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Effects of palmitoylethanolamide on the cutaneous allergic inflammatory response in *Ascaris* hypersensitive Beagle dogs

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ABSTRACT

Palmitoylethanolamide (PEA) is an endogenous lipid mediator with anti-inflammatory and anti-hyperalgesic properties. The main objective of the present study was to evaluate the effects of PEA on the cutaneous allergic inflammatory reaction induced by different immunological and non-immunological stimuli in hypersensitive dogs. Six spontaneously *Ascaris* hypersensitive Beagle dogs were challenged with intradermal injections of *Ascaris suum* extract, substance P and anti-canine IgE, before and after a single oral administration of PEA at doses of 3, 10 and 30 mg/kg.

A significant reduction in wheal area induced by both antigen and anti-canine IgE challenge was observed after PEA administration. No significant differences were observed between the two higher doses studied, suggesting that the 10 mg/kg dose had exerted the maximum inhibitory effect. When blood levels of PEA were compared with the effects at different times, an evident correlation was obtained. However, the anti-inflammatory effects of PEA were more long-lasting than their plasma concentrations. The intradermal injection of substance P did not reveal any skin reaction (wheal or erythema formation) at any of the concentrations tested. In conclusion, PEA might constitute a new therapeutic strategy for the treatment of allergic inflammatory skin diseases in companion animals.

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Introduction

The incidence of spontaneous allergic reactions in dogs (atopic dermatitis) appears to be increasing (Nødtvedt et al., 2006). Although management combines different interventions depending on the particular aetiology, the most widespread therapeutic approach still relies on glucocorticoids, which can lead to a variety of adverse effects especially with chronic treatments (Blois et al., 2009). Therefore, it is important to develop new effective mechanism-oriented approaches for controlling allergic and inflammatory processes associated with a number of skin diseases in the dog and cat.

The skin mast cell (MC) plays a pivotal role in both innate and allergic immune responses through the release of bioactive mediators in response to a wide range of biological, chemical, microbial and physical stimuli, (Hammerberg et al., 2001; von Ruedorffer et al., 2003; Wuersch et al., 2006; De Mora et al., 2006; Metz et al., 2008; Hershko and Rivera, 2009; Hofmann and Abraham,

2009; Metz and Maurer, 2009). MC degranulation leads to an initial early phase reaction with the subsequent induction of transcription of inflammatory cytokines and chemokines, which contribute to the late phase reactions through the recruitment of inflammatory cells, such as lymphocytes and eosinophils, and may ultimately result in chronic skin inflammation (Holgate et al., 1993; Olivry et al., 1996; Galli, 1997). The modulation of the early phase allergic reaction can thus become an effective mechanism-based strategy to reduce the development of the inflammatory mediator cascade and to control the more damaging chronic phase.

Recent studies have shown the existence of new endogenous lipid mediators with anti-inflammatory and antinociceptive properties (Lambert et al., 2002; Re et al., 2007). Palmitoylethanolamide (PEA) is one of these lipids and belongs to the autacoid local injury antagonist (ALIA) amide family (Jack, 1996; Lambert et al., 2002). PEA is a long chain *N*-acylethanolamine that can be found in low concentrations in most organisms, including yeasts, plants and mammals (Muccioli et al., 2009; Chapman, 2004; Ozalp and Barraso, 2009). It is produced on demand during inflammatory and degenerative processes (Sagar et al., 2009) and its local elevation is considered to exert protective roles (Re et al., 2007).

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In cats with eosinophilic granuloma, Scarpella et al. (2001) showed an improvement of clinical signs and lesions after 1 month of treatment with PEA. Another study carried out by Marsella et al. (2005) demonstrated that after 7 days of treatment, PEA was able to delay but not prevent the development of clinical signs of atopic dermatitis in a canine experimental model. More recently, PEA was found to counteract dinitrofluorobenzene (DNFB)-induced dermatitis in rats (Petrosino et al., 2010). These anti-inflammatory and anti-allergic effects may be due, at least in part, to the ability of PEA to down-modulate mediator release from canine skin MCs (Cerrato et al., 2010). Despite these encouraging in vitro results, so far no studies have investigated the in vivo effect of PEA on acute cutaneous reactions in dogs.

