



Adelmidrol increases the endogenous concentrations of palmitoylethanolamide in canine keratinocytes and down-regulates an inflammatory reaction in an in vitro model of contact allergic dermatitis

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ABSTRACT

This study aimed to investigate potential new target(s)/mechanism(s) for the palmitoylethanolamide (PEA) analogue, adelmidrol, and its role in an in vitro model of contact allergic dermatitis. Freshly isolated canine keratinocytes, human keratinocyte (HaCaT) cells and human embryonic kidney (HEK)-293 cells, wild-type or transfected with cDNA encoding for *N*-acylethanolamine-hydrolysing acid amidase (NAAA), were treated with adelmidrol or azelaic acid, and the concentrations of endocannabinoids (anandamide and 2-arachidonoylglycerol) and related mediators (PEA and oleoylethanolamide) were measured. The mRNA expression of PEA catabolic enzymes (NAAA and fatty acid amide hydrolase, FAAH), and biosynthetic enzymes (*N*-acyl phosphatidylethanolamine-specific phospholipase D, NAPE-PLD) and glycerophosphodiester phosphodiesterase 1, was also measured. Brain or HEK-293 cell membrane fractions were used to assess the ability of adelmidrol to inhibit FAAH and NAAA activity, respectively. HaCaT cells were stimulated with polyinosinic-polycytidylic acid and the release of the pro-inflammatory chemokine, monocyte chemoattractant protein-2 (MCP-2), was measured in the presence of adelmidrol.

Adelmidrol increased PEA concentrations in canine keratinocytes and in the other cellular systems studied. It did not inhibit the activity of PEA catabolic enzymes, although it reduced their mRNA expression in some cell types. Adelmidrol modulated the expression of PEA biosynthetic enzyme, NAPE-PLD, in HaCaT cells, and inhibited the release of the pro-inflammatory chemokine MCP-2 from stimulated HaCaT cells. This study demonstrates for the first time an 'entourage effect' of adelmidrol on PEA concentrations in keratinocytes and suggests that this effect might mediate, at least in part, the anti-inflammatory effects of this compound in veterinary practice.

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Introduction

Chronic allergic skin diseases represent a growing burden, and more than 20% of the animals are referred to a veterinary doctor for a dermatological problem (Hill et al., 2006). Although topical anti-inflammatory treatments are considered the reference standard for managing focal or multifocal skin lesions in atopic dermatitis (Olivry et al., 2010), only few alternatives to topical steroids are available. The search for valuable treatment options to be safely applied for long

durations has recently focused on the cutaneous endocannabinoid system (Lambert, 2007; Biro et al., 2009; Kupczyk et al., 2009), including cannabinoid receptors, the endocannabinoids (anandamide and 2-arachidonoylglycerol), structurally similar bioactive amides (such as palmitoylethanolamide, PEA), and their biosynthetic and catabolic enzymes (Fezza and Maccarrone, 2014).

The main physiological function of the endocannabinoid system in the skin is to preserve the local homeostasis and the complex cellular dynamics (Biro et al., 2009). Recent studies have suggested the existence of the endocannabinoid system in the canine skin (Campora et al., 2012) and it has been shown that PEA concentrations in the skin change during canine atopic dermatitis (Abramo et al., 2014). Moreover, PEA was recently found to afford

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significant therapeutic benefit in dogs with experimental hypersensitivity (Cerrato et al., 2012a) and spontaneous atopic dermatitis (Noli et al., 2014).

Adelmidrol is an analogue of PEA. Unlike PEA, which is highly lipophilic, adelmidrol is suitable for topical application because it exhibits both hydrophilic and lipophilic features (Cerrato et al., 2012b). Adelmidrol is the di-ethanolamide derivative of azelaic acid, a natural substance, topically effective for human inflammatory skin disorders (Nazzaro-Porro, 1987) and able to modulate the inflammatory response in human keratinocytes (Mastrofrancesco et al., 2010).

Several mechanisms of action have been proposed for PEA (Petrosino et al., 2010a), including: (1) the direct stimulation of an as-yet uncharacterised cannabinoid type 2-like receptor (Conti et al., 2002; Farquhar-Smith and Rice, 2003); (2) an 'entourage effect' (Di Marzo et al., 2001; Petrosino et al., 2015); and (3) the direct stimulation of particular molecular targets (Lo Verme et al., 2005). By contrast, the mechanism of action of adelmidrol has been investigated only in part (De Filippis et al., 2009). Yet, topical application of adelmidrol alleviates chronic inflammatory skin conditions in both humans (Pulvirenti et al., 2007) and dogs (Mantis et al., 2007; Cerrato et al., 2012b; Fabbri and Leone, 2013).

The aim of the present study was to investigate the mechanism(s) of action of adelmidrol and its potential effect in an in vitro model of contact allergic dermatitis (CAD).

Materials and methods

Cell cultures and treatments

Canine keratinocytes, immortalised human keratinocytes (HaCaT) cells and human embryonic kidney (HEK)-293 cells, either wild-type (HEK-WT) or stably transfected with cDNA encoding for human recombinant *N*-acylethanolamine acid amidase (NAAA; HEK-NAAA), were cultured (Appendix: Supplementary material).

