Ultramicronized PEA-OXA was synthesized as follows: Under a nitrogen atmosphere, 3.0 g of *N*-(2-hydroxyethyl)palmitamide was suspended at 0 °C in 20 ml of thionyl chloride and stirred first for 30 min at 0 °C and then at room temperature for 15 h. The solution obtained was taken to dryness under vacuum, and the residue purified by crystallization from 15 ml of *tert*-butyl methyl ether, isolated and vacuum dried. The crystallized product was suspended in 20 ml of anhydrous toluene and 1.3 g of potassium *tert*-butoxide then added. This mixture was heated at 40 °C for 2 h and then cooled at 4 °C. The resulting solution was extracted 3 times with 6 ml water and the washes discarded. The resulting organic layer was dried under vacuum and the final residue distilled under high vacuum at about 0.5 mm Hg. The fraction which distilled at about 175 °C was separated, solidified at room temperature and stored under nitrogen. The final yield of PEA-OXA was about 92%, with the following characteristics: molecular formula C18H35NO; C = 76.81%, H = 12.53%, N = 4.94%, O = 5.68%; Mr 281.5; ESI–MS: 282 (MH +); uncorrected melting point 46–48 °C; solubility: poorly soluble in water, >10 mg/ml in ethanol (Fig. 2).

2.4. Animals

Male Sprague–Dawley rats (200–230 g, Envigo, Italy).were housed in a controlled environment and provided with standard rodent chow and water. Experiments were conducted in conformity with the Public Health Service policy on Humane Care and Use of Laboratory Animals, and in compliance with Italian regulations on the protection of animals used for experimental and other scientific purposes (D.M. 116192) as well as with EEC regulations (O.J. of E.C. L 358/1 12/18/1986). The study reported here was approved by the University of Messina Institutional Animal Care and Use Committee.

2.5. Carrageenan-induced paw edema

Paw edema was induced by subplantar injection of CAR (0.1 ml of a 1% suspension in 0.85% saline) into the right hindpaw on rats [31]. The animals were killed and paws were collected 6 h after CAR injection.

2.6. Experimental groups

Rats were randomly allocated into the following groups:

(i) CAR + saline (vehicle): rats were subjected to CAR-induced paw edema (*N* = 10);

(ii) CAR + ultramicronized PEA-OXA (10 mg/kg) dissolved in 2.5% carboxymethylcellulose (CMC) (2.5% p/p in water): same as the CAR + saline group but PEA-OXA (10 mg/kg, oral) was administered 30 min before CAR (N = 10);

(iii) CAR + ultramicronized PEA-OXA (3 mg/kg) dissolved in 2.5% CMC (2.5% p/p in water): same as the CAR + saline group but PEA-OXA (3 mg/kg, oral) was administered 30 min before CAR (N = 10);

(iv) CAR + ultramicronized PEA-OXA (1 mg/kg) dissolved in 2.5% CMC (2.5% p/p in water): same as the CAR + saline group but PEA-OXA (1 mg/kg, oral) was administered 30 min before CAR (N = 10);

(v) CAR + ultramicronized PEA (10 mg/kg) dissolved in 2.5% CMC: same as the CAR + saline group but ultramicronized PEA (10 mg/kg, oral) was administered 30 min before CAR (N = 10);

(vi) CAR + ultramicronized PEA (3 mg/kg) dissolved in 2.5% CMC: same as the CAR + saline group but ultramicronized PEA (3 mg/kg, oral) was administered 30 min before CAR (N = 10);

(vii) CAR + ultramicronized PEA (1 mg/kg) dissolved in 2.5% CMC: same as the CAR + saline group but ultramicronized PEA (1 mg/kg, oral) was administered 30 min before CAR (N = 10);