The main objective of the present study was to evaluate the potential inhibitory effects of PEA, administered orally in a single dose, on the skin reaction induced by different immunological and non-immunological stimuli (anti-canine IgE, *Ascaris suum* antigen and substance P) in hypersensitive Beagle dogs. In addition, we correlated the anti-inflammatory activity of PEA with its plasma levels at different times after oral treatment.

Materials and methods

Drugs, chemicals, and reagents

PEA was supplied by Innovet in the ultramicrosized form. *Ascaris suum* extract was purchased from Greer Laboratories. Anti-canine IgE was purchased from Bethyl Laboratories. Evans blue dye, Substance P acetate salt hydrate and histamine diphosphate salt were obtained from Sigma-Aldrich.

Animals

Six spontaneously hypersensitive Beagle dogs with mean bodyweights of 14.0 ± 0.6 kg were used. The animals were fasted overnight before oral administration of the tested drug. All experiments and procedures were performed in accordance with European regulations governing the care and treatment of laboratory animals and were approved by the Animal Care and Use Committee of the Universitat Autònoma de Barcelona.

Experimental protocol

The method used was based on the measurement of the wheal area induced by intradermal injections of *A. suum* extract solution, substance P (SP) and anti-canine IgE in the skin before and after oral administration of an aqueous suspension of PEA in carboxymethylcellulose (0.5%) at three different doses (3, 10, 30 mg PEA/kg).

Intradermal challenge

Before any treatment administration, intradermal injections were performed to determine basal conditions (baseline values). In order to better visualize wheal areas, a 2% w/v Evans blue dye solution in saline was given IV (0.4 mL/kg) 30 min before the first intradermal injections of stimuli. Then 40 μ L of *A. suum* extract (0.1 mg/mL), SP (0.1, 1 and 10 nM) and anti-canine IgE (1 μ g/mL) were administered using a Hamilton syringe (type 701LT) in the shaved lateral thoracic area of the awoken dogs. Wheal areas were measured 10 min after each injection by applying an acetate sheet over the reaction site, thus allowing us to draw an outline of the wheal with indelible ink. Histamine (0.01 mg/mL) and saline were also injected as positive and negative controls, respectively.

Each dog was then given a single dose of PEA with a flexible oesophageal tube with a washout period of 1 week between different PEA doses (3, 10, 30 mg/kg). *A. suum* extract, anti-canine IgE, SP, saline and histamine were injected again 1, 2, 4, 8 and 24 h after PEA administration. Wheal areas were measured with an image analyzer MIP 4 ADVANCED (Digital Image System) and the percentage of inhibition was calculated for each dog, each time and each dose.

A control study was also conducted in each dog without treatment to determine whether cutaneous wheal responses induced by successive stimuli intradermal injections were maintained uniform over time (8 h). To guarantee test reproducibility all experiments were performed by the same investigator blinded to treatment.

PEA extraction and detection by high-pressure liquid chromatography (HPLC)

Plasma levels study was performed after the completion of the efficacy study.

The objective was to evaluate whether PEA levels were quantifiable and whether metabolites were produced. The higher dose of PEA was selected and only the male dogs ($n = 4$) were used to avoid possible hormone-dependent variability.

