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Ultramicronized palmitoylethanolamide counteracts the effects of compound 48/80 in a canine skin organ culture model

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Background – Ultramicronized palmitoylethanolamide (PEA-um) has been reported to reduce pruritus and skin lesions in dogs with moderate atopic dermatitis and pruritus.

Hypothesis/Objectives – A canine *ex vivo* skin model was used to investigate the ability of PEA-um to counteract changes induced by compound 48/80, a well-known secretagogue that causes mast cell degranulation.

Animals – Normal skin was obtained from three donor dogs subjected to surgery for reasons unrelated to the study.

Methods – Cultured skin biopsy samples in triplicate were treated with 10 and 100 μg/mL compound 48/80, without or with 30 μM PEA-um. Mast cell (MC) degranulation, histamine release into the culture medium, local microvascular dilatation, epidermal thickness, keratinocyte proliferation and epidermal differentiation markers were evaluated.

Results – Exposure of the skin organ culture to PEA-um 24 h before and 72 h concomitantly to compound 48/80 resulted in a significant decrease of degranulating MCs. PEA-um also reduced the histamine content in the culture medium by half, although the effect did not reach statistical significance. PEA-um significantly counteracted vasodilation induced by 100 μg/mL compound 48/80. Finally, PEA-um alone did not induce changes in epidermal thickness, differentiation markers, keratinocyte proliferation, MC density and/or degranulation.

Conclusions and clinical importance – Collectively, these results support the protective action PEA-um on the skin of dogs undergoing allergic changes.

Introduction

Palmitoylethanolamide (PEA) is a bioactive lipid compound found in mammalian cells and tissues, including canine skin, and whose production increases in response to a variety of stressful stimuli.[1–3] For example, in the lesioned skin of dogs affected with atopic dermatitis (AD), PEA levels are more than 30-fold higher than in normal nonatopic skin.[4] Such increases are generally considered to be a mechanism to maintain or restore cellular homeostasis in the face of external stressors.[5] A growing literature documents the beneficial effects of exogenously administered PEA in both *in vitro* and *in vivo* models of acute and chronic inflammation.[1,2] These include a protective role of PEA against skin inflammation in different *in vivo* models of allergic dermatitis.[6,7] An open-label clinical study demonstrated the efficacy of orally administered ultramicronized PEA (PEA-um, a formulation of PEA with smaller particle size[8]) in reducing pruritus and skin lesions in dogs affected with AD.[9]

Mast cells (MCs) are resident immune cells prevalent at sites exposed to the environment, such as skin, where they often localize at high density in the superficial dermis. Their cytoplasmic secretory granules store a variety of bioactive mediators, such as biogenic amines, neuropeptides, cytokines and proteases.[10–13] MCs are a key player in innate and acquired immune responses, particularly in allergic reactions, including human and canine AD,[4,10,14,15] and are known to regulate skin vasodilation.[14,16] On activation, MCs undergo degranulation and release their granule content into the extracellular microenvironment.[17,18] The classical mode of activation is antigen cross-linking to antigen-specific IgE bound on the MC surface; furthermore, they also respond to basic secretagogues including compound 48/80.[19,20] The action

Abbreviations: TEM, Transmission electron microscopy; PEA, Palmitoylethanolamide.

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of compound 48/80 depends on its interaction with specific MC surface receptors associated with allergic reactions, and it is used to elucidate the mechanisms and efficacy of anti-allergic medications.[21,22]

The beneficial effects of PEA seem to be related, at least in part, to its ability to down-modulate MC activation and degranulation,[23,24] through a mechanism originally known as ALIA (Autacoid Local Injury Antagonism).[25,26] Investigations have identified direct and indirect receptor targets of PEA,[27,28] including cannabinoid receptors,[29] that are found in different tissues including the skin of healthy and AD dogs.[30–32] Moreover, the so-called "entourage effect" is also likely involved in PEA actions, where there is enhancement of the anti-inflammatory effects exerted by different lipid compounds of the endocannabinoid family.[33]

Our study investigated downregulation by PEA-um of the degranulation of cutaneous MCs and related vasodilation, using an organ culture model of canine skin,[34,35] based on ethical use of research animals.[36]

Methods

Animals

Normal skin was obtained from three adult, client-owned female spayed dogs subjected to surgical procedures unrelated to dermatological conditions (mastectomy) with no history of chemotherapy or other drug treatments. A written informed consent was obtained from each dog's owner prior to surgery. Before general anaesthesia with propofol (4 mg/kg, intravenous) and 2% isoflurane in oxygen, hair was clipped and the surgical site aseptically prepared. All samples were taken from the lower abdominal area to minimize variations due to body location, and were collected at the periphery of the surgical site in order to avoid pathological changes.

Dog skin organ culture and treatments

During the surgical procedure, a skin strip was collected from each dog and promptly immersed in fresh isolation medium (99% Williams' E medium-L-glutamine-free supplemented with 1% antibiotic antimycotic solution containing 1% 10,000 IU penicillin/10 mg streptomycin and 25 μg amphotericin B per mL). The strip was kept at 4°C until used (within 2 h). Skin organ cultures were prepared by collecting 21 full-thickness 4 mm skin biopsy punches from each skin strip after gentle surgical removal of excess subcutaneous fat. Three of the 21 biopsies were immersed in 10% buffered formalin solution (pH 7.4) and regarded as Day 0 samples. The remaining 18 biopsies were used for cultures established in triplicate (three biopsies per time point/treatment). Skin biopsies were placed into six-well multiplates containing Williams' E medium supplemented with 1% 10,000 IU/mL penicillin/10 mg/mL streptomycin, 0.1% 10 μg/mL insulin, 0.02% 10 ng/mL hydrocortisone and 1% 200 mM L-glutamine. All reagents were from Sigma Aldrich S.r.l. (Milan, Italy).