Keratinocytes (9×10^4 cells/cm²) were treated with adelmidrol (10 μ M, Epitech Group) or vehicle (methanol 0.05%, Ctrl) for 24 h, or also with azelaic acid (10 μ M) for 24 h (HaCaT), at 37 °C with 5% CO₂. To investigate if increased PEA concentrations could be controlled by NAAA, PEA concentrations were measured in HEK-NAAA cells and compared to HEK-WT cells after adelmidrol treatment (10 μ M) for 40 min and 24 h. The resulting cells and supernatants were processed for the quantification of anandamide (AEA), 2-arachidonoylglycerol (2-AG), and PEA and oleylethanolamide (OEA).

Quantification of AEA, 2-AG, PEA, and OEA concentrations

Cells and supernatants were homogenised in a solution containing 10 pmol of [³H]₈-AEA and 5 pmol of [³H]₅-2-AG, [³H]₄-PEA and [³H]₂-OEA. The lipid-containing organic phase was pre-purified by open-bed chromatography on silica gel (Bisogno et al., 1997) and analysed by LC-APCI-MS (Marsicano et al., 2002). AEA, 2-AG, PEA and OEA amounts (pmol) were normalised per mg of extracted lipids (Appendix: Supplementary material).

NAAA assay

HEK-NAAA and HEK-WT cells were homogenised in Tris-HCl 20 mM (pH 7.4) and centrifuged at 800 g for 10 min and at 12,000 g for 30 min at 4 °C, in sequence. The 12,000 g pellet (membranes) was suspended in phosphate-buffered saline (PBS; pH 7.4), subjected to two cycles of freezing and thawing and allowed to react with [1,2-¹⁴C]-*N*-palmitoylethanolamine (Saturnino et al., 2010) in a solution containing vehicle or increasing concentrations of adelmidrol (Appendix: Supplementary material). The quantification of [1,2-¹⁴C]-ethanolamine was carried out by using a liquid scintillation analyser.

Fatty acid amide hydrolase (FAAH) assay

AEA hydrolysis was measured by incubating the 10,000 g membrane fraction of whole rat brain with *N*-arachidonoyl-[¹⁴C]-ethanolamine and vehicle or increasing the concentrations of adelmidrol (Appendix: Supplementary material). After incubation, the reaction was terminated as described for the NAAA assay.

Real-time PCR

The effect of adelmidrol on the mRNA expression of catabolic and biosynthetic proteins was studied by comparison of absolute transcriptional expression of FAAH,

Table 1

Primer sequences of FAAH, NAPE-PLD, NAAA and GDE-1 enzymes.

Enzymes	Forward primer	Reverse primer
FAAH	5'-TCAGAGAAGAGGTCTACAC-3'	5'-GAGGGCATGGTATAGTTG-3'
NAPE-PLD	5'-GTCCTTATCAGTCACAAC-3'	5'-CCATCTCACTCATTACC-3'
NAAA	5'-TTAAGAATGGGCAGATT-3'	5'-CCTTTATCTCGTTCATCA-3'
GDE-1	5'-ACAGACTCAGGAATGATT-3'	5'-AGACCACACTATTATTATACAG-3'
RNApol	5'-AACCAGAAGCGAATCACC-3'	5'-AACGGCGAATGATGATGG-3'
β2M	5'-GTGTGAACCATGTGACTT-3'	5'-GCATCTTCAACCTCCAT-3'

FAAH, fatty acid amide hydrolase; NAPE-PLD, *N*-acyl phosphatidylethanolamine-specific phospholipase D; NAAA, *N*-acylethanolamine-hydrolysing acid amidase; GDE-1, glycerophosphodiester phosphodiesterase-1; RNApol, RNA polymerase II subunit; β2M, β-2-microglobulin.

NAAA, *N*-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) and glycerophosphodiester phosphodiesterase 1 (GDE1) in HaCaT and HEK-WT cells vs. the expression of these enzymes after adelmidrol treatment. In HEK-NAAA cells, only NAAA expression was investigated after adelmidrol treatment.

Total RNA was purified, quantified and reverse transcribed as previously described (Grimaldi et al., 2009). Quantitative real-time PCR was performed (Appendix: Supplementary material). For each target, all mRNA sequences¹ were aligned and common primers were designed (Table 1).

Cell viability

Cell viability was measured in HaCaT, HEK-WT and HEK-NAAA cells treated with adelmidrol (10 μ M) or vehicle and using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay (Appendix: Supplementary material).

CAD in HaCaT cells

We also investigated if the effect of adelmidrol on PEA and 2-AG concentrations could result in an anti-inflammatory action in an in vitro model of CAD, a skin disorder that commonly affects dogs. As primary canine keratinocytes are not amenable to multiple pharmacological experiments, we used HaCaT cells for this purpose.

HaCaT cells (1×10^5 cells/cm²) were stimulated with polyinosinic-polycytidylic acid (poly-[I:C]; 100 μ g/mL, Invivogen), or vehicle (water) and incubated for 6 h at 37 °C with 5% CO₂. Poly-[I:C]-stimulated HaCaT cells were treated with either vehicle (methanol 0.05%), adelmidrol (10, 50, and 100 μ M), or PEA (10 μ M) and incubated for the indicated times. After 6 h the supernatants were used for ELISA.

ELISA

The human monocyte chemotactic protein-2 (MCP-2) concentrations were measured from cell supernatants derived from poly-[I:C]-stimulated HaCaT cells, in the presence of vehicle or adelmidrol or PEA, using the human MCP-2 ELISA kit protocol and according to the manufacturer's instructions (RayBiotech).

Results

Effect of adelmidrol on endocannabinoids and related compounds in keratinocytes

The concentrations of PEA and 2-AG were significantly increased in canine keratinocytes treated with adelmidrol (10 μ M for 24 h) when compared to vehicle. No significant effect was observed on AEA and OEA (Fig. 1).

