



Immunopharmacology and inflammation

Palmitoylethanolamide reduces inflammation and itch in a mouse model of contact allergic dermatitis



Massimo Vaia^a, Stefania Petrosino^{b,c,d}, Daniele De Filippis^a, Luana Negro^a, Andrea Guarino^a, Rosa Carnuccio^a, Vincenzo Di Marzo^{b,c,*}, Teresa Iuvone^{a,c,*}

^a Department of Pharmacy, University of Naples Federico II, Via D. Montesano 49, 80131 Napoli, Italy

^b Istituto di Chimica Biomolecolare (ICB), Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, 80078 Pozzuoli, NA, Italy

^c Endocannabinoid Research Group, Italy

^d Epitech Group S.p.A., Via Luigi Einaudi 13, 35030 Saccolongo, PD, Italy

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ABSTRACT

In mice, 2,4-dinitrofluorobenzene (DNFB) induces contact allergic dermatitis (CAD), which, in a late phase, is characterized by mast cell (MC) infiltration and angiogenesis. Palmitoylethanolamide (PEA), an endogenous anti-inflammatory molecule, acts by down-modulating MCs following activation of the cannabinoid CB₂ receptor and peroxisome proliferator-activated receptor- α (PPAR- α). We have previously reported the anti-inflammatory effect of PEA in the early stage of CAD. Here, we examined whether PEA reduces the features of the late stage of CAD including MC activation, angiogenesis and itching. After sensitization to DNFB, female C57BL/6J mice underwent to three DNFB challenges at days 5, 12 and 19 and treatments were given at each challenge and for two more days. CAD was expressed as Δ increase in ear thickness between challenged and unchallenged mice. PEA (5 mg/kg/i.p.) reduced: i) the DNFB-induced Δ increase; ii) the number of MCs per tissue area; iii) the expression of VEGF and its receptor Flk-1. These effects were reversed by co-administration of AM630 (1 mg/kg/i.p.), a CB₂ antagonist, but not GW6471 (1 mg/kg/i.p.), a PPAR- α antagonist. Finally, PEA reduced the number of ear scratchings 48 h after DNFB challenge and this effect was reversed by both CB₂ and PPAR- α antagonists, suggesting the involvement of both receptors. PEA, by reducing the features of late stage CAD in mice, may be beneficial in this pathological condition.

1. Introduction

Contact allergic dermatitis (CAD) is an inflammatory condition of the skin characterized by erythema, itching, edema and thickening occurring after repeated exposure to irritants or allergens. Repeated applications of a contact sensitizing hapten cause epidermal hyperplasia, accumulation of CD4⁺ T cells and mast cells (MCs), and elevated serum levels of antigen-specific immunoglobulin E (IgE) (Kitagaki et al., 1995).

MCs are tissue-localized immune competent cells, which are principally involved in immunological skin diseases (Navi et al., 2007). Upon activation, MCs release inflammatory mediators including histamine, cytokines and proteases (Wernersson and Pejler, 2014). Skin lesions after repeated hapten application are reduced in histamine-deficient compared to wild-type mice (Seike et al., 2005). Moreover, MCs recruit neutrophils to the skin site through the release of MC-derived tumor necrosis factor (TNF)- α in 3-nitrochlorobenzene-induced CAD (Suto et al., 2006). Finally, a MC chymase inhibitor

attenuates dermatitis in mice (Watanabe et al., 2002).

Angiogenesis plays a critical role in murine skin after dinitrofluorobenzene (DNFB) challenge through MC-released vascular endothelial growth factor (VEGF) (Watanabe et al., 2004). Moreover, nerve growth factor (NGF) is produced by numerous cells laying in the skin, including keratinocytes and MCs (Tron et al., 1990; Leon et al., 1994). NGF promotes skin innervation and plays an important role in the sensitization of nerve fibers (Stucky et al., 1999) and neurogenic inflammation (Horigome et al., 1994). Increased NGF levels act on MCs inducing the release of histamine *in vitro* (Tachibana et al., 1990). Histamine is an important transmitter of itch (Magerl et al., 1990) and increased histamine signaling has been reported in dinitrochlorobenzene-induced delayed-type hypersensitivity in guinea pigs (Simone et al., 1987).