Skin samples were kept at 37°C in a humidified incubator with 5% CO_2. Medium was replaced after 24 h (Day 1) and then at Day 2 (concomitantly with treatments) and Day 4. After 24 h from culture establishment (Day 1) three triplicates were treated overnight with a final concentration of 30 μM PEA-um (Innovet Italia S.r.l.; Milan, Italy) suspended in an aqueous solution of Pluronic F68 10% (v/w) (Sigma-Aldrich). The concentration of PEA-um was chosen based on previous studies.[23,26] At Day 2, compound 48/80 was added at a final concentration of either 10 or 100 μg/mL for 72 h (days 2–5) with or without concomitant treatment with PEA-um (Figure 1).

Samples were collected at Day 5. Biopsies were fixed in Karnovsky solution (2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Samples were then divided in two halves using a microtome blade under a stereomicroscope. The first half was processed for methacrylate resin inclusion (JB-4 Embedding Kit, Polysciences, Inc.; Warrington, PA, USA) for MC assessment and to visualize skin microvessels. The second half was routinely processed for paraffin embedding.

Tissue fragments (2 mm^3) were obtained from samples treated with either vehicle or compound 48/80 alone: these fragments underwent OsO_4 post-fixation for inclusion in epoxy resin (Epon-Araldite, Sigma-Aldrich) for transmission electron microscopy. The experimental design is summarized in Figure 1.

Evaluation of MC degranulation

Morphometric evaluation

Two 1 μm thick sections, obtained from samples embedded in methacrylate resin, were stained with toluidine blue to visualize metachromatic MCs. A total of 18 sections per treatment were analysed (i.e. two sections for each of the three dogs' triplicate biopsies). Perivascular and interstitial nucleated MCs were counted under light microscopy at ×400 magnification in consecutive microscopic fields. The entire skin biopsy was examined. Degranulating MCs were defined as cells associated with 6–8 metachromatic granules outside the cell membrane at ×400 magnification, as described previously.[25,37,38] Resting and degranulating MCs were counted separately. The total number of MCs/mm^2 (i.e. MC density) and number of degranulating MCs/mm^2 were recorded.

Transmission electron microscopy (TEM)

Ultrathin sections obtained from epoxy resin-embedded samples were mounted on Formvar/Carbon 200 mesh copper grids (Ted Pella, Inc.; Redding, CA, USA) and observed by TEM after double contrast with 2% uranyl acetate and lead citrate. Previously published ultrastructural criteria for MC identification were used.[18]

Histamine release

At Day 5, histamine released into the culture medium upon different treatments was measured by radioimmunoassay (histamine, RIA kit, Beckman Coulter S.r.l.; Milan, Italy). Briefly, after acylation of histamine in experimental samples and calibration standards, an incubation step with known concentrations of ^{125}I-labelled histamine in the anti-histamine antibody coated tubes was performed. Following incubation, the contents of the tubes were aspirated and bound radioactivity measured in a gamma counter. Histamine values (nM/L) in the unknowns were determined by interpolation from the standard curve.

Evaluation of vasodilation

Two nonsequential, 1 μm thick sections, obtained from each sample embedded in methacrylate resin and stained with toluidine blue, were used for morphometric evaluation of local vasodilation. A total of 18 sections per treatment were analysed (i.e. two sections for each of the three dogs' triplicate biopsies). Subepidermal microvessel size was measured in the uppermost layer of the dermis. The Region of Interest (ROI) width was the entire skin biopsy, whereas its height was 100 μm deep from the epidermal basement membrane in order to include capillaries emerging from the superficial vascular plexus of the skin and excluding arterioles and venules. In the ROI area, vessel lumina were traced manually using dedicated software for computer-assisted image analysis (NIS-Elements Br Microscope Imaging Software, Nikon Instruments; Calenzano, Italy). For each traced vessel lumen, the following semi-automated measures were collected: equivalent diameter (i.e. the adjusted diameter for irregular circular shapes), area, perimeter and twice-the-apothem (a measure related to the ratio between luminal area and perimeter). Because capillary lumen is represented geometrically by either a circular or elliptical shape, the choice of using all four measures was made in an attempt to represent the capillary condition as closely as possible to the natural complexity of skin vessels.

Given that, (i) the internal endothelial tube of capillaries in the superficial skin might approximate the diameter of one erythrocyte, (ii) canine erythrocytes measure 6 μm in cytological specimens and