Adelmidrol (10 μ M) also induced a significant increase in PEA concentrations in HaCaT cells after 24 h, without altering the concentrations of the other mediators (Fig. 2A). In HaCaT cells treated with azelaic acid (10 μ M for 24 h), no significant effect on AEA, 2-AG, PEA and OEA concentrations was observed (Fig. 2B).

Adelmidrol exhibited a bell-shaped dose-response curve since no significant effects on PEA concentrations were found when the compound was tested at 1, 20 and 50 μ M (data not shown).

¹ See: <http://www.ncbi.nlm.nih.gov/gene/> (accessed 31 October 2015).

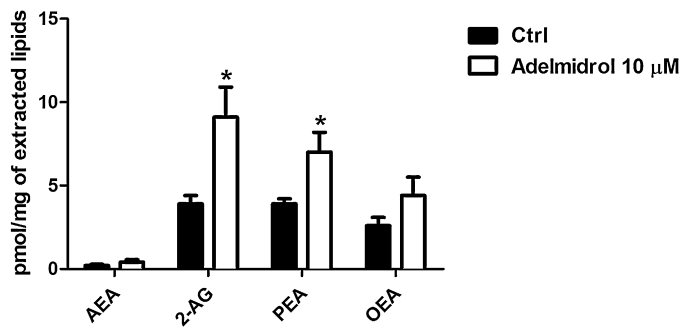


Fig. 1. Endogenous concentrations of anandamide (AEA), 2-arachidonoylglycerol (2-AG), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) in canine keratinocytes treated with adelmidrol 10 µM or vehicle (Ctrl) for 24 h. Data are means $\pm$ SE of $n = 10$ separate determinations. * $P < 0.05$ for Ctrl vs. Adelmidrol 10 µM. Student's t test was used.

Effect of adelmidrol on PEA concentrations in HEK-WT and HEK-NAAA cells

PEA concentrations were significantly increased in HEK-WT cells treated with adelmidrol 10 µM, compared to vehicle, although only after 24 h (Fig. 3A). Again, no significant effect on PEA concentrations was found with adelmidrol at 1, 20, and 50 µM (data not shown). In HEK-NAAA cells, baseline PEA concentrations were significantly lower than in HEK-WT cells, in agreement with the role of active NAAA in the tonic degradation of this mediator. PEA concentrations were significantly decreased in HEK-NAAA cells treated

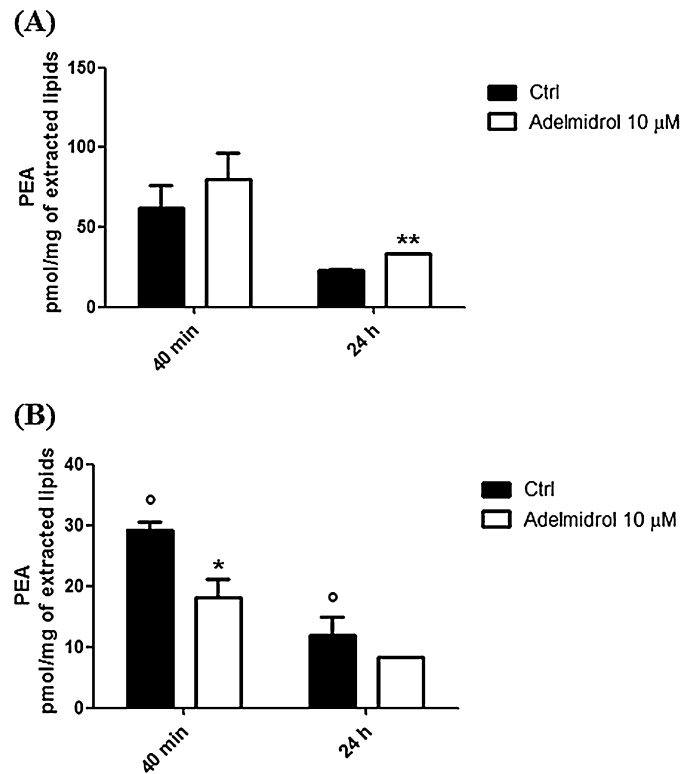


Fig. 3. (A) Endogenous concentrations of palmitoylethanolamide (PEA) in human embryonic kidney wild-type (HEK-WT) cells treated with Adelmidrol 10 µM or vehicle (Ctrl) for 40 min and 24 h. (B) Endogenous concentrations of PEA in HEK cells stably transfected with *N*-acylethanolamine hydrolysing acid amidase (NAAA) cDNA (HEK-NAAA), treated with Adelmidrol 10 µM or vehicle (Ctrl) for 40 min and 24 h. Data are means $\pm$ SE of $n = 6$ separate determinations. ** $P < 0.01$; * $P < 0.05$ for Ctrl vs. Adelmidrol 10 µM. ° $P < 0.05$ vs. corresponding baseline concentrations in HEK-WT cells. One-way ANOVA was used followed by 'Newman-Keuls multiple comparison test'.