Palmitoylethanolamide (PEA) is an endogenous lipid mediator (Facci et al., 1995) exhibiting anti-inflammatory, anti-nociceptive, anti-angiogenic properties (Petrosino et al., 2010a). PEA through the peroxisome proliferator activated receptor (PPAR)- α produces anti-

* Corresponding authors at: Istituto di Chimica Biomolecolare (ICB), Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, 80078 Pozzuoli, NA, Italy.
E-mail address: vdimarzo@icb.cnr.it (V. Di Marzo).

nociceptive and anti-inflammatory effects especially at the central nervous system (CNS) level (D'Agostino et al., 2007). We have previously demonstrated that the early phase of CAD, occurring 5–7 days after the sensitization to DNFB, is attenuated by PEA through transient receptor potential vanilloid 1 (TRPV1) activation/desensitization at the keratinocyte level (Petrosino et al., 2010b). The pathophysiology of CAD, extensively investigated using DNFB as contact sensitizer in mouse models, is characterized by infiltration of inflammatory cell, including eosinophils in peripheral blood, and activation/proliferation of MCs (Navi et al., 2007). Moreover, mice lacking cannabinoid receptors of type 1 and 2 (CB₁ and CB₂) develop an exacerbated ear swelling in response to DNFB, suggesting that the endocannabinoid system tonically attenuates CAD (Karsak et al., 2007). Indeed, DNFB increases the levels of the endocannabinoids, anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), as well as those of PEA in the ear skin of DNFB-treated mice after the I challenge (Petrosino et al., 2010b; Karsak et al., 2007). Exogenously administered PEA exhibits a protective effect on keratinocyte-sustained inflammation after the 1st and 2nd challenge of CAD *via* TRPV1 receptors (Petrosino et al., 2010b).

The aim of the present study was to investigate the effect of exogenous administered PEA in the development of the allergic (3rd) phase of CAD, which in the DNFB mouse model occurs at day 21 after sensitization.

2. Materials and methods

2.1. Chemicals

2,4-dinitrofluorobenzene (DNFB) was purchased from Sigma-Aldrich (Milano, Italy). PEA was a gift from Epitech group S.p.A. (Padova, Italy). Mouse anti-VEGF was from R&D System Inc. (Minneapolis, MN), mouse anti-VEGF_r2(fl k-1) and mouse anti- β actin were from Santa Cruz Biotechnology (Dallas, Texas). AM630, a CB₂ receptor antagonist, and the PPAR- α antagonist, GW6471, were from Tocris Biosciences (Bristol, U.K.), toluidine blue was purchased from Sigma Aldrich (Saint Louis, MO), hematoxylin and eosin were both from Kaltek srl (Padova, Italy).

2.2. DNFB-induced CAD in mice and drugs treatments

Female C57BL/6J mice (obtained from Harlan Nossan, Milano, Italy) weighing 25–30 g and 8–10 week old were used in all experiments. Animals were housed under specific conditions with a 12-h light/ dark cycle and free access to standard rodent food and water. CAD experiments were performed as previously described (Karsak et al., 2007). Animals were sensitized by painting 50 μ l of DNFB (0.2% v/v in an acetone/olive oil 4:1 solution) on the shaved abdomen for two consecutive days. Then, mice were challenged by painting both sides of one ear with 10 μ l of 0.3% DNFB on days 5, 12 and 19. Ear swelling was determined 24 h, 48 h and 72 h after the III challenge (day 20, 21, 22) by measuring the difference between the DNFB exposed and the untreated ear using an engineer's micrometer. Further experiments, as prompted by the ear swelling results, were performed at 48 h after III challenge, *i.e.* day 21. At this day, after a behavioral test, animals were euthanized with CO₂, the ears were removed and soon after the explantation, the samples were immediately either stored in 4% formaldehyde for histological analysis or stored at –80 °C for further biochemical studies. PEA (5 mg/kg), AM630 (1 mg/kg), GW6471 (1 mg/kg) and the association of PEA (5 mg/kg) and AM630 (1 mg/kg) or of PEA (5 mg/kg) and GW6471 (1 mg/kg) were administered by intraperitoneal injection (*i.p.*) as reported in Fig. 1. Doses were chosen according to previous studies (Petrosino et al., 2010b; Di Filippo et al., 2004; Esposito et al., 2012).

All experimental procedures were approved by the Local Ethical Commission for Animal Care and Use at the University of Naples

“Federico II”, and met the guidelines and protocols approved by the European Community (2010/63/UE). All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to perform statistically valid analysis.