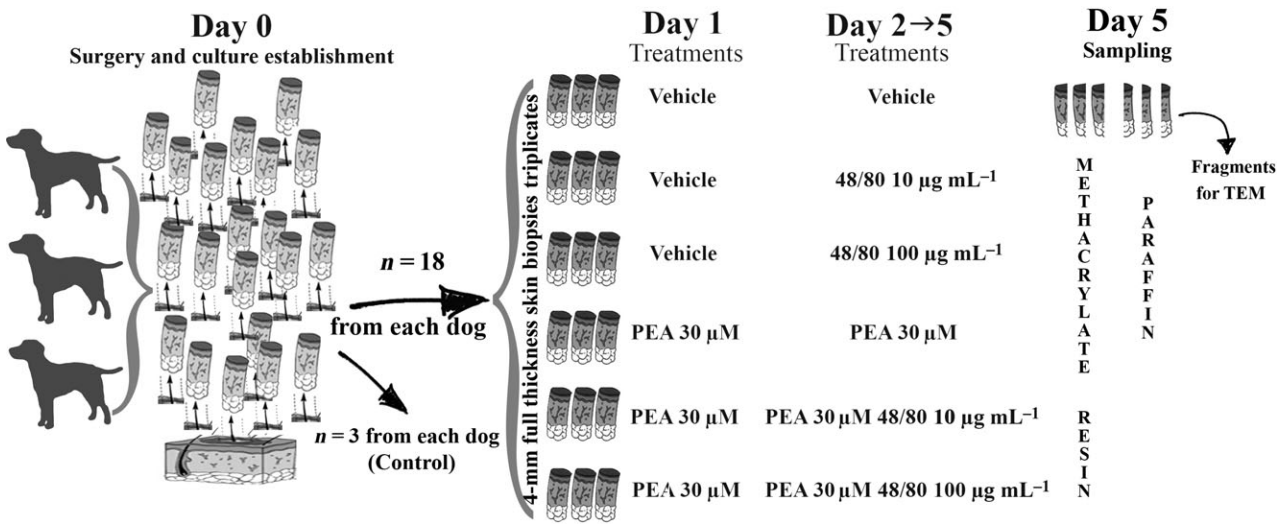

Figure 1. Scheme illustrating the experimental design of the study. Skin strips obtained from three dogs served to generate a total of 21 full-thickness biopsies per dog. Eighteen biopsies (from each dog) were used for experiments; the remaining three biopsies (per dog) served as Day 0 control samples. At Day 1 three subsets of triplicates were treated with 30 μM ultramicronized palmitoylethanolamide (PEA-um) that was continued up to Day 5. Compound 48/80 was added at either 10 or 100 μg/mL from days 2 to 5 in order to stimulate mast cell degranulation.

tissue processing might reduce the mean erythrocyte diameter, and (iii) in humans the descending capillary loops are 1.5 μm larger than the ascending ones, we devised three groups of capillaries based on their diameter to perform *post hoc* statistical analysis. The first group included vessels with a diameter smaller than 6 μm, the second included vessels with lumina ranging from 6 to 7.5 μm, and the third included vessels larger than 7.5 μm. This last group was taken as the cut-off value to assess percentage of dilated capillaries.

Evaluation of PEA effects on epidermal thickness, differentiation markers and keratinocyte proliferation

Five μm thick sections, obtained from paraffin-embedded samples, were either stained with haematoxylin & eosin or used for immunohistochemical analyses. Changes induced by treatment with PEA-um were assessed using three morphological features: epidermal thickness and differentiation markers, and keratinocyte proliferation.

Epidermal thickness

Epidermal thickness was measured on 20–400× captured fields per time point, by manually tracing 16 segments at regular intervals per captured field with NIS-Elements Br Microscope Imaging Software (NIS-Elements Br Microscope Imaging Software, Nikon Instruments). Segments were perpendicular to the basement membrane and extended from the germinative layer to the beginning of the stratum corneum. Measurements were expressed in μm.

Epidermal differentiation markers

In order to assess epidermal differentiation cytokeratin 10 (CK10), cytokeratin 14 (CK14) and loricrin were assayed. Indirect immunofluorescence was performed as follows: superfrost-Plus mounted sections were deparaffinized and rehydrated; epitope retrieval was carried out at 120°C in a pressure cooker for 3 min with a Tris/EDTA buffer pH 9.0. Nonspecific binding was blocked by incubation with Phosphate Buffered Saline (PBS) + 2% bovine serum albumin (BSA) (Vector Labs; Burlingame, CA, USA) (45 min at room temperature (RT)). Slides were then incubated overnight at 4°C with either a cocktail of the mouse monoclonal anti-CK10 antibody (Cat. N° ab9026, Abcam plc; Cambridge, UK), rabbit polyclonal anti-loricrin antibody (Cat. N° ab24722, Abcam plc), mouse monoclonal anti-CK14 antibody (Cat. N° ab7800, Abcam plc) or rabbit polyclonal anti-loricrin antibody (Cat. N° ab24722, Abcam plc) diluted in PBS +1% BSA. Sections were then rinsed in PBS for 3 × 10 min, followed by incubation with fluorescein anti-mouse antibody (Cat. N° FI-2000, Vector Labs) for CK14 and CK10, and fluorescein anti-rabbit antibody (Cat. N° FI-1000, Vector Labs) for loricrin. Slides were then mounted with Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear counterstaining (Vectashield mounting medium with DAPI, Vector Labs).

Keratinocyte proliferation

Ki67 immunostaining was performed to evaluate keratinocyte proliferation. Briefly, superfrost-Plus mounted sections were deparaffinized and rehydrated. Epitope retrieval was performed as described above. Quenching of endogenous peroxidases was carried out by incubation with 1% H_2O_2 in 0.1 M phosphate buffer pH 7.4 (PBS) for 5 min, at room temperature. Nonspecific binding was prevented by incubating slides with 5% normal goat serum (Vector Labs) for 1 h at RT. Slides were then incubated with mouse monoclonal anti-Ki67 primary antibody (Cat. N° sc-101861, Santa Cruz Biotechnology; TX, USA) 1:200 in PBS overnight at 4°C. Subsequently they were rinsed in PBS for 3 × 10 min, followed by incubation with a biotinylated goat anti-mouse IgG antibody (Cat. N° BA-9200, Vector Labs), diluted 1:300 in PBS. Sections were again rinsed in PBS for 3 × 10 min. Staining was visualized by incubating sections in diaminobenzidine solution (Vector Labs), followed by dehydration, clearing and mounting with a permanent mounting medium. Five 250× fields were photographed per time point per animal and Ki67-positive cells counted, data being expressed as percentage of basal cells.