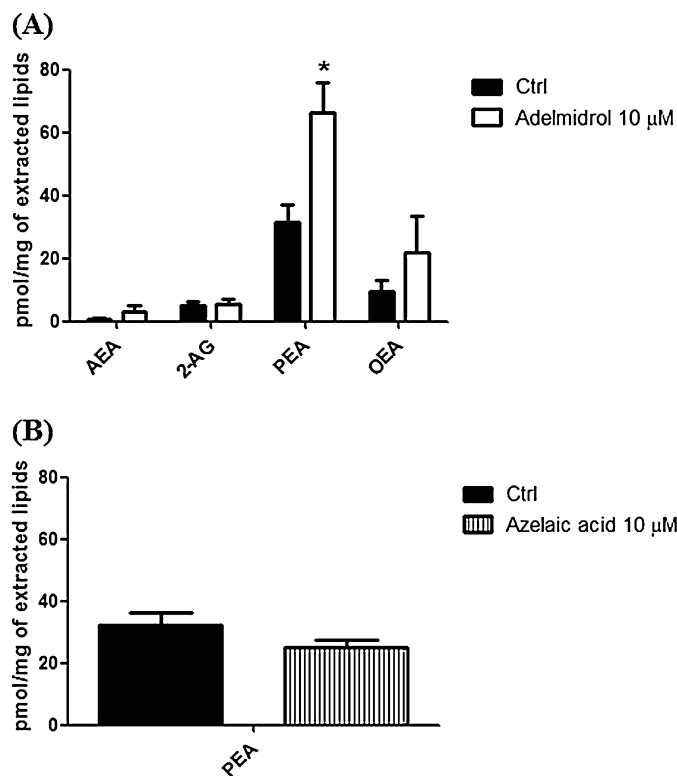


Fig. 2. (A) Endogenous concentrations of anandamide (AEA), 2-arachidonoylglycerol (2-AG), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) in human keratinocyte (HaCaT) cells treated with adelmidrol 10 µM or vehicle (Ctrl) for 24 h. Data are means $\pm$ SE of $n = 6$ separate determinations. * $P < 0.05$ for Ctrl vs. Adelmidrol 10 µM. Student's t test was used. (B) Endogenous concentrations of PEA in HaCaT cells treated with azelaic acid 10 µM or Ctrl for 24 h. Data are means $\pm$ SE of $n = 6$ separate determinations.

with adelmidrol for 40 min and there was a trend ($P = 0.07$) after 24 h, compared to vehicle (Fig. 3B).

Effect of adelmidrol on the activity of PEA catabolic enzymes

Enzymatic assays performed in rat brain and HEK cell membrane fractions showed no effect of adelmidrol on FAAH and NAAA activity, respectively (Table 2).

Effect of adelmidrol on the mRNA expression of PEA catabolic and biosynthetic enzymes

In terms of absolute transcriptional expression, HaCaT and HEK-WT cells showed a strong expression of GDE-1 (about 22–24 Cq), a moderate and comparable expression of both FAAH and NAPE-PLD

Table 2
Lack of significant inhibition of FAAH and NAAA activity by adelmidrol.

IC ₅₀ on FAAH ^a	Max tested on FAAH (% of inhibition ^b)	IC ₅₀ on NAAA ^c	Max tested on NAAA (% of inhibition ^b)
>10 µM	10 µM (11.4 $\pm$ 1.7)	>10 µM	10 µM (21.2 $\pm$ 3.2)

IC₅₀, half maximal inhibitory concentration; FAAH, fatty acid amide hydrolase; NAAA, *N*-acylethanolamine-hydrolysing acid amidase; HEK-NAAA, human embryonic kidney cells stably transfected with NAAA cDNA.

^a Rat brain membranes.

^b Data are means $\pm$ SD of $n = 3$ separate determinations and are expressed as maximum inhibition observed at a 10 µM concentration.

^c HEK-NAAA cells.