2.3. PEA and endocannabinoid measurement in the ears of DNFB-sensitized and challenged mice

Ears were homogenized in chloroform/methanol/Tris–HCl 50 mM pH 7.4 (2:1:1, v/v) containing 10 pmol of [²H]₈-anandamide, 5 pmol of [²H]₅-2-AG and [²H]₄-PEA, as internal standards. The lipid-containing organic phase was dried down, weighed and pre-purified by open-bed chromatography on silica gel. Fractions obtained by eluting the column with 9:1 (v: v) chloroform/methanol were analyzed by liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS) by using a Shimadzu HPLC apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2020) quadrupole MS *via* a Shimadzu APCI interface. LC–APCI–MS analyses were carried out in the selected ion monitoring mode, using *m/z* values of molecular ions +1 for deuterated and un-deuterated compounds, respectively as follows: 356 and 348 (AEA), 384.35 and 379.35 (2-AG), 304 and 300 (PEA). Tissue levels were calculated on the basis of the area ratio with the internal deuterated standard signal areas, and amount in pmols normalized per g or mg of tissue and compared by ANOVA followed by the Bonferroni's test; *n* =9 mice per group were used for these experiments.

2.4. Histological analysis

The tissues from ears of mice were collected on day 21 and immediately fixed in 4% neutral buffered formalin and routinely processed for histology. Serial sections (7 μ) of paraffin embedded tissues were cut. For each tissue, 20 sections were cut and used for the staining procedures. For histological evaluation of the number of MCs, the sections were deparaffinised and incubated for 2 min with toluidine blue solution (1% toluidine blue dissolved in ethanol 70%) in NaCl solution 10%, washed in distilled water, dehydrated and mounted with Entellan. The MC count was carried out in the total section using a 20X objective with a Leica DMRB microscope, and the section area was measured using the ImageJ software. For the determination of the number of vessels, the sections were deparaffinized, stained with haematoxylin and eosin, washed in distilled water for 10 min, dehydrated and mounted with Entellan. The count of MCs and vessels was carried out in the total section using a 20 $\times$ or 40 $\times$ objective, respectively, with a Leica DMRB microscope. The section area was measured using the ImageJ software. Quantitative evaluations were performed by a researcher blind to the experiment.

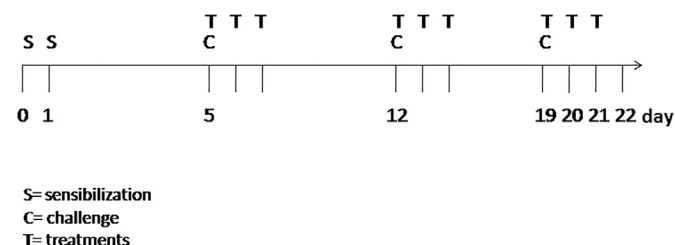


Fig. 1. Protocol for CAD induction in mice. Animals were sensitized with DNFB at day 0 and 1 (S) by painting their shaved abdomen; repeated challenges (C) were carried out by painting the right ear of each mouse with DNFB at day 5 (I challenge, early irritation phase), 12 (II challenge) and 19 (III challenge, late allergic phase). Treatments (T) with PEA (5 mg/kg *i.p.*), PEA+AM630 (1 mg/kg *i.p.*), PEA+GW6471 (1 mg/kg *i.p.*) were performed for three days from the challenge. At the doses used (selected based on previous studies), AM630 and GW6471 were *per se* inactive (data not shown).

2.5. Western blot analysis

Western blot was performed on samples of homogenized ears. Briefly, frozen samples of ears were lysed in 150 μ l of ice-cold hypotonic lysis buffer and incubated on ice for additional 45 min. The total protein extract was obtained by centrifugation at 14,000 rpm for 10 min at 4 °C. Samples (50 μ g/ml) were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred onto nitrocellulose membrane and incubated with one of the following antibodies: mouse anti-VEGF (1:1000 v/v;), mouse anti- FLK-1 (1:1000 v/v), mouse anti- β -actin (1:1000 v/v). The membranes were then incubated with the appropriate peroxidase-conjugated secondary antibodies (1:2000 v/v; PerkinElmer, Massachusetts, USA). The immunocomplexes were visualised by the Enhanced chemiluminescence (ECL) method (Santa Cruz Biotechnology, USA) and acquired by an imaging system (ImageQuant™ 400, GE Healthcare Life Sciences). Densitometric analyses were conducted using the ImageQuant software (Bio-Rad).

2.6. Assessment of scratching behavior

Pruritus induced by DNFB in mice was measured by counting the number of ear scratchings at 48 h after the III challenge with DNFB, in accordance with the other parameters studied and in agreement with the method of Kuraishi et al. (1995). Briefly, mice for each group (4 animals/cage) were moved to the observation room and left there for about 10 min for acclimation. For the observation of scratching, the animals were recorded using a video camera under unmanned conditions for 1 h and analyzed by the same observatory blinded for the treatments. Scratching only of the DNFB challenged ear with the hind paws was counted according to Kuraishi et al. (1995).