Statistical analysis

Data were analysed using SAS v9.2 (SAS Institute; Cary, NC, USA). The response to treatments was analysed using a generalized linear mixed model, with the animal being the random effect. The Tukey-Kramer Honest Significant Difference (HSD) adjusted test for multiple comparisons was used as *post hoc* analysis and *P*-values were adjusted accordingly. The chi-square test was used in *post hoc* analysis to compare the distribution of vessel diameter classes among treatments. The level of significance was set at $P < 0.05$. Data are expressed as mean ± standard error (SE), unless otherwise stated.

Results

PEA-um and compound 48/80-induced MC degranulation

Morphometric evaluation

Mast cells were present in the subepidermal area (i.e. in the dermis below the epidermal basement membrane

(superficial vascular plexus)), along the profile of adnexal structures (middle periadnexal vascular plexus) and in the interfollicular dermis. In resting (nondegranulating) MCs the granules were confined to the cytoplasm. Cells showed a smooth surface, with granules either densely grouped in the cytoplasm (often overlapping the nucleus) and sparse or grouped towards the cell periphery. Degranulating MCs were recognized by their irregular fuzzy cellular membrane and for being surrounded by sparse metachromatic granules (Figure 2a).

Compound 48/80, at both concentrations, induced a highly statistically significant MC degranulation (Figure 2b, $P < 0.0001$), without affecting MC density. When PEA-um was added 24 h before and 72 h concomitant to either 10 or 100 μg/mL of compound 48/80, a statistically significant reduction in the number of degranulating MCs per mm^2 was achieved ($P < 0.005$ and $P < 0.01$, respectively; Figure 2b). Moreover, at both concentrations of compound 48/80 the number of degranulating MCs following PEA-um treatment did not show any significant difference compared to vehicle. Total MC number did not differ among treatments. Detailed measures are reported in Table S1.

Transmission electron microscopy (TEM)

At the ultrastructural level resting, as well as actively degranulating, MCs were detected (Figure 2c). The typical resting MC had a round nucleus, a cytoplasm full of round to elliptical electron-dense bodies (granules) and was surrounded by a cell membrane with numerous narrow surface folds. Degranulating MCs were recognizable by their circular nucleus, large cytoplasm with either lack or rare presence of granules and presence of membrane folds. Actively degranulating MCs showed a large amount of granule remnants, extracellular dense bodies of the same size and appearance as MC granules. TEM analysis also showed the presence of normal keratinocytes, fibroblasts and other connective tissue cells (Figure 2c).

Histamine release

Treatment with PEA-um consistently reduced the level of histamine in the culture medium in response to both the lower (−57%) and higher (−53%) concentrations of compound 48/80, although the effect failed to reach statistical significance (Figure 2d).

PEA-um and compound 48/80-induced capillary bed vasodilation

Three main microvascular plexuses, namely the deep, medium and superficial, could be observed in all specimens analysed. The superficial plexus was found to lie parallel or oblique to the skin surface; above it isolated or few grouped capillaries (n = 2–3) were seen at the dermo-epidermal junction. Capillaries were recognized for their small size, thin vascular wall and presence of surrounding pericytes or veil cells. Identification was confirmed by the frequent visualization of an erythrocyte within the lumen (Figure 3a).

Measurements collected through computer-assisted image analysis, relative to the capillary bed, are reported in Table S1. Compound 48/80 at both concentrations induced a statistically significant increase ($P < 0.001$) in all measured values including area, perimeter, equivalent diameter and twice-the-apothem (Figure 3a). Treatment with PEA-um reduced the vasodilation induced by compound 48/80, reaching statistical significance ($P < 0.001$) for the higher concentration. Moreover, treatment with PEA-um reduced microvascular changes to values not statistically different from vehicle and, as far as area and twice-the-apothem were concerned, could be considered statistically equivalent to the latter ones ($P > 0.97$). As depicted in Figure 3b, PEA-um normalized microvessel diameter distribution ($P < 0.0001$) which had been altered by compound 48/80 treatment ($P < 0.0001$).

PEA-um effects on epidermal thickness, differentiation markers, keratinocyte proliferation, and MC density and degranulation

Morphological analysis of samples treated either with vehicle or PEA-um showed a normal skin composed of viable epidermis, dermis and adnexal structures comparable to skin collected at Day 0. Epidermis was normally stratified with clearly visible granular and cornified layers. Focal minimal hyperplasia was sometimes qualitatively recorded in samples treated with PEA-um, an observation that was not confirmed by morphometry when epidermal thickness was assessed. Epidermal thickness (μm) in samples treated with vehicle (20.97 ± 3.61) did not differ from those treated with PEA-um (20.29 ± 1.69). Similarly, no statistical differences were observed in the number of Ki67-positive cells, being 4.49 ± 0.91 and 4.51 ± 0.89 in samples treated with vehicle and PEA-um, respectively. K10, K14 and Loricrin showed a distribution comparable between samples treated with vehicle or PEA-um, resembling the distribution commonly seen in normal skin. Treatment with PEA-um did not induce any change in MC density or degranulation compared to vehicle ($P = 0.99$).