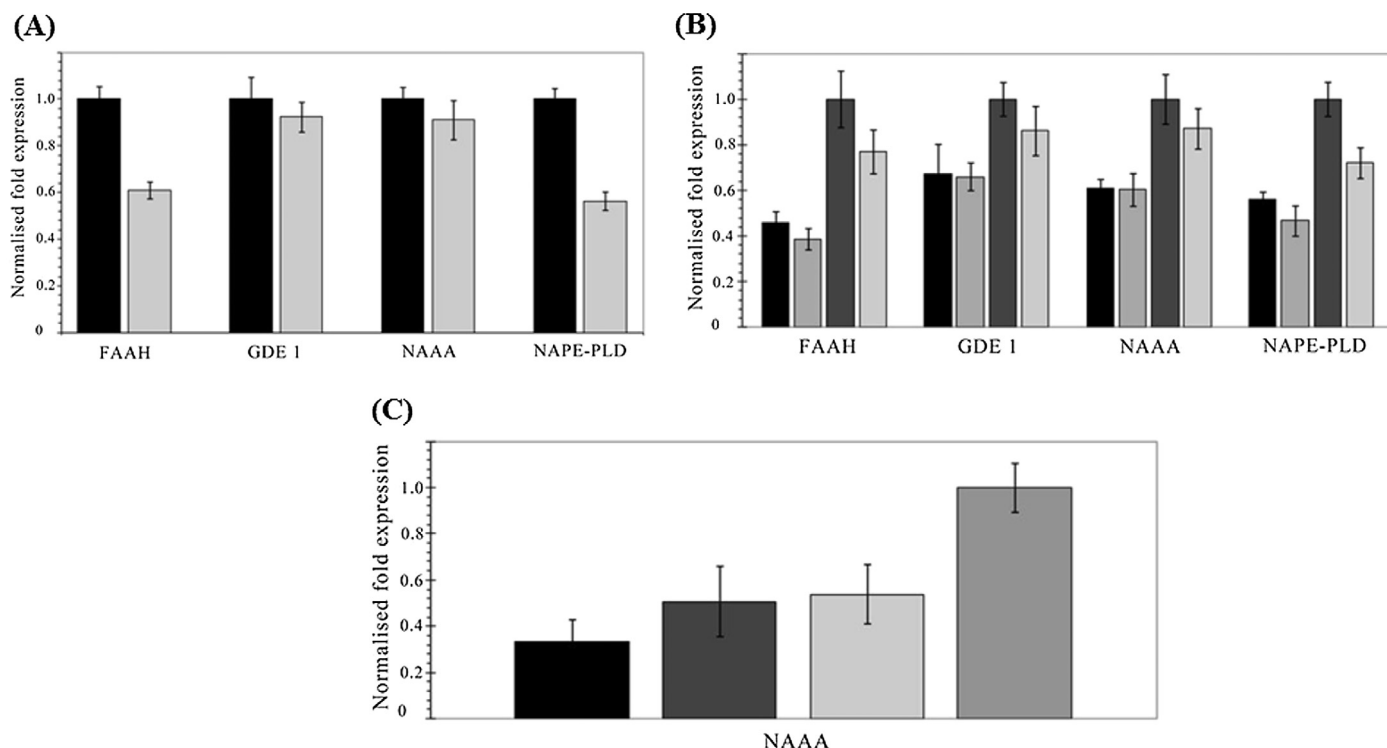


Fig. 4. (A) Normalised fold expression of fatty acid amide hydrolase (FAAH), glycerophosphodiester phosphodiesterase-1 (GDE-1), *N*-acylethanolamine hydrolysing acid amidase (NAAA) and *N*-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) mRNAs in human keratinocyte (HaCaT) cells. Real-time PCR analysis was performed in cells treated by vehicle or by Adelmidrol for 24 h of cell culture. For all the targets relative expression was scaled relative to the control condition put = 1. (B) Normalised fold expression of FAAH, GDE-1, NAAA and NAPE-PLD mRNAs in human embryonic kidney wild-type (HEK-WT) cells. Real-time PCR analysis was performed in cells treated by vehicle or by Adelmidrol for 40 min of cell culture and in cell treated by vehicle or by Adelmidrol for 24 h of culture. For all the targets relative expression was scaled relative to the highest condition considered as = 1. (C) Normalised fold expression of NAAA mRNA in HEK cells stably transfected with NAAA cDNA (HEK-NAAA). Real-time PCR analysis was performed in cells treated by vehicle or by Adelmidrol for 40 min of cell culture and in cell treated by vehicle or by Adelmidrol for 24 h of cell culture. Relative expression was scaled relative to the highest condition considered as = 1.

(about 27–29 Cq), and only a very faint expression of NAAA (over 30 Cq; Figs. 4A, B). This suggests that PEA biosynthesis in both HaCaT and HEK-WT cells is mostly due to GDE-1, whereas PEA inactivation is likely mediated mostly by FAAH.

HaCaT cells cultured for 24 h with adelmidrol showed no alteration of NAAA or GDE-1 mRNA concentrations, whereas both FAAH and NAPE-PLD mRNAs were significantly down-regulated by adelmidrol (Fig. 4A), thus potentially explaining the stimulatory effect on PEA concentrations (Fig. 1), considering that NAPE-PLD is not the most important PEA biosynthetic enzyme in these cells. In HEK-WT cells, an up-regulation of FAAH and, to a lesser extent, NAAA mRNA concentrations were observed as a function of culture time (Fig. 4B). These data parallel the observed PEA decrease (Fig. 3A) as a function of culture time. Adelmidrol did not significantly modify these concentrations, thus suggesting that in these cells its effect on PEA concentrations is not due to alterations in biosynthetic or catabolic enzyme expression.

In HEK-NAAA cells, although these cells already significantly overexpressed the enzyme (data not shown), a further up-regulation of NAAA was observed after 24 h adelmidrol treatment (Fig. 4C), which might explain in part why the compound tended to reduce PEA concentrations in these cells at this time point. These data suggest a possible interference of adelmidrol in the mechanisms that regulate the transcriptional concentrations of NAAA in HEK-NAAA cells.

Effect of adelmidrol on cell viability

No cytotoxic effect of adelmidrol was observed at any times and in any of the cell types used (Fig. 5).

Adelmidrol inhibits the release of MCP-2 in HaCaT cells

Adelmidrol (10 and 50 μ M) strongly reduced MCP-2 concentrations (Fig. 6). The same effect was observed when poly-(1:C)-challenged HaCaT cells were co-stimulated with PEA 10 μ M (Fig. 6). No effect was observed on MCP-2 release in non-challenged HaCaT cells (Fig. 6).

Discussion

The main findings of the present study are that adelmidrol increases PEA and 2-AG concentrations in canine keratinocytes and inhibits inflammatory response in keratinocytes. Since both PEA and 2-AG exert anti-inflammatory and anti-allergic effects in the skin (Karsak et al., 2007; Petrosino et al., 2010b; Cerrato et al., 2012a; Kendall and Nicolaou, 2013), these results suggest that adelmidrol potentiates an inherent cellular protective mechanism by increasing the availability of endogenous anti-inflammatory lipids. This mechanism is particularly interesting as it has potential applications in the topical treatment of canine inflammatory, chronic skin diseases associated with allergies. Currently, glucocorticoid creams and tacrolimus ointment are the main options available and, although effective, they are associated with adverse events (e.g. cutaneous atrophy) and slow onset of response, respectively. The bio-pharmacological pathway reported herein might underlie a natural disease-oriented mechanism for adelmidrol and suggests its potential use within the topical management of allergic-inflammatory skin conditions in dogs.