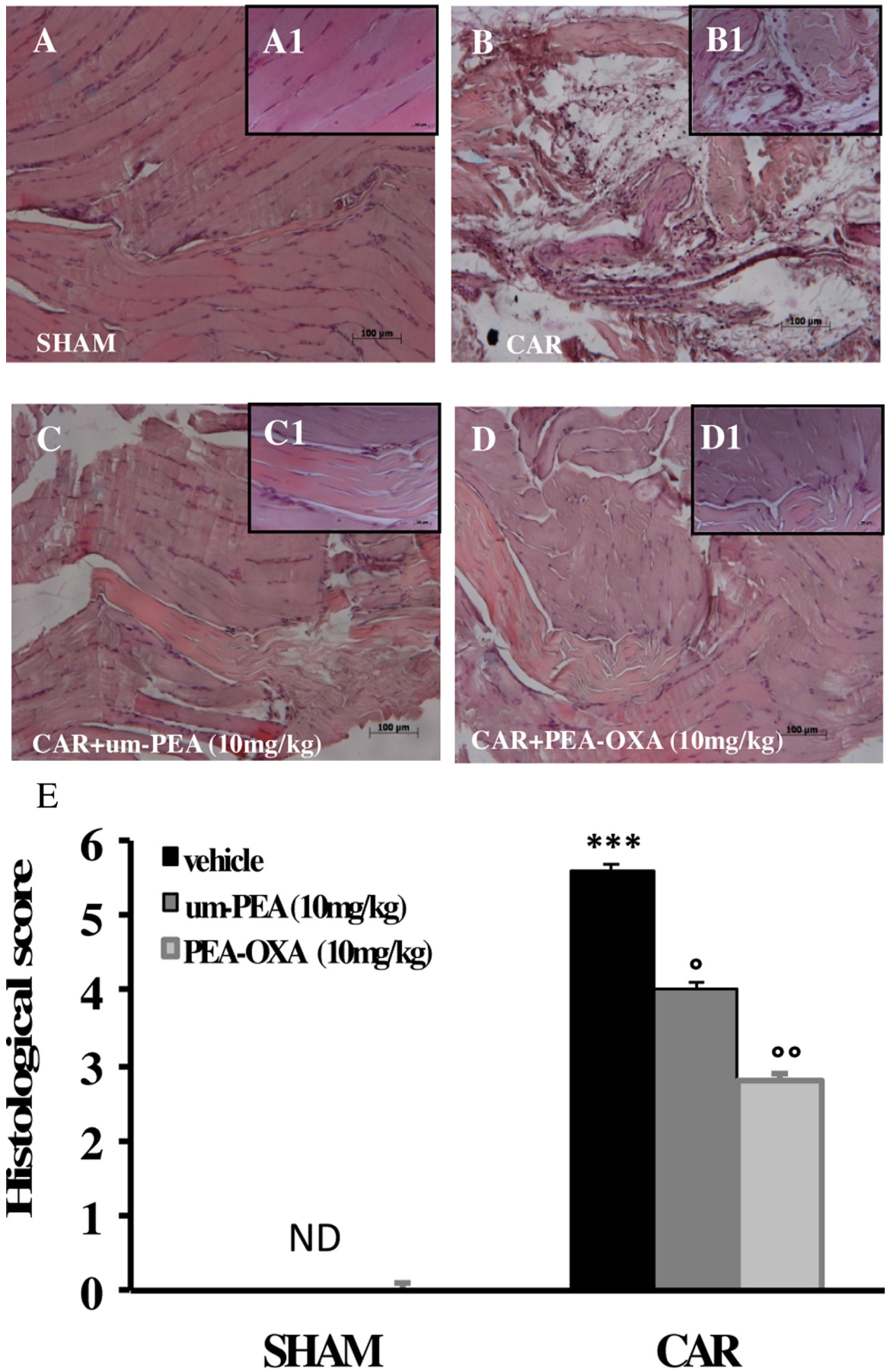

Fig. 3. Anti-inflammatory effects of orally administered PEA-OXA and ultramicronized PEA following intraplantar injection of carrageenan (CAR)

The sham-operated group underwent the same surgical procedures as the CAR group, except that saline or drugs were administered instead of CAR ($N = 10$ for all experimental groups).

The doses and the route of administration of ultramicronized PEA and PEA-OXA were chosen based on a dose-response carried out in our lab and described in a recent study [27].

2.7. Histological examination of the CAR-inflamed hindpaw

For histological examination, paw biopsies were taken 6 h following the intraplantar injection of CAR. Tissue samples from the hindpaw pads were removed with a scalpel, fixed in Dietric solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated through a graded series of ethanol and then embedded in Paraplast (Sherwood Medical). Tissue sections (thickness 7 μm) were deparaffinized with xylene, stained with haematoxylin/eosin and observed under light microscopy (Dialux 22 Leitz). All histological analyses were performed by an observer blinded to the treatment protocol. The degree of inflammation was evaluated according to [32] with a score from 0 to 5, defined as follows: 0 = no inflammation, 1 = mild inflammation, 2 = mild/moderate inflammation, 3 = moderate inflammation, 4 = moderate/severe inflammation and 5 = severe inflammation.