2.7. Statistical analysis

The mice that underwent to experimental procedures were $n=7-12$ per group. One-way ANOVA followed by the Bonferroni post-hoc test was used to determine statistical differences between the groups, and a value of $P < 0.05$ was used as threshold for statistical significance.

3. Results

3.1. Endocannabinoid levels in DAC

The levels of the endocannabinoids, AEA and 2-AG, and of PEA, were analyzed in the ear skin from mice with CAD. In the late stage of CAD, 2-AG levels were significantly reduced by DNFB challenge in comparison to controls, whereas AEA and PEA levels were not

significantly altered as compared to controls. PEA (5 mg/kg, i.p.) was able to significantly increase only the levels of 2-AG in the ear tissue (Fig. 2). A trend towards increased PEA levels was observed in the DNFB+PEA group, although this is likely the result of the administration of PEA and its delivery in the ear skin (Fig. 2)..

3.2. Effect of PEA on ear thickness

The increase of ear skin thickness induced by DNFB was evaluated by measuring the difference (Δ) in ear thickness between the right (DNFB challenged) and left (unchallenged) ear of each mouse 24–48–72 h after the 3rd challenge with DNFB. Marked increases in the thickness of ear skin occurred 24 h after the DNFB challenge, reaching a maximum 72 h after the challenge; PEA (5 mg/kg i.p.) treatment significantly reduced the Δ increase at all time points (by 31% at 24 h, 40% at 48 h, 20% at 72 h) (Fig. 3A). However, since the percent reduction was higher at 48 h, all other experiments were performed on tissues recovered 48 h after DNFB challenge. In order to investigate whether PEA effect was due to activation of CB₂ and/or PPAR- α receptors, we used selective antagonists. The effect of PEA at reducing ear thickness in DNFB-challenged mice was reversed, at 48 h, by co-administration of AM630 (1 mg/kg i.p.), a CB₂ receptor antagonist, but not GW6471 (1 mg/kg i.p.), a PPAR- α receptor antagonist (Fig. 3B)..

3.3. Effect of PEA on MC number

The effect of PEA on MC number was evaluated by histological analysis 48 h after the III challenge. A representative image of DNFB-treated (a) and DNFB + PEA-treated ear tissue sections (b) is shown in Fig. 4 A. An increased presence of MCs (86.4 ± 4.9) was observed in DNFB-challenged ear tissues in comparison with the unchallenged ears (15 ± 1.8), whereas PEA (5 mg/kg i.p.) treatment significantly reduced MC number (51.4 ± 0.9) (Fig. 4 B). The effect of PEA on MC number was significantly reversed by co-administration with AM630 (1 mg/kg i.p.), but not by GW6471 (1 mg/kg i.p.) (Fig. 4 B)..

3.4. Effect of PEA on protein expression of VEGF and its receptor Flk-1

The effect of PEA on the protein expression of VEGF and its receptor Flk-1 was evaluated 48 h after the III challenge by Western blot analysis. DNFB challenge induced an increased protein expression of VEGF and its receptor Flk-1 in the ear tissues compared with unchallenged ears. PEA (5 mg/kg i.p.) caused a significant reduction of both VEGF and Flk-1 protein expression compared with the DNFB group. The effect of PEA was reversed by the co-administration with AM630 (1 mg/kg i.p.), but not GW6471 (1 mg/kg i.p.) (Fig. 5)..