Discussion

In the present study we used a canine skin organ culture model to show that PEA-um is able to counteract MC degranulation induced by administration of compound 40/80 and the subsequent vascular response. Although the effects of PEA in several inflammatory settings have been shown to depend, at least in part, upon the down-modulation of MC degranulation;[39–43] most available data rely on biochemical quantification of the released mediators,[24,43] focus on MC lines,[26,28] or primary MCs using *in vitro* conditions;[23] so they are outside the constitutive 3D environment of the skin. Here we present, to the best of the authors' knowledge for the first time, the use of a canine *ex vivo* model to study the degranulating state of MCs *in situ,* in response to a well-known secretagogue and document the effect of PEA-um.

Our findings demonstrate the ability of compound 48/80 to induce MC degranulation in a canine skin organ culture model, thus confirming a previous study that suggested compound 48/80 could be useful as part of a model for canine hypersensitivity and for the development of new approaches to controlling such reactions.[44]

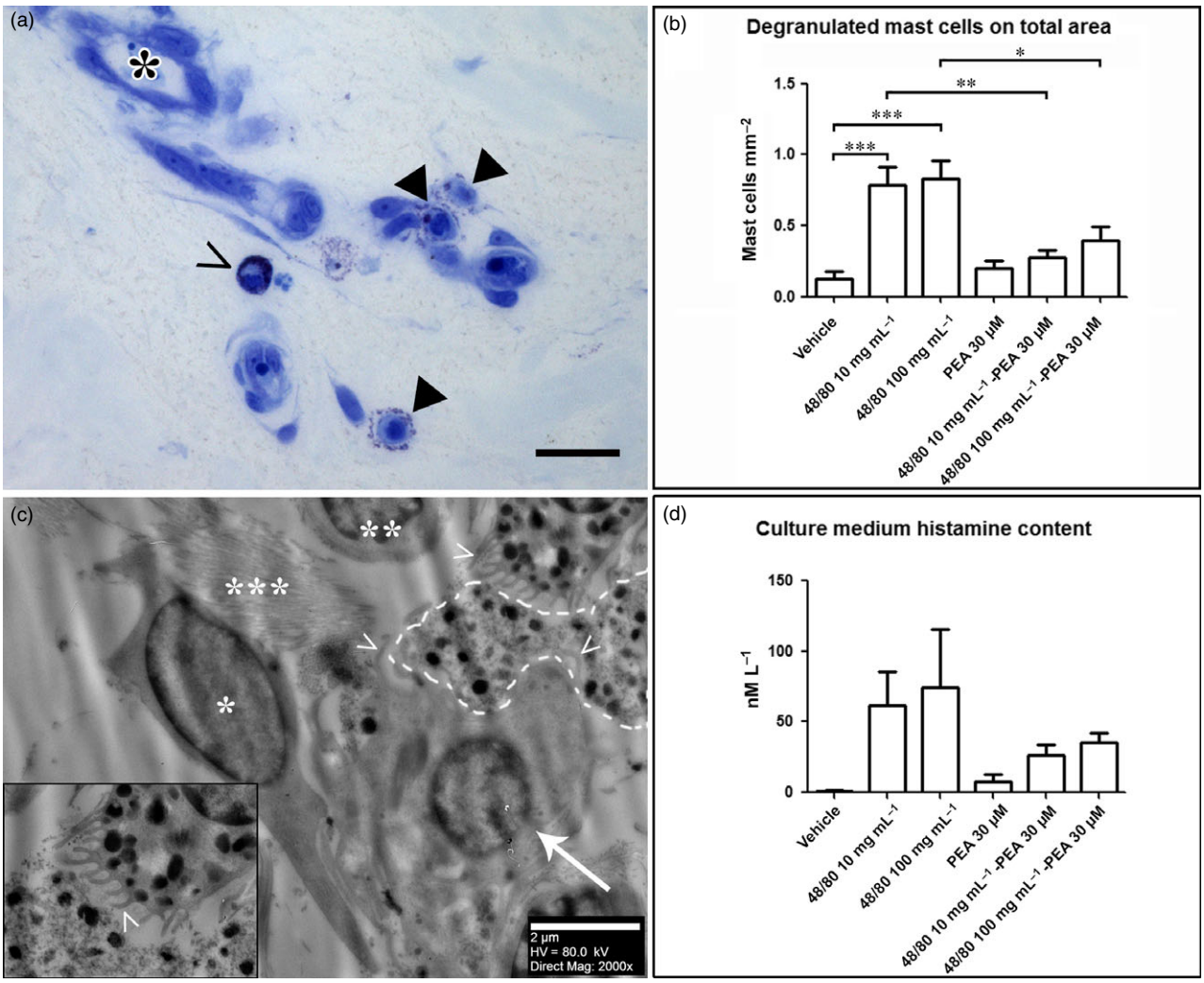

Figure 2. Photomicrographs of degranulating mast cells (MCs); effect of ultramicronized palmitoylethanolamide (PEA-um) in counteracting compound 48/80-induced MC degranulation; histamine modulation by different treatments. (a) Perivascular resting, i.e. nondegranulating (empty arrowhead) and degranulating MCs showing granules surrounding the plasma membrane (full arrowheads); asterisk indicates a blood vessel lumen; toluidine blue staining, scale bar = 20 μm. (b) Histogram showing the effect of PEA-um (30 μM) in counteracting compound 48/80-induced MC degranulation (*$P < 0.005$; **$P < 0.01$; ***$P < 0.0001$). Mean values are reported with standard error. (c) Electron micrographs focusing on a degranulating MC (white arrow); extracellular granules are enclosed by the dotted white line; a granulated MC is present on the top right corner (enlarged in inset); empty arrowheads indicate MC membrane folds; a fibroblast (*), a lymphocyte (**) and collagen fibres (***) are also shown. (d) Histogram reporting mean values of histamine levels measured in culture medium at Day 5: although not statistically significant, differences are analogous to the recorded number of degranulating MCs (b).