Determination of PEA plasma levels was performed after 0, 1, 2, 4 and 8 h of oral PEA administration (30 mg/kg dosing). Blood samples were obtained through a catheter inserted into a cephalic vein. The samples were collected at pre-established times. To determine PEA plasma levels, blood samples were collected and placed into spray-coated lithium-heparin tubes (BD Vacutainer). Afterwards, blood samples were centrifuged at 1881 g for 10 min and the collected plasma was stored at -80°C until analysis using HPLC. Plasma was Dounce homogenized and extracted with chloroform/methanol/Tris HCl 50 mM pH 7.5 (2:1:1, v/v) containing internal standards. The lipid extract was pre-purified by open-bed chromatography on silica columns eluted with increasing concentrations of methanol in chloroform. After extraction and purification, PEA-containing fractions were subjected to isotope-dilution liquid chromatography-atmospheric pressure chemical ionization-mass spectrometric analysis (LC-APCI-MS) by using a Shimadzu HPLC apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2010) quadrupole MS via a Shimadzu APCI interface as described previously (Matias et al., 2002). The concentrations of PEA are given as pmol/mL blood.

Data analysis

Wheal areas are expressed in mm^2 as means $\pm$ standard deviation (mean $\pm$ SD) from six experiments. Results are expressed as the percentage of inhibition observed at any time (t_n area) in comparison with the area obtained in the same dog before starting the treatment (baseline area).

Wheal inhibition was calculated with the following formula,

$$\% \text{ inhibition} = \frac{\text{baseline area (mm}^2\text{)} - t_n \text{ area (mm}^2\text{)}}{\text{baseline area (mm}^2\text{)}} \times 100$$

Plasma levels of PEA (30 mg/kg) are expressed in pmol/mL as means $\pm$ SD from four experiments.

Statistical analysis was performed using SAS v9.1.3 (SAS Institute). Statistical significance level was set at 0.05. Response variables were summarized using mean, SD, coefficient of variation (CV) and sample size when necessary. Multiple comparisons correction was performed using Dunnett's correction when comparisons were performed to a control level or Tukey's correction for pair wise comparisons.

Results

The expected side effects of the oral administration of PEA at the established doses (3, 10, 30 mg/kg) were excitation and anxiety or sedation and drowsiness. The veterinary control confirmed that PEA did not produce any side effects.

Mean wheal areas induced by successive histamine, anti-canine IgE and *A. suum* extract injections in untreated dogs were measured to assess the repeatability of the wheal area over time. Despite the differences observed between dogs (CV > 10%), no differences were observed within-dogs (CV < 5%). For this reason the inhibitory effects in each dog were calculated from they own basal value. Table 1 showed the wheal areas obtained with all stimuli in each animal at different times.

The intradermal injection of SP did not develop any skin reaction at the tested concentrations (0.1, 1 and 10 nM), even when the evaluation was performed at different times after challenge (5, 10 and 20 min). Therefore, the inhibitory effect of PEA after SP challenge was not evaluated.

Table 1

Cutaneous wheal areas obtained with repeated intradermal injections of histamine, anti-IgE and *Ascaris suum* during 8 h without treatment.

AREA (mm^2)			
Animal	Histamine Mean $\pm$ SD	Anti-IgE Mean $\pm$ SD	<i>Ascaris suum</i> Mean $\pm$ SD
A	68.3 $\pm$ 2.3	45.9 $\pm$ 1.0	42.9 $\pm$ 1.0
B	57.5 $\pm$ 1.3	49.3 $\pm$ 1.0	45.3 $\pm$ 2.3
C	65.2 $\pm$ 2.3	46.9 $\pm$ 1.4	58.9 $\pm$ 1.3
D	91.5 $\pm$ 3.7	63.0 $\pm$ 1.4	40.7 $\pm$ 1.0
E	66.0 $\pm$ 2.0	45.8 $\pm$ 1.1	56.8 $\pm$ 1.4
F	70.1 $\pm$ 1.8	56.0 $\pm$ 1.0	66.0 $\pm$ 1.3

Fig. 1a and b show the percentage inhibition observed after a single oral PEA administration (3, 10 and 30 mg/kg) on wheal areas induced by *A. suum* antigen and anti-canine IgE, respectively. Wheal areas induced by *A. suum* antigen were evaluated. The higher studied doses of PEA significantly reduced the antigen-induced skin reaction 1, 2 and 4 h after PEA administration. The maximum inhibitory effects (29.3 ± 8.7 and $32.3 \pm 10.5\%$) were observed at the 10 and 30 mg/kg doses and at 1 h and 2 h after compound administration, respectively ($P < 0.01$; Fig. 1a).