2.8. Myeloperoxidase (MPO) activity

MPO activity, an index of polymorphonuclear cell accumulation, was determined as previously described [33]. At the specified time following the intraplantar injection of CAR, tissue samples from the hindpaw pads were collected and 5 mm pieces obtained by means of a tissue punch. Each tissue piece was then homogenised in a solution containing 0.5% hexadecyltrimethylammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000*g* (4 °C). An aliquot of the supernatant was then reacted with a solution of 1.6 mM tetramethylbenzidine and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide min^{-1} at 37 °C and was expressed in milliunits per gram wet tissue weight.

2.9. Thermal hyperalgesia

Hyperalgesic response to heat was determined by the Hargreaves Method using a plantar test (Ugo Basile, Comeria, Italy) [34] with a cut-off latency of 20 s used to prevent tissue injury. Data obtained were converted to percent maximal possible antinociceptive effect (%MPE) as follows: (response latency − baseline latency)/(cut-off latency − baseline latency) × 100.

2.10. Statistical evaluation

All values are expressed as mean ± standard error of the mean [6] of N observations. For in vivo experiments, N represents the number of animals. For experiments involving histology or immunohistochemistry, the figures shown are representative at least three experiments (histological or immunohistochemical labeling) performed on different experimental days on tissue sections collected from all animals in each group. The results were analysed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. A p value of less than 0.05 was considered significant.

Table 1
Quantification of PEA and PEA-OXA content in samples of green coffee.

Sample	PEA (μg/g)	PEA-OXA (μg/g)
India 14	2.83 ± 0.78	<LOQ
India 15	3.42 ± 1.28	<LOQ
Brazil 14	7.55 ± 1.75	0.046 ± 0.015
Brazil 15	11.94 ± 0.91	0.046 ± 0.010
Mexico 14	5.06 ± 1.17	0.026 ± 0.003
Mexico 15	7.80 ± 0.35	0.058 ± 0.009

Concentrations of PEA and OXA-PEA were determined by the standard addition method, and values averaged from 3 measurements run on different extracted samples. LOQ, limit of quantification. See Section 2 for further details.

Table 2
Quantification of PEA and PEA-OXA content in sample of roasted coffee.

Sample	PEA (μg/g)	PEA-OXA (μg/g)
Brazil 15	7.20 ± 0.30	0.022 ± 0.002

Concentrations of PEA and OXA-PEA were determined by the standard addition method and values averaged from 3 measurements run on different extracted samples. See Section 2 for further details.

Table 3
Quantification of PEA and PEA-OXA content in samples of Moka infusion.

Sample	PEA (μg/g)	PEA-OXA (μg/g)
Brazil 15 a	1.13 ± 0.25	0.060 ± 0.005
Brasil 15 b	1.30 ± 0.30	0.010 ± 0.004
Brasil 15 c	0.56 ± 0.12	0.006 ± 0.002

Concentrations of PEA and PEA-OXA were determined in three different Moka infusions by the standard addition method. Values are averaged from three measurements run on each extracted sample. Contents are expressed as "per gram" of coffee powder exposed to infusion. See Section 2 for further details.

3. Results

3.1. PEA-OXA is a natural constituent of coffee beans

N-Acylethanolamines constitute a class of bioactive lipid molecules present in animal and plant tissues [16]. Among the NAEs, PEA has attracted much attention due to its anti-inflammatory, analgesic and neuroprotective activities [12–15]. Although PEA has no action on its main catabolic enzyme NAAA, a recent report suggests that its oxazoline form might modulate NAAA [27]. Because saturated and unsaturated NAEs occur in desiccated seeds primarily as 16- and 18-carbon species with PEA and *N*-linoleoylethanolamine being the most abundant [35], we examined coffee samples for the presence of PEA-OXA. Neither PEA nor PEA-OXA has been described previously in coffee beans. PEA-OXA was clearly identifiable in both green and roasted coffee beans (Tables 1 and 2, respectively) and Moka infusions (Table 3) from several sources, although amounts were substantially lower than values for PEA. PEA-OXA was chemically synthesized (see Section 2) and subsequently tested for pharmacological efficacy in an established model of CAR-induced inflammation.

into the rat hind paw: histological analysis. Histological evaluation was performed by hematoxylin/eosin staining. (A) Sham; (B) intraplantar injection of CAR into the rat hind paw; (C) CAR + ultramicronized PEA ('um-PEA') (10 mg/kg) (c); CAR + PEA-OXA (10 mg/kg). Insets A1 through D1 are higher-resolution images of the respective panels. Ultramicronized PEA and PEA-OXA were administered orally 30 min before CAR injection, and all animals were killed 6 h after CAR injection. (E) Histological scores for the various treatment groups. (g) Myeloperoxidase (MPO) activity in paw tissues from the various treatment groups. Ultramicronized PEA and PEA-OXA both produced significant improvements. PEA-OXA showed a greater effect than ultramicronized PEA. See Section 2 for further details. Values are means ± SEM (N = 10). ***$p < 0.001$ vs sham; °$p < 0.05$ and °°$p < 0.01$ vs CAR alone.