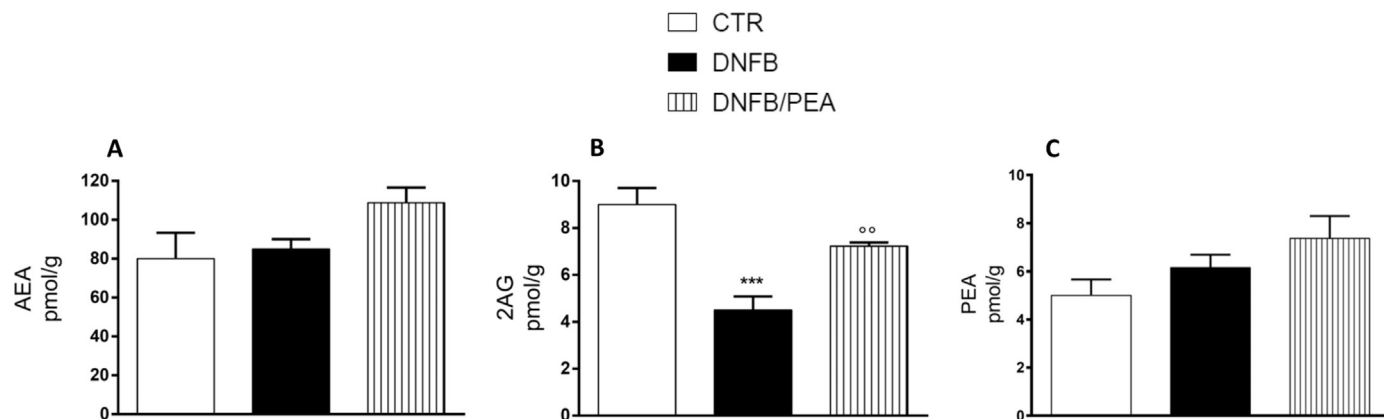


Fig. 2. Levels of the endocannabinoids, AEA (A) and 2-AG (B), and of PEA (C) in tissue homogenates of ears from mice sensitized with DNFB and un-challenged (CTR), challenged (DNFB), challenged and treated with PEA (5 mg/kg i.p.) (DNFB/PEA). The columns represent the means $\pm$ S.E.M. of 9 animals per group. DNFB vs CTRL *** $P < 0.001$; DNFB/PEA vs DNFB ** $P < 0.01$.

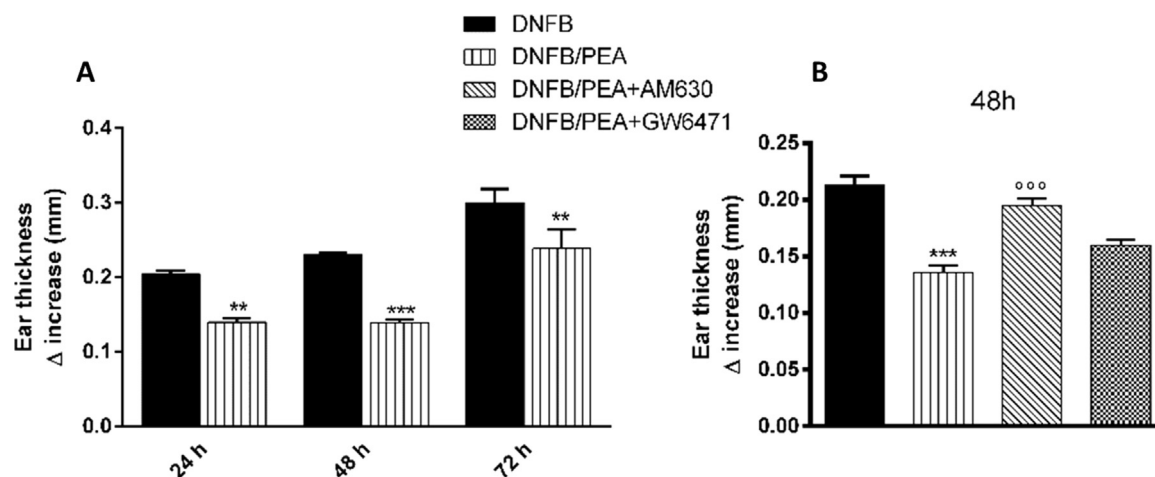


Fig. 3. (A) Time course of DNFB-induced CAD in mice, evaluated as Δ increase, i.e. the difference between the thickness of the right (DNFB-challenged) and left (*un*-challenged) ear of the same mouse 24, 48, 72 h after the 3rd challenge. The columns represent the mean $\pm$ S.E.M. of 7–12 animals per group. DNFB/PEA vs DNFB $^{**}P < 0.01$; $^{***}P < 0.001$. (B) Effect of AM630 (1 mg/kg i.p.), a CB₂ antagonist, and GW6471 (1 mg/kg i.p.), a PPAR- α antagonist, on PEA (5 mg/kg i.p.) inhibition of DNFB-induced CAD in mice. CAD was evaluated 48 h after the 3rd challenge. The columns represent the means $\pm$ S.E.M. of 7–12 animals per group. DNFB/PEA vs DNFB $^{***}P < 0.001$; DNFB/PEA+AM630 vs DNFB/PEA $^{ooo}P < 0.001$.