Our observations on the ability of PEA-um to significantly decrease compound 48/80-induced MC degranulation agree with previous results on the physiological control of MCs exerted by endocannabinoids which are a family of endogenous mediators acting on cannabinoid receptors, the main member being anandamide, of which PEA is a congener.[3,45,46] The endocannabinoid anandamide has been shown to moderate secretagogue-activated MCs *in situ* using different human organ culture models including scalp hair follicles and nasal polyps).[45,47]

The effect of PEA-um also resulted in a significant control of superficial vasodilation induced by compound 48/80. This was not surprising, because MCs are located proximal to blood vessels, and their release of preformed and newly synthesized mediators is known to promote local vascular dilation and permeability,[14,48] sustaining the typical wheal and flare reactions,[49,50] One can thus hypothesize that downregulation exerted by PEA-um on secretagogue-activated MCs resulted in a lower release of vasoactive mediators from MCs and consequently decreased vasodilation. The ability of PEA-um to reduce by around 50% the histamine content in culture medium is consistent with this hypothesis, even though the effect failed to reach statistical significance. This observation may depend on the time of sampling or the small number of samples used. Although further studies on the present *ex vivo* model are merited, one should keep in mind that PEA-um has been reported to decrease MC-derived vasoactive mediators, both *in vitro* and *in vivo*.[23,28,40,43,51] Moreover, the effect of systemic administration of PEA-um on clinical signs sustained by dilation of cutaneous capillary vessels, including erythema, and the wheal and flare reaction, has been demonstrated in dogs.[7,9]

In the present *ex vivo* model, exposing skin organ cultures to PEA-um for 96 h did not elicit any change in either

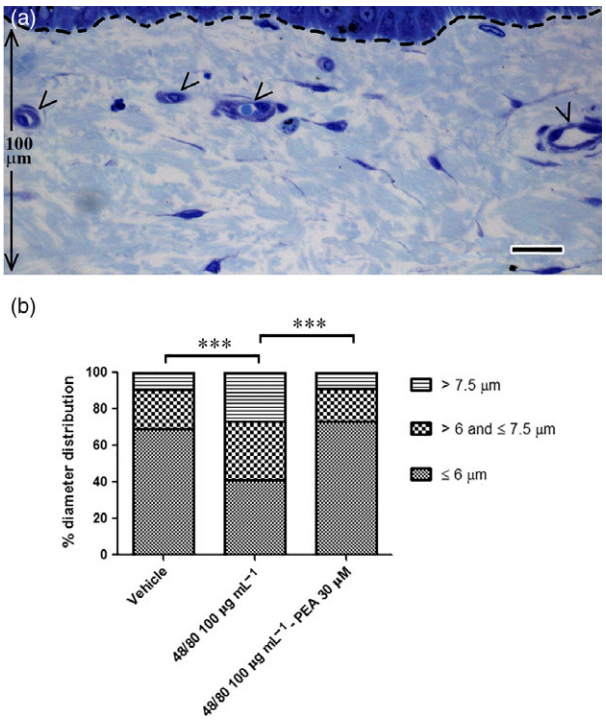

Figure 3. Photomicrographs showing the superficial dermis where microvessel size was recorded morphometrically and histogram showing the microvessel diameter distribution. (a) Toluidine blue stained section showing the Region of Interest where microvascular dilatation was evaluated morphometrically (100 µm below the dermo-epidermal junction, the latter shown by the dotted line); arrowheads indicate blood capillaries, scale bar = 20 µm. (b) Treatment with compound 48/80 100 (µg/mL) increased the percentage of dilated vessels (>7.5 µm); this latter effect was reverted by ultramicronized palmitoylethanolamide (PEA-um) (30 µM) treatment (***$P < 0.0001$).

the epidermal or dermal skin compartments, in terms of epidermal thickness or keratinocyte proliferation, the same pattern of keratinocyte differentiation markers, unchanged MC density and degranulation state, compared to vehicle-treated samples. These findings are consistent with results of previous studies on the lack of PEA-um cytotoxicity and its overall *in vivo* safety profile.[1,9,52]

Our findings suggest that PEA-um effectively downmodulated compound 48/80-induced MC degranulation in the model used. However, some limitations should be considered when interpreting the data. First, as an *ex vivo* study, one should take into consideration the possible lack of correlation between *ex vivo* and *in vivo* results. This issue is difficult to address, mainly for two reasons. First, very few data are available at present on the pharmacokinetic profile (PK) of PEA-um. One factor may be the endogenous presence (and endogenous changes) of PEA levels in the mammalian body,[1] which likely render PK studies more difficult and require its isotopic labelling. Second, the mechanism of action of PEA appears to invoke multiple pathways, both of a direct nature (e.g. receptor–ligand interaction), as well as those based on the so-called entourage effect.[33,53] Conceivably, PEA actions might not be directly related, both in terms of plasma/tissue concentration and timing, to its PK features.

The small number of dogs in our study imposed limits, although the statistical unit was the biopsy, not the dog. Furthermore, the results were independent of the donor because the dog was used as random effect in the generalized linear mixed model adopted for the statistical analysis. A final inherent limit of the present investigation concerns the fact that time-course changes could not be followed due to the "single sample" feature of the study. Observations made at Day 5 are not from a Day 2 biopsy three days later, but from a "sister biopsy", originally cultivated to be processed at Day 5.