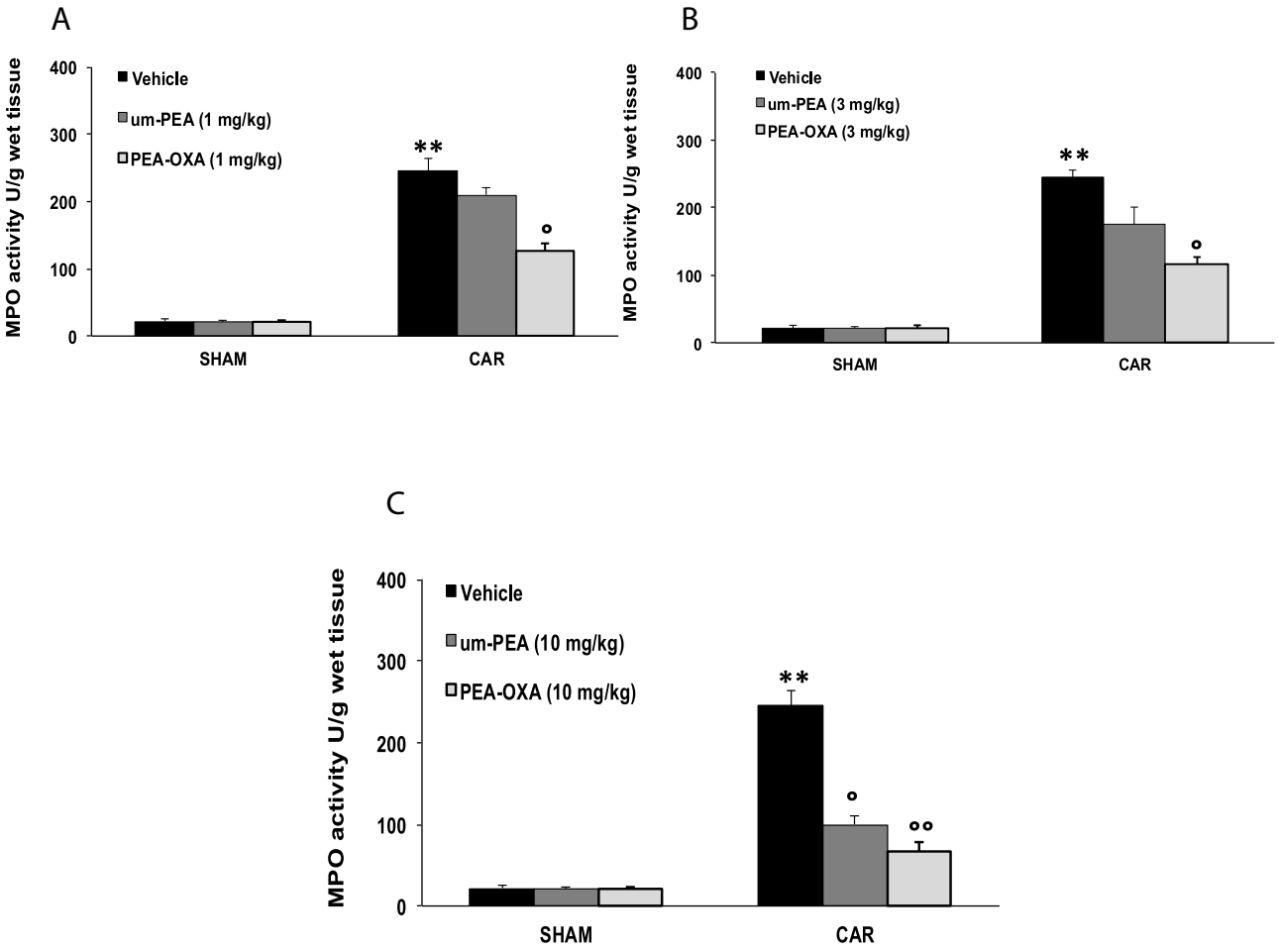

Fig. 4. Orally administered PEA-OXA and ultramicronized PEA limit the increase in myeloperoxidase (MPO) activity following intraplantar injection of carrageenan (CAR) into the rat hind paw. PEA-OXA and ultramicronized PEA ('um-PEA') (1, 3, 10 mg/kg each) were orally administered 30 min before CAR injection; all animals were killed 6 h after CAR injection. Ultramicronized PEA and PEA-OXA each significantly improved the rise in MPO activity in CAR-injected rats. PEA-OXA showed a greater effect than ultramicronized PEA also at lower doses. See Section 2 for further details. Values are means ± SEM (N = 10). $^{**}p < 0.01$ vs sham; $^{\circ}p < 0.05$ and $^{\circ\circ}p < 0.01$ vs CAR.

3.2. Histological analyses of rat paw tissues

To evaluate histologically the anti-inflammatory effect of PEA-OXA (10 mg/kg), samples of paw tissue from each experimental group was examined by hematoxylin/eosin staining. Control rats showed no histologic damage (Fig. 3A,E). Significant damage was observed 6 h after CAR injection into the right hind paw with a marked accumulation of infiltrating inflammatory cells (Fig. 3B,E), compared to sham control. Inflammatory cell infiltration was significantly decreased with ultramicronized PEA treatment (Fig. 3C,E). Moreover, treatment with PEA-OXA (Fig. 3D,E) not only produced a significant reduction in histological alterations in CAR-injected rats, but also yielded a significantly greater reduction in score compared to ultramicronized PEA.

3.3. Effects of PEA-OXA on MPO activity in CAR-treated rats