3.5. Effect of PEA on scratching

DNFB-sensitized mice showed, 48 h after the III challenge, increased scratching (measured for 1 h) of the DNFB-treated ear in comparison to the untreated one. PEA (5 mg/kg) treatment dramatically reduced this scratching behavior. This effect, similar to the other endpoints mentioned above, was reversed by co-administration with AM-630 (5 mg/kg). However, in this case, the effect of PEA was significantly attenuated also by GW6471 (Fig. 6).

4. Discussion

CAD is an inflammatory skin disease with high socio-economic

impact as it can profoundly affect the quality of life. The most used drugs are corticosteroids, whose use is limited by side effects often occurring after long-term treatments. Therefore, new pharmacological tools to approach these skin diseases are needed. In the present study, we focused our attention on the late (3rd) phase of CAD, which is characterized by allergic as well as chronic-inflammatory features, and on the possible therapeutic use of PEA. In fact, this compound, marketed for the treatment of chronic pain and inflammation in various disorders, because of its down-regulatory action on overactive MCs, is an endogenous anti-inflammatory, anti-nociceptive and anti-angiogenic mediator (D'Agostino et al., 2007; Cipriano et al., 2015). Here, we show that 2-AG levels are significantly reduced in ear skin from mice at the late stage of CAD, and that exogenously administered

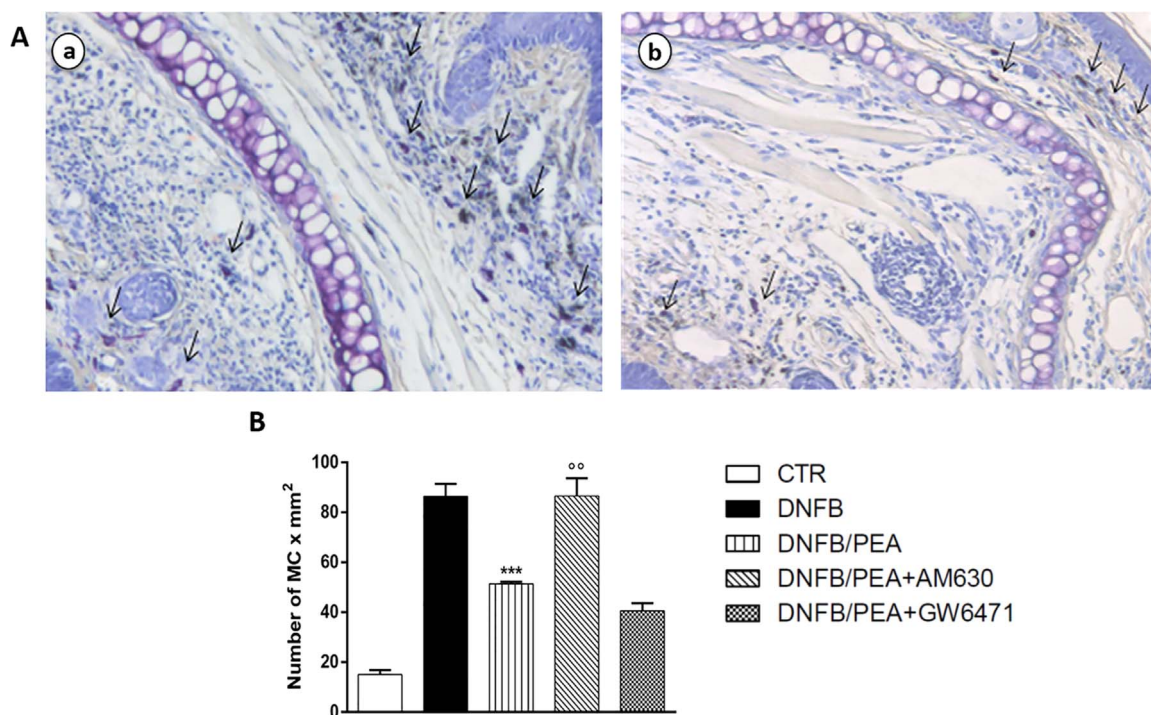


Fig. 4. Effect of PEA on the number of MCs. (A) Histological analysis of (a) DNFB-challenged ear tissue (DNFB) and (b) DNFB-challenged + PEA (DNFB/PEA) ear tissue sections stained with toluidine blue. Arrows indicate MCs. (B) MC number per area (mm²) in ear tissue of non-DNFB-challenged mice (white bar) or of DNFB-challenged mice treated with vehicle (black bar), PEA (5 mg/kg i.p., vertically striped bar), PEA + AM630 (1 mg/kg i.p., diagonal striped bar) or PEA + GW6471 (1 mg/kg i.p., checked bar). The bars represent the means $\pm$ S.E.M. of 7–12 animals per group. DNFB/PEA vs DNFB $^{***}P < 0.001$; DNFB/PEA+AM630 vs DNFB/PEA $^{ooo}P < 0.001$. At the doses used, AM630 and GW6471 were *per se* inactive (data not shown).