Similar results were found when intradermal challenge was performed with anti-canine-IgE. The higher tested doses of PEA resulted in a significant reduction of wheal areas 1, 2 and 4 h after PEA administration, but the most effective inhibition was reached 2 h after treatment at the 10 and 30 mg/kg doses, with significant mean inhibition percentages of $32.4 \pm 9.4\%$ and $31.7 \pm 14.9\%$, respectively ($P < 0.01$; Fig. 1b).

Table 2

Plasma concentrations of palmitoylethanolamide (PEA) after oral administration at 30 mg/kg ($n = 4$).

Time (h)	PEA plasma levels (pmol/mL)				Mean $\pm$ SD
	Dog A	B	C	D	
0	9.5	5.2	10.8	18.9	11.1 ± 5.7
1	76.5	37.9	52.5	50.6	$54.4 \pm 16.1^*$
2	76.0	55.1	72.4	32.6	$59.0 \pm 18.9^*$
4	11.6	8.4	10.0	10.8	10.2 ± 1.4
8	14.4	9.1	15.4	9.7	12.2 ± 3.2

* $P < 0.01$.

Table 2 provides the concentration of PEA observed in blood samples obtained after a single oral dose of PEA (30 mg/kg) in

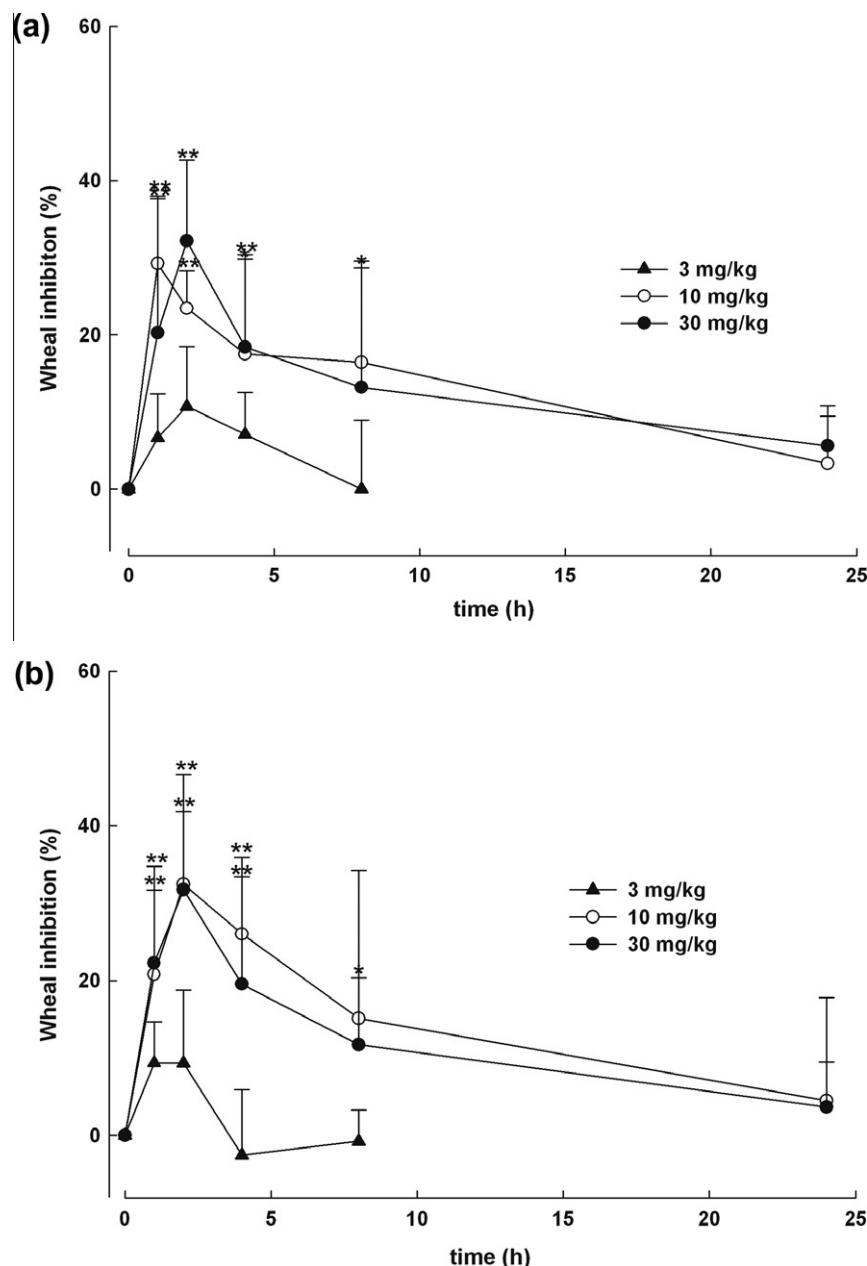


Fig. 1. Mean inhibition percentage ($\pm$ SD) ($n = 6$) of wheal areas induced in conscious hypersensitive Beagle dogs by *Ascaris suum* extract (a) and anti-canine IgE (b) at several times after oral administration of palmitoylethanolamide (PEA) at 3, 10 and 30 mg/kg. * $P < 0.05$ and ** $P < 0.01$ vs. baseline values.

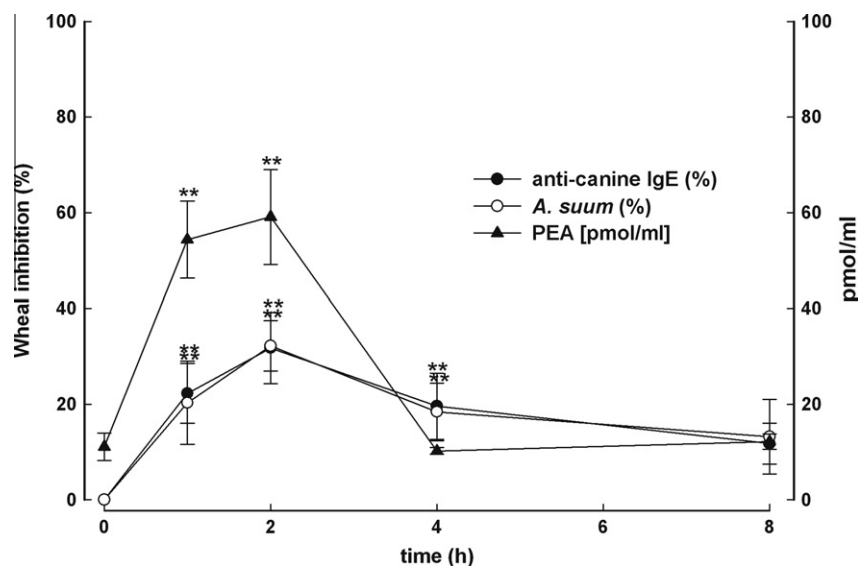


Fig. 2. Mean plasma levels ($\pm$ SD) of palmitoylethanolamide (PEA) ($n = 4$) and mean inhibition percentage ($\pm$ SEM) of wheal areas ($n = 6$) induced in conscious hypersensitive Beagle dogs by *Ascaris suum* extract and anti-canine IgE at several times after oral administration of PEA at 30 mg/kg. ** $P < 0.01$ vs. baseline values.