The development of histological damage was associated with increased infiltration of neutrophils as shown by an increase in MPO activity, a peroxidase enzyme released by neutrophils and considered a marker of neutrophilic infiltration (Fig. 4) [36]. The administration of either ultramicronized PEA (10 mg/kg) or PEA-OXA (10 mg/kg) each significantly reduced MPO activity (Fig. 4C), although PEA-OXA was significantly better than the former. Moreover, ultramicronized PEA-OXA at lower doses of 1 and 3 mg/kg showed a greater beneficial effect compared to ultramicronized PEA (Fig. 4A, B).

3.4. Effects of PEA-OXA on thermal hyperalgesia in CAR-treated rats

CAR injection in the right hindpaw caused a time-dependent development of thermal hyperalgesia (Fig. 5). Oral administration of ultramicronized PEA (10 mg/kg) produced a significant inhibition of the CAR-induced thermal hyperalgesia (Fig. 5). Moreover, oral treatment with ultramicronized PEA-OXA (10 mg/kg) was more efficacious than ultramicronized PEA (Fig. 5**).**

4. Discussion

Innate immune system-derived cells such as microglia, macrophages and mast cells generate a diversity of lipid-derived mediators that are involved in the response to tissue damage and inflammation [37]. While these molecules may stimulate or suppress pain and inflammation, a growing body of evidence indicates that these host-defense cells can also release bioactive lipids that attenuate rather than provoke pain and inflammation. Among the analgesic and anti-inflammatory lipids released from these cells are the fatty acid ethanolamides. Two members of this family, PEA and oleoylethanolamide, have emerged as important regulators of nociception and inflammation, with the great majority of studies