The present canine *ex vivo* allergic model demonstrated that PEA-um limited degranulation of skin MCs and the related microvascular response. Although further studies are clearly warranted, these data further support the ALIA (autacoid local injury antagonism) effect of PEA-um with MC downmodulation under hyperactivated conditions; they shed new light on the mechanism(s) of anti-allergic and anti-inflammatory action of this bioactive endocannabinoid-like compound.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Detailed data on mast cell morphometric analysis, histamine quantification and histological assessment of vasodilation.

Résumé

Contexte – Le PEA-um (ultramicronized palmitoylethanolamide) a été décrit comme réduisant le prurit et les lésions cutanées chez les chiens atteints de prurit et de dermatite atopique modérés.

Hypothèses/Objectifs – Un modèle de peau canine *ex vivo* a été utilisé pour étudier la capacité du PEA-um à empêcher les changements induits par le composé 48/80, un sécrétagogue bien connu qui entraine une dégranulation des mastocytes.

Sujets – La peau normale a été obtenue de trois chiens donneurs au cours de chirurgies dont les raisons étaient indépendantes de l'étude.

Méthodes – Les échantillons de biopsies cutanées cultivées ont été traitées avec 10 et 100 μg/mL du composé 48/80, avec ou sans 30 μM PEA-um. La dégranulation des mastocytes, la libération d'histamine dans le milieu de culture, la dilatation microvasculaire locale, l'épaisseur épidermique, la prolifération des kératinocytes et les marqueurs de différentiation épidermique ont été évalués.

Résultats – L'exposition des cultures de peau au PEA-um24h avant et 72h concomitamment au composé 48/80 a résulté en une diminution significative de dégranulation des mastocytes. Le PEA-um a également réduit de moitié le contenu en histamine dans le milieu de culture bien que l'effet n'ai pas été statistiquement significatif. Le PEA-um a significativement contré la vasodilatation induite par 100 μg/mL du composé 48/80. Enfin, le PEA-um n'a pas entrainé de changement dans l'épaisseur épidermique, les marqueurs de différentiation, la prolifération kératinocytaire, la densité des mastocytes et/ou leur dégranulation.

Conclusions et importance clinique – Collectivement, ces résultats supportent l'action protectrice du PEA-um sur la peau des chiens allergiques.

Resumen

Introducción – Se ha publicado que la palmitoiletanolamida ultramicronizada (PEA-um) reduce el prurito y las lesiones cutáneas en perros con dermatitis atópica moderada y prurito.

Hipótesis/Objetivos – Se usó un modelo cutáneo *ex vivo* canino para investigar la capacidad de PEA-um para contrarrestar los cambios inducidos por el compuesto 48/80, un secretagogo bien conocido que causa la degranulación de los mastocitos.

Animales – Se obtuvo piel normal de tres perros donantes sometidos a cirugía por razones no relacionadas con el estudio.

Métodos – Se trataron muestras de biopsia de piel cultivada por triplicado con 10 y 100 μg/mL de compuesto 48/80, sin o con 30 μM de PEA-um. Se evaluó la degranulación de los mastocitos (MC), la liberación de histamina en el medio de cultivo, la dilatación microvascular local, el espesor epidérmico, la proliferación de queratinocitos y los marcadores de diferenciación epidérmica.

Resultados – La exposición del cultivo de piel orgánica a PEA-um 24 h antes y 72 h al mismo tiempo que el compuesto 48/80 resultó en una disminución significativa de la degranulación de MC. PEA-um también redujo el contenido de histamina en el medio de cultivo a la mitad, aunque el efecto no alcanzó significación estadística. PEA-um contrarrestó significativamente la vasodilatación inducida por 100 μg/mL de compuesto 48/80. Finalmente, el PEA-um solo no indujo cambios en el grosor epidérmico, marcadores de diferenciación, proliferación de queratinocitos, densidad y/o degranulación de MC.

Conclusiones e importancia clínica – Colectivamente, estos resultados apoyan la acción protectora de PEA-um en la piel de perros sometidos a cambios alérgicos.

Zusammenfassung

Hintergrund – Es wurde bereits beschrieben, dass ultrakleines Palmitoylethanolamin (PEA-um) Juckreiz und Hautläsionen bei Hunden mit mäßiger atopischer Dermatitis und Juckreiz vermindern kann.

Hypothese/Ziele – Ein canines *ex vivo* Hautmodell wurde verwendet, um die Fähigkeit von PEA-um zu untersuchen, den durch Compound 48/80 - einem gutbekannten Sekretionsförderer - induzierten Veränderungen, welche eine Mastzelldegranulation verursachen, entgegenzuwirken.

Tiere – Es wurde normale Haut von drei Spenderhunden entnommen, die aus Gründen, die mit der Studie nichts zu tun hatten, operiert wurden.

Methoden – Es wurden kultivierte Hautbiopsieproben im Triplikat mit 10 und 100 μg/ml Compound 48/80, ohne oder mit 30 μM PEA-um behandelt. Es wurden die Mastzelldegranulation (MC), der Histaminaustritt ins Kulturmedium, die lokale mikrovaskuläre Dilatation, die epidermale Dicke, die Keratinozytenproliferation und epidermale Differenzierungsmarker untersucht.