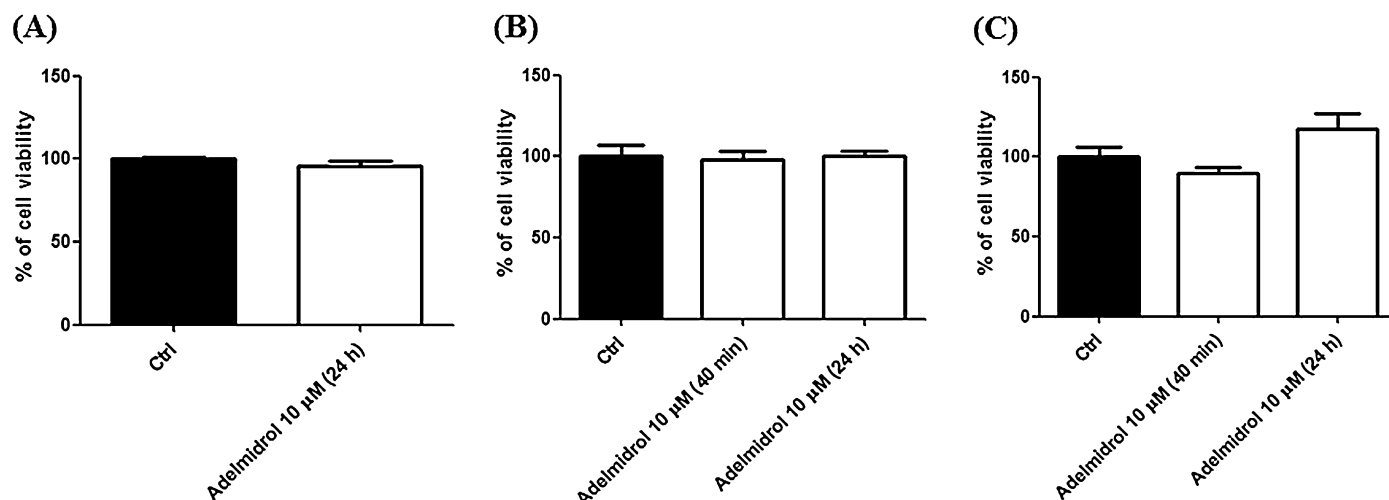


Fig. 5. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay in (A) human keratinocyte (HaCaT) cells, (B) human embryonic kidney wild-type (HEK-WT) cells and (C) HEK cells stably transfected with *N*-acylethanolamine hydrolysing acid amidase (NAAA) cDNA (HEK-NAAA), treated with Adelmidrol 10 µM or vehicle (Ctrl) for 40 min and 24 h. Data are means $\pm$ SE of $n = 6$ separate determinations.

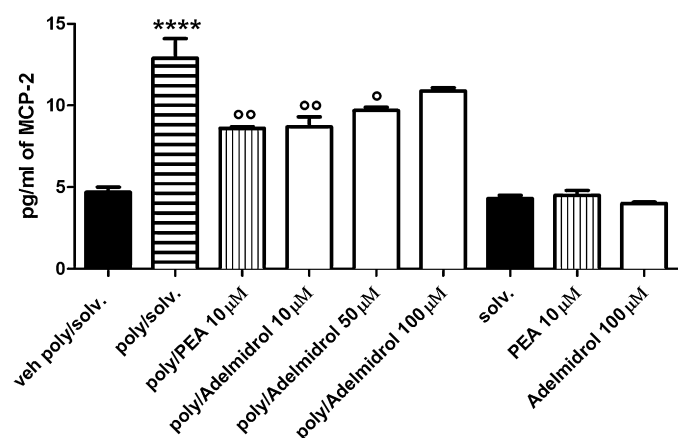


Fig. 6. ELISA for monocyte chemoattractant protein-2 (MCP-2) in the supernatants of polyinosinic-polycytidylic acid (poly-[I:C])-stimulated human keratinocyte (HaCaT) cells (100 µg/mL, poly/solvent) in presence of vehicle or Adelmidrol (10, 50, and 100 µM) or PEA (10 µM) for 6 h. Data are means $\pm$ SE of $n = 6$ separate determinations. **** $P < 0.0001$ for veh poly/solvent vs. poly/solvent; °° $P < 0.01$ for poly/solvent vs. poly/PEA 10 µM and poly/Adelmidrol 10 µM; ° $P < 0.05$ for poly/solvent vs. poly/Adelmidrol 50 µM. Data are expressed as pg/mL of MCP-2 and one-way ANOVA was used followed by 'Newman-Keuls multiple comparison test'.

Adelmidrol has proven therapeutic benefit in allergic skin conditions. A complete resolution in 80% of the children affected by mild atopic dermatitis, and a significantly reduced antigen-induced skin wheal response in spontaneous hypersensitive Beagle dogs, were observed following topical treatment with adelmidrol, for a time period of 4 weeks and 6 days, respectively (Pulvirenti et al., 2007; Cerrato et al., 2012b). Moreover, privately-owned dogs with atopic dermatitis and chronic pruritus (i.e. lasting longer than 4 weeks) treated for 30 days with topical adelmidrol showed a statistically significant decrease in pruritus severity and erythema, and a better quality of life (Fabbrini and Leone, 2013).