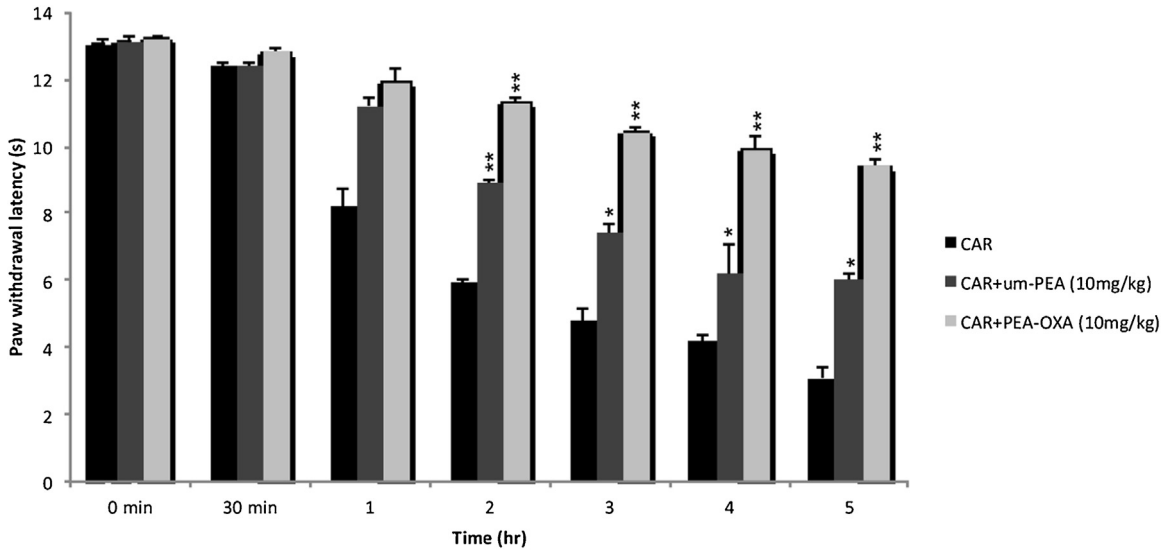

Fig. 5. Orally administered PEA-OXA and ultramicronized PEA reduce thermal hyperalgesia following intraplantar injection of carrageenan (CAR) into the rat hind paw. Ultramicronized PEA and PEA-OXA each significantly reduced thermal hyperalgesia induced by CAR injection in rats. PEA-OXA showed a greater effect than ultramicronized PEA. See Section 2 for further details. Values are means ± SEM (N = 10). $^{**}p < 0.01$ vs CAR; $^{*}p < 0.05$ vs CAR.

to-date being focused on PEA [10–15]. Here we describe the occurrence of the oxazoline of PEA in a plant source, its synthesis, and anti-inflammatory activity when orally administered in a rat paw CAR model of inflammatory pain. PEA-OXA reduced tissue damage and thermal hyperalgesia caused by CAR intraplantar injection in the right hindpaw in a more significant way compared to PEA. These results were in agreement with a recent study which demonstrated a significant reduction of paw volume by PEA-OXA treatment [27]. In addition, the accumulation of infiltrating inflammatory cells with the increase in hindpaw MPO activity was also limited in CAR-injected rats treated with PEA-OXA, and showing a more beneficial effect also at lower doses of 1 and 3 mg/kg compared to PEA.

N-Acylethanolamines are not only widely distributed throughout the animal kingdom [38–43], but they also comprise minor but ubiquitous components of plant tissues, being especially abundant in some desiccated seeds. The fatty acids have up to three double bonds and 12–18 carbon atoms, linoleic acid being the predominant species [44]. Their total contents in seeds range from about 0.2–40 mg/g fresh weight with no relationship to phylogeny, their concentration being depleted during germination and seedling development. Although present in much smaller amounts than PEA, PEA-OXA was isolated from both green and roasted coffee beans and Moka infusions, with samples originating from wide-ranging sources such as Brazil, Mexico and India. To the best of our knowledge, the present observations are the first report of PEA-OXA in the plant kingdom.

In preclinical models of inflammatory pain, inhibition of the lysosomal cysteine amidase NAAA increases tissue levels of PEA while reducing hyperalgesic responses and the production of pro-inflammatory cytokines [37]. PEA levels are tightly regulated by actions of the biosynthetic enzyme *N*-acylphosphatidylethanolamine-specific phospholipase D and NAAA [45]. Because PEA is believed to play a key role in the maintenance of tissue homeostasis [11,12,15] through its rapid on-demand synthesis and equally rapid degradation [27], a complete and prolonged pharmacological block may upset this balance and limit the cell's ability for further on-demand PEA production. An alternative approach to a frank inhibition of NAAA may be the use of molecules which modulate NAAA activity, thus allowing for a finer 'tuning' of PEA levels. Indeed, it has been suggested that PEA-OXA may function as a putative modulator of NAAA [28]. It will be interesting in future studies to compare further the analgesic efficacy of PEA and PEA-OXA and, in particular, the ability of PEA-OXA to render PEA more effective at (sub)threshold doses in an inflammatory setting.

Conflict of interest

Dr. Salvatore Cuzzocrea, researcher on the study team, is co-inventor on patent WO2013121449 A8 (Epitech Group SpA) which deals with compositions and methods for the modulation of amidases capable of hydrolysing *N*-acylethanolamines useable in the therapy of inflammatory diseases. Moreover, Dr Cuzzocrea is also a co-inventor with Epitech group on the following patent:

1. EP 2 821 083.
2. MI2014 A001495.
3. 102015000067344.

No other authors have conflict of interests.

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