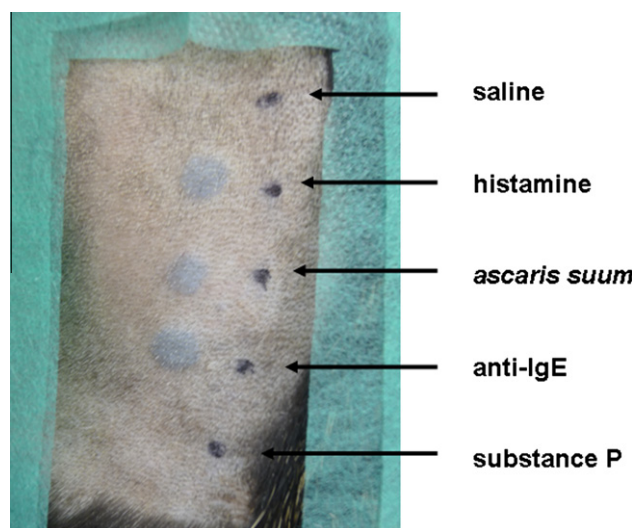


Fig. 3. Skin wheals induced in hypersensitive beagle dogs 20 min after an intradermal injection of 40 μ L of *Ascaris suum* extract, anti-canine IgE, histamine, substance P and saline solution.

the four Beagle dogs evaluated. Blood samples were collected before and 1, 2, 4 and 8 h after treatment. As shown in Table 2, PEA returned to basal values 4 h after treatment.

Fig. 2 shows the average PEA plasma levels measured after a single oral dose of 30 mg/kg. The highest concentrations of PEA in plasma (54.4 ± 16.1 pmol/mL and 59.0 ± 19.8 pmol/mL) were found 1 h and 2 h after dosing and these concentrations were statistically different from basal values (10 pmol/mL). Fig. 3 shows the wheal areas induced by different stimuli and controls in hypersensitive Beagle dogs skin. Inhibitory effects observed at these times (0, 1, 2, 4 and 8 h) are also represented to obtain a pharmacokinetic–pharmacodynamic correlation.

Discussion

In the present study the anti-allergic and anti-inflammatory properties of PEA, an endogenous lipid mediator belonging to the ALIamide family, were evaluated after a single oral treatment in *A. suum* hypersensitive Beagle dogs. This is the first time that the

efficacy of ultramicrosized PEA in reducing immediate skin reaction in this canine model of skin allergy is reported. Spontaneous hypersensitivity to *A. suum* antigens in Beagle dogs provides an established model of allergic dermatitis (De Mora et al., 2006). Following intradermal exposure to *A. suum* extract, an immediate MC-mediated wheal and pruritus develops (Brazis et al., 1998; Queralt et al., 1998; Brazis et al., 2006; De Mora et al., 2006; Torres et al., 2006). Afterwards, a marked cutaneous eosinophilia is observed, indicative of the development of a late phase allergic reaction (Torres et al., 2003).

Treatment of *A. suum* hypersensitive Beagle dogs with a single dose of ultramicrosized PEA resulted in significant reduction of skin wheal areas induced by *A. suum* extract and anti-IgE antibody. These data are in agreement with an earlier experimental study, showing that PEA inhibits skin inflammation in a model of atopic dermatitis in mice, i.e. the allergic dermatitis induced by DNFB (Petrosino et al., 2010). Moreover, that study also reported that PEA down-regulates the expression and levels of the inflammatory cytokine MCP-2 (monocyte chemotactic protein-2), a chemokine produced by challenged keratinocytes (Petrosino et al., 2010) and responsible for the recruitment of MCs (Romagnani, 2002).

In a previous study performed in our laboratory, PEA was found to reduce the release of histamine, prostaglandin (PG) D₂ and tumour necrosis factor (TNF)- α from immunologically challenged canine skin MCs (Cerrato et al., 2010). Interestingly, TNF α was also reduced by PEA treatment in a dose-dependent manner in an experimental model of chronic skin inflammation (α -carra-genin-induced granuloma) in rats (De Filippis et al., 2010). It can thus be speculated that the effect of PEA relies on its ability to down-modulate the degranulation of skin MCs and the consequent release of inflammatory mediators and vasoactive amines, with histamine being deeply involved in the wheal and flare reaction in dogs (Roßbach et al., 2009).