Adelmidrol belongs to the family of aliamides, the most studied member of which is PEA (Re et al., 2007). The first hypothesis to explain the mechanism of action of PEA and related aliamides was formulated in the mid-1990s, when the ALIA acronym (autacoid local injury antagonism) was introduced to indicate that these endogenous *N*-acylethanolamines are locally antagonistic to cell injury

processes (Aloe et al., 1993; Levi-Montalcini et al., 1996). In particular, it was suggested that aliamides protect against injury through the down-modulation of excessive mast cell degranulation (Aloe et al., 1993; Mazzari et al., 1996). In dogs and cats, hyper-reactive mast cells have been repeatedly confirmed as one of the main cellular targets of PEA (Scarpella et al., 2001; Abramo et al., 2006; Miolo et al., 2006; Re et al., 2007). In experimental settings, PEA was also shown to control the inflammatory response of other cell types (Petrosino et al., 2010b; Esposito and Cuzzocrea, 2013; Skaper et al., 2014a). Furthermore, multiple mechanisms of action have been reported for PEA, which is now considered a multi-target lipid mediator with anti-inflammatory and pain-relieving properties (Maione et al., 2013; Skaper et al., 2014b).

Little is known about the mechanism(s) of action of adelmidrol. Mast cell down-modulation is the only mechanism reported until now for this compound in both dogs (Abramo et al., 2008; Cerrato et al., 2012b) and experimental animals (De Filippis et al., 2009). In the present study, we investigated new possible target(s)/mechanism(s) for adelmidrol and demonstrated, for the first time, that this aliamide is able to act on human and canine keratinocytes *in vitro*.

In particular, the concentrations of both PEA and 2-AG increased in response to adelmidrol in canine keratinocytes, while only PEA concentrations were increased in human keratinocytes. This species difference in response agrees with a recent observation by some of us (Petrosino et al., 2015) that PEA elevates 2-AG concentrations strongly in canine keratinocytes and significantly less efficiently in HaCaT cells. Based on the present findings, we speculate that the ability of adelmidrol to increase PEA concentrations in both human and canine keratinocytes, as well as 2-AG concentrations in canine keratinocytes, represents the so-called 'entourage effect'. Also, PEA was shown to exert an entourage effect, by being able to increase the endogenous concentrations of AEA (Di Marzo et al., 2001) and 2-AG (Petrosino et al., 2015). Thus, PEA and adelmidrol seem to share similar mechanisms. Conversely, we observed that azelaic acid was unable to increase the concentrations of PEA in HaCaT cells. As a result, the higher concentrations of PEA in response to the treatment with adelmidrol could only be attributable to this compound and not to its possible hydrolysis to azelaic acid.

Since it is well known that PEA inactivation to palmitic acid and ethanolamine can be catalysed specifically by NAAA (Ueda et al.,

1999), but also by FAAH (Cravatt et al., 1996), we investigated whether the increase of PEA concentrations, observed in response to adelmidrol treatment, depended on changes of the activity or expression of these two catabolic enzymes, or on the expression of PEA biosynthetic and catabolic enzymes. Therefore, we first measured the concentrations of PEA in HEK-NAAA cells compared to HEK-WT cells and then tested adelmidrol as possible inhibitor of the expression and activity of such catabolic enzymes. We found that, as in keratinocytes, PEA concentrations were increased in HEK-WT cells treated with adelmidrol. In contrast, PEA concentrations were decreased in HEK-NAAA cells after treatment with the compound, possibly due to the stimulatory action of adelmidrol on NAAA expression observed in this study. However, no direct inhibitory activity of adelmidrol was observed on NAAA and FAAH enzymatic activity. These findings indicate that adelmidrol is able to increase the endogenous concentrations of PEA in several cell types and show that the 'entourage effect' can be under negative control by, but does not depend on changes of, the main enzyme catalysing PEA inactivation, i.e. NAAA.

It is possible that, in HaCaT cells, the stimulatory effect of adelmidrol on PEA concentrations might have been due its down-regulation of another degradative enzyme for PEA, i.e. FAAH. On the other hand, in these cells the compound did not alter the concentrations of NAAA mRNA, which were considerably lower than those of FAAH pre-treatment. Adelmidrol also did not affect the expression of GDE-1, which, of the two main *N*-acylethanolamine biosynthetic enzymes (Okamoto et al., 2004), was the one most strongly expressed in HaCaT cells. This suggests that the 'entourage effect' exerted by adelmidrol on PEA concentrations in these cells does not depend on the stimulation of PEA biosynthetic pathways.

Finally, our group has recently reported the role of PEA in reducing allergic inflammation both in dogs (Cerrato et al., 2012a; Noli et al., 2014) and experimental models of CAD (Petrosino et al., 2010b). In particular, we found that PEA down-regulates the expression and concentrations of the inflammatory chemokine, MCP-2, in HaCaT cells when stimulated with poly-(I:C) (an injury mimic agonist of the toll-like receptor 3; Petrosino et al., 2010b). Based on these results, we also investigated whether adelmidrol is able, like PEA, to inhibit the allergic inflammatory response in keratinocytes. Indeed, adelmidrol reduced MCP-2 concentrations in stimulated HaCaT cells and, most importantly, this effect was comparable to that observed in response to PEA. Chemokines play a key role in the pathogenesis of canine and human atopic dermatitis (Maeda et al., 2002; Narbutt et al., 2009). The ability of adelmidrol to reduce MCP-2 release from activated keratinocytes might underlie the beneficial effects observed in dogs with experimental and spontaneous atopic dermatitis following the topical application of this compound (Cerrato et al., 2012b; Fabbri and Leone, 2013).