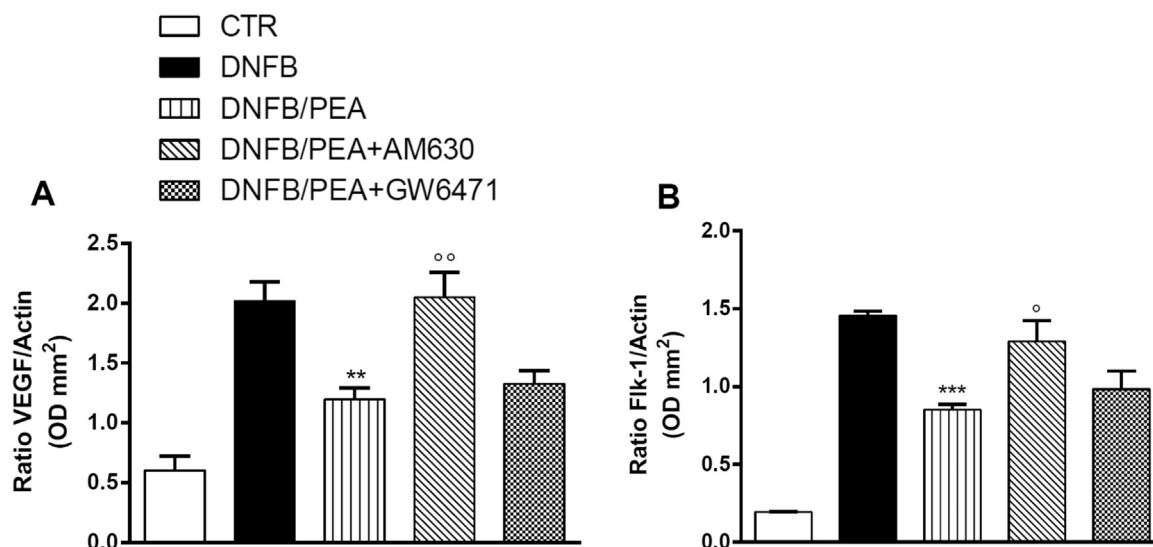


Fig. 5. Effect of PEA on (A) VEGF and (B) its receptor Flk-1 protein expression in ear tissue from DNFB-challenged mice treated with PEA (5 mg/kg i.p.) and PEA plus either AM630 (1 mg/kg i.p.) or GW6471 (1 mg/kg i.p.). The columns represent the means $\pm$ S.E.M. of 7–12 animals per group. DNFB/PEA vs DNFB *** P < 0.001 and ** P < 0.01; DNFB/PEA + AM630 vs DNFB/PEA ^{°°} P < 0.01 and [°] P < 0.05. At the doses used, AM630 and GW6471 were *per se* inactive (data not shown).

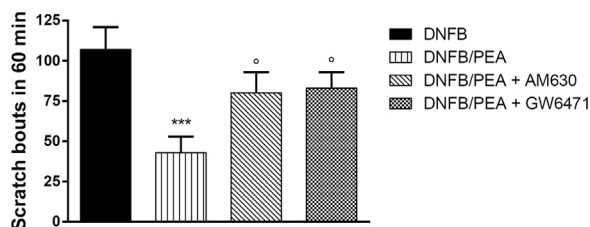


Fig. 6. Effect of PEA on ear scratching behavior in DNFB-induced CAD. DNFB-challenged mice were treated with PEA (5 mg/kg i.p.) with and without AM630 (1 mg/kg i.p.) or GW6471 (1 mg/kg i.p.). The columns represent the means $\pm$ S.E.M. of 12 animals per group. DNFB/PEA vs DNFB *** P < 0.001; DNFB/PEA + AM630 and DNFB/PEA + GW6471 vs DNFB/PEA [°] P < 0.05. At the doses used, AM630 and GW6471 were *per se* inactive (data not shown).