The maximal inhibition of anti-canine IgE-induced wheal was achieved 2 h after oral treatment with a single dose of 3, 10 or 30 mg/kg PEA, with the mean inhibitory effects of the two higher doses being $32.4 \pm 9.4\%$ and $31.7 \pm 14.9\%$, respectively. Similar results were found when an intradermal challenge was performed with *A. suum* extract. Although significant inhibition was observed with each of the three doses of PEA, no differences were observed between the two higher doses, both in the anti-IgE and the *A. suum* experiments. This suggests the existence of a plateau at the 10 mg/

kg dose, which could explain the maximum effects of PEA achieved at this concentration. These results are in agreement with those observed in a recent study in an acute inflammation model (carra-geenin-induced paw oedema) in rats (Wise et al., 2008). A significant anti-inflammatory effect of PEA was observed after systemic treatment at 12.5, 25 and 50 mg/kg, but no differences were observed among the doses studied. Moreover, this optimal dose for PEA to exert anti-inflammatory effects (i.e. 10 mg/kg), irrespective of the route of administration, has also been shown in previous studies (Mazzari et al., 1996; Conti et al., 2002; Costa et al., 2002).

The intradermal injection of SP did not induce any skin reaction (wheal or erythema formation). Several experiments were conducted to confirm the lack of cutaneous reaction following SP challenge (Fig. 3). Moreover, the skin reaction was evaluated 5, 10 and 20 min after challenge. These results are in disagreement with a previous study in healthy and atopic dogs where inflammatory reactions were observed using lower concentrations of SP (0.1, 0.5 and 1 nM) (Marsella and Nicklin, 2001). In that study, however, no significant difference was detected in the wheal areas between the three tested concentrations of SP and, more importantly, lower responses were reported to occur in atopic dogs compared with clinically normal dogs. A possible explanation for the apparent discrepancy between these results and our findings could reside in the postulated increase in cutaneous SP concentrations in the skin of hypersensitive Beagle dogs used in the present study and the consequent decrease in reactivity to SP injections. According to Marsella and Nicklin (2001), this would suggest a similarity between the model used and naturally occurring atopic disease.

In order to correlate the observed anti-allergic activity of PEA with its blood levels, plasma samples were taken during 24 h after oral treatment. The absorption of PEA was very fast and the peak plasma concentration was found between 1 and 2 h after administration. Blood levels of PEA decreased rapidly and returned to basal levels 4 h after administration. Plasma concentration of PEA and anti-inflammatory effects exhibited a parallel time-course during the 8 h following oral treatment. It is interesting to note that although PEA plasma levels returned to basal value 4 h after administration, the inhibitory effects lasted up to 8 h after treatment. The rapid return to basal levels may be due to amidases that are known to rapidly inactivate PEA to palmitic acid and ethanolamine (Cravatt et al., 1996; Ueda et al., 2001). On the other hand, differences between the rapid elimination of PEA and the slower decrease of the inhibitory effects may be related to the ability of PEA to up-regulate the levels or enhance the action of other endogenous bioactive compounds, as has been suggested previously (Lambert and Di Marzo, 1999; Hoareau et al., 2009).

Conclusions

Treatment of hypersensitive dogs with a single oral dose of ultra-micronized PEA resulted in a significant reduction of allergic wheal reactions in the skin. The dose of PEA exerting the maximum anti-allergic effect was 10 mg/kg. The anti-inflammatory effect of PEA outlasted its plasma levels. Further studies are needed to understand better the long-lasting mechanisms involved in the anti-inflammatory and anti-allergic effects of PEA. Clinical studies comparing PEA treatment with other classic therapeutic agents, such as glucocorticoids, are necessary to confirm the efficacy of this treatment in allergic dogs.

Conflict of interest statement

Alda Miolo is an employee of CeDIS (Science Information and Documentation Centre), Innovet Italia srl. Maria Federica della

Valle is a science consultant for Innovet Italia srl. This work was supported in part by Innovet Italia srl.

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