Ergebnisse – Eine Exponierung der Hautorgankultur zu PEA-um 24h vor und 72h während dem Compound 48/80 ergab eine signifikante Reduzierung der degranulierten MCs. PEA-um reduzierte ebenfalls den Histamingehalt im Kulturmedium um die Hälfte, obwohl die Wirkung keine statistische Signifikanz erreichte. PEA-um wirkte der Vasodilatation, die durch 100 μg/mL von Compound 48/80 induziert wurde, signifikant entgegen. Letztendlich konnte PEA-um keine Veränderungen der epidermalen Dicke, der Differenzierungsmarker, der Keratinozytenproliferation, der MC Dichte und/oder ihre Degranulation induzieren.

Schlussfolgerungen und klinische Bedeutung – Insgesamt bestärken diese Ergebnisse die Schutzfunktion von PEA-um für die Haut von Hunden, die allergischen Veränderungen unterliegt.

要約
背景 – ウルトラミクロ化パルミトイルエタノールアミド(PEA-um)は、中等度のアトピー性皮膚炎および痒みを伴う犬の掻痒および皮膚病変を軽減することが報告されている.
仮説/目的 – 犬のex vivo皮膚モデルを用いて、PEA-umが、化合物48/80(肥満細胞の脱顆粒を引き起こすことが知られている分泌促進物質)によって誘導される変化を抑制できるかどうかを調べた.
供与動物 – 正常な皮膚を本研究に無関係の理由で手術を受けた3頭のドナー犬より採取した.
方法 – 培養皮膚生検試料に10μg/mLあるいは100μg/mLの化合物48/80を添加し、30μMのPEA-um有り無しの条件下で処理した。実験は3ど肥満細胞(MC)脱顆粒、培養培地へのヒスタミン放出、局所微小血管拡張、表皮の厚さ、ケラチノサイト増殖および表皮分化マーカーを評価した.
結果 – 3セットの培養皮膚組織に化合物48/80を添加し、PEA-umに暴露する24時間前および72時間後で比較すると、脱顆粒MCが有意に減少した。統計的な有意差は認められなかったが、PEA-umは培地中のヒスタミン含量を半減させた。PEA-umは、100μg/ mLの化合物48/80によって誘導された血管拡張を中和した。PEA-um単独では、表皮の厚さ、分化マーカー、ケラチノサイトの増殖、MC密度および/または脱顆粒の変化を誘導しなかった.
結論および臨床的な重要性 – これらの結果より、PEA-umはアレルギー性変化を起こしている犬の皮膚に対してプロアクティブな効果を示すことが示唆された.

背景 — 据报道,超微乳化棕榈酰乙醇酰胺(PEA-um)可以减轻中度异位性皮炎和瘙痒患犬的瘙痒和皮肤病变.
假设/目的 — 使用犬体外皮肤模型,探究PEA-um消解化合物48/80引起的变化的能力,化合物48/80是一种众所周知的引发肥大细胞脱颗粒的促分泌素.
动物 — 从接受外科手术的三只供体犬上获取正常皮肤,手术原因均与本研究无关.
方法 — 培养的皮肤活检样品一式三份,使用或不用30μM PEA-um处理,再分别加入10和100μg/mL化合物48/80作用。最终,对肥大细胞(MC)脱颗粒程度,培养基中组胺释放量,以及局部微血管扩张、表皮厚度、角质细胞增殖和表皮分化标志物进行评估.
结果 — 将皮肤培养物暴露于PEA-um中,处理24h,再加入化合物48/80,共同作用72h,能明显降低肥大细胞脱颗粒程度。尽管作用不具有统计学意义,但PEA-um仍能使培养基中组胺含量减少一半。PEA-um能显著对抗由100μg/mL化合物48/80引起的血管舒张。 最后,单独使用PEA-um并不会引起表皮厚度、分化标志物、角质细胞增殖、MC密度和/或脱颗粒的变化.
结论和临床意义 — 总之,以上结果证明PEA-um对过敏患犬的皮肤具有保护作用.

Resumo
Contexto – A palmitoietanolamida ultramicronizada (PEA-um) tem sido associada à redução do prurido em lesões cutâneas de cães apresentando dermatite atópica moderada e prurido.
Hipótese/Objetivos – Um modelo de pele canino *ex vivo* foi utilizado para investigar a habilidade de PEA-um de neutralizar as alterações induzidas por um composto 48/80, um conhecido secretagogo que causa degranulação de mastócitos.
Animais – Pele normal foi obtida de três cães doadores submetidos à cirurgia por motivos não relacionados ao estudo.
Métodos – As amostras de pele cultivadas em triplicata foram tratadas com 10 e 100 μg/mL do composto 48/80, com ou sem 30 μM de PEA-um. Foram avaliados: degranulação de mastócitos (MC), liberação de histamina no meio de cultura, microvasodilatação local, espessura epidérmica e marcadores de proliferação de queratinócitos e diferenciação epidérmica.
Resultados – A exposição da cultura de pele a PEA-um 24 horas antes e 72 horas concomitantemente com o composto 48/80 resultou em redução significativa na degranulação de MCs. PEA-um também reduziu à metade a concentração de histamina no meio de cultura, ainda que este efeito não tenha alcançado diferenças estatísticas significativas. A PEA-um neutralizou significativamente a vasodilatação induzida por 100 μg/mL do composto 48/80. Finalmente, PEA-um isoladamente não induziu alterações na espessura epidérmica, nos marcadores de diferenciação, na proliferação de queratinócitos, na densidade de MC e/ou degranulação.
Conclusões e importância clínica – Coletivamente, estes resultados confirmam a ação protetora de PEA-um na pele de cães passando por alterações alérgicas.