PEA is able to restore the normal levels of this anti-inflammatory endocannabinoid. Concomitantly, PEA reduces mice ear thickness and the number of MCs in the late phase of CAD. Thus, PEA anti-inflammatory effect is exhibited in the late phase of DNFB-induced CAD, in which MCs are deeply involved, whereas PEA exerts its anti-inflammatory effect in keratinocytes in the early (1st) phase (Petrosino et al., 2010b). Indeed, PEA has already been shown to possess the ability to reduce both acute and chronic inflammation by down-modulating activated MCs and through the inhibition of the release of pro-inflammatory mediators from these cells (Skaper et al., 2013; De Filippis et al., 2008a). It is interesting to note how the effects of exogenous PEA in CAD are due to: 1) indirect TRPV1 channel activation/desensitization in the 1st phase of CAD, when the endogenous levels of PEA as well as those of 2-AG in ear skin are increased (Petrosino et al., 2010b); 2) increased levels of 2-AG and indirect activation of CB₂ receptors (see below) in the 3rd phase of CAD (present study), when the endogenous levels of PEA are not significantly elevated and those of 2-AG are instead reduced.

CAD is characterized also by neo-angiogenesis, which is required to allow for the cellular trafficking necessary to sustain chronicity, and to provide oxygen and nutrients to damaged tissues (Colville-Nash et al., 1995). Accordingly, VEGF and its endothelial cell receptors (VEGFRs), which play a major role in angiogenesis, were shown to be involved in the pathogenesis of contact hypersensitivity skin reactions (Yamamoto et al., 2007). Our study, for the first time, demonstrates that PEA is able to inhibit the expression of VEGF and its receptor Flk-1 in the ear skin of DNFB-challenged mice.

Previous studies have reported that pruritus shares much of its

neural substrates and signaling characteristics with those of another nocifensive modality, that is pain. Many of the same receptor systems shown to modulate analgesia (*e.g.*, the serotonin and opioid systems) are being examined also for their involvement in itch, often within the framework of the anti-nociception-enhancing perception of itch (Paus et al., 2006). Here, we show that PEA is able also to significantly reduce ear scratching in DNFB-challenged mice, thus possibly contributing to counteract the vicious neurogenic inflammatory components leading to chronic CAD.

The action of PEA, on both ear thickness and related MC activation as well as neo-angiogenesis was counteracted by a CB₂ receptor antagonist but not by a PPAR α antagonist. Therefore, also in view of the observation that PEA increased 2-AG levels in the ear skin of DNFB-challenged mice, we propose that PEA exerts its anti-inflammatory and anti-angiogenic actions *via* indirect activation of CB₂ receptors due to a stimulatory effect on 2-AG levels. Accordingly, some of us have shown that PEA, *in vitro* and *in vivo*, strongly enhances 2-AG levels (Petrosino et al., 2016), and CB₂ receptor activation can reduce angiogenesis in models of skin inflammation (De Filippis et al., 2008b; Guabiraba et al., 2013), as well as during liver cirrhosis (Huang et al., 2012) and fibrosis (Reichenbach et al., 2012), or (Pisanti et al., 2009), including melanoma (Blázquez et al., 2006). These findings establish a link between MC-induced inflammation and angiogenesis in CAD as in other inflammatory disorders, and point to PEA as an ideal modulator of both processes. Interestingly, the anti-pruritogenic action of PEA is antagonized not only by a CB₂, but also by a PPAR α antagonist. This suggests that other non-MC-mediated components play a major role in this model of CAD-induced itch. It is likely that sensory neuron-mediated mechanisms of pain, whereupon PEA has been shown to act mostly *via* PPAR α (Lo Verme et al., 2005; Khasabova et al., 2012), participate in DNFB-induced itch, particularly during the 3rd phase, when MC-derived mediators sensitize these neurons. This would explain why the amelioration of DNFB-induced scratching behavior by PEA is antagonized by both AM630 and GW6471.

In conclusion, we have shown here that PEA, apart from counteracting the 1st phase of DNFB-induced CAD in mice *via* a keratinocyte-mediated mechanism, is also able, perhaps more importantly, to reduce the 3rd phase mostly *via* a MC-mediated mechanism. This makes of this compound a suitable treatment for CAD, and possibly other types of inflammatory skin diseases (Noli et al., 2015; Yuan et al., 2014).

Conflict of interest

SP is an employee of Epitech Group S.p.A. (Italy). The other authors declare that they have no conflict of interests.

Author Contributions

TI, VD, DDF and RC conceived and designed the experiments. MV, SP, LN and AG performed the experiments. MV, SP and DDF analyzed the data and prepared the figures. TI, RC and VD wrote the manuscript. All of the authors approved the final manuscript.

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