Our data suggest that adelmidrol reduces inflammatory responses in the skin, such as chemokine production following allergic stimulation, partially through the increase in endogenous concentrations of the anti-inflammatory compound PEA, demonstrated here for the first time. Thus, adelmidrol might exert an anti-inflammatory effect by acting as an 'entourage compound'.

Conclusions

Although the exact mechanism of action of adelmidrol is still not completely understood, our study has provided important new data. In particular, we have shown that adelmidrol: (1) increases PEA concentrations in canine and human keratinocytes ('entourage effect'), this effect not being due to its hydrolysis to azelaic acid; (2) is not an inhibitor of NAAA and FAAH, although, under certain circumstances, it can modulate the expression of these two PEA catabolic enzymes and the biosynthetic enzyme, NAPE-PLD; (3) is not a cy-

tototoxic compound; and (4) reduces the concentrations of MCP-2 in stimulated keratinocytes. These data substantiate the use of adelmidrol-containing topical preparations (currently available in the human and veterinary market) as adjuncts in the treatment of allergic-inflammatory disorders, especially of dermatologic nature.

Conflict of interest statement

SP, MF and MA are employees of Epitech Group. MFdV is a scientific cofounder of and consultant for Innovet Italia. RV receives unrestricted research support from Epitech Group. None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.tvjl.2015.10.060.

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1 **Supplementary material**

2 *Drugs, chemicals and reagents*

3 Adelmidrol was provided from Epitech Group. Cell culture media, antibiotics and azelaic
4 acid were purchased from Sigma-Aldrich. Deuterated standards were purchased from Cayman
5 Chemicals. Polyinosinic-polycytidylic acid (poly-[I:C]) was purchased from Invivogen. The human
6 Monocyte Chemotactic Protein-2 (MCP-2) ELISA kit was purchased from RayBiotech.

7

8 *Cell cultures and treatments*

9 Canine keratinocytes were isolated from skin fresh biopsies obtained from surgeries due to
10 other purposes, in healthy dogs at the Veterinary Hospital of the Universitat Autònoma de
11 Barcelona. Biopsies were cut in small fragments and enzymatically digested with 0.05% trypsin-
12 0.02% Ethylene-Diamine-Tetraacetic Acid (EDTA) solution during 30 min at 37 °C. Trypsin
13 supernatant was filtered twice with cell strainers (100 µm and 40 µm pore size, respectively, BD
14 Biosciences) and centrifuged at 300 *g* for 5 min to recover canine keratinocytes. Canine
15 keratinocytes (1×10^6 cells/cm²) were cultured in Dulbecco's Modified Eagle Medium
16 (DMEM)/Ham's F12 (3:1) supplemented with hydrocortisone (0.4 µg/mL), insulin (5 µg/mL),
17 adenine (24 µg/mL), triiodothyronine (1.3 ng/mL), cholera toxin (7 ng/mL), epidermal growth
18 factor (0.01 µg/mL) and 10% Fetal Calf Serum (FCS) in an humidified 5% CO₂ atmosphere at 37
19 °C.

20

21 Immortalised human keratinocytes (HaCaT) cells were purchased from CLS GmbH and
22 cultured in DMEM supplemented with glutamine (2 mM), penicillin (400 U/mL), streptomycin (50
23 mg/mL) and 10% Fetal Bovine Serum (FBS) in an humidified 5% CO₂ atmosphere at 37 °C.

24

Human embryonic kidney (HEK)-293 cells, either wild-type (HEK-WT) or stably transfected with cDNA encoding for human recombinant *N*-acylethanolamine acid amidase (NAAA) (HEK-NAAA), were a kind gift of Allergan Inc. HEK-WT and HEK-NAAA cells were cultured in Eagle's Minimum Essential Medium (EMEM) medium supplemented with glutamine (2 mM), penicillin (400 U/mL), streptomycin (50 mg/mL) and 10% FBS in an humidified 5% CO₂ atmosphere at 37 °C. Transfected cells were selected using blasticidin (2 mM).

31

Quantification of AEA, 2-AG, PEA and OEA levels

Cells and supernatants were homogenised in a solution of chloroform/methanol/Tris-HCl 50 mM pH 7.4 (2:1:1 by vol.) containing 10 pmol of [²H]₈-AEA and 5 pmol of [²H]₅-2-AG, [²H]₄-PEA and [²H]₂-OEA as internal deuterated standards. The lipid-containing organic phase was pre-purified by open-bed chromatography on silica gel (Bisogno et al., 1997) and fractions obtained by eluting the column with a solution of chloroform/methanol (90:10 by vol.) were analysed by liquid chromatography-atmospheric pressure chemical ionisation-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.

41

LC-APCI-MS analyses of AEA, 2-AG, PEA and OEA were carried out in the selected ion monitoring (SIM) mode (Marsicano et al., 2002), using *m/z* values of molecular ions +1 for deuterated and undeuterated compounds, respectively as follows: 356 and 348 (AEA), 384.35 and 379.35 (2-AG), 304 and 300 (PEA), 328 and 326 (OEA). AEA, 2-AG, PEA and OEA levels were calculated on the basis of their area ratio with the internal deuterated standard signal areas and their amounts (pmol) were normalised per mg of extracted lipids.

48

NAAA assay

The membranes were used as the enzyme preparation to be used in the assay. The enzyme preparation (50 µg/sample) was allowed to react at 37 °C for 30 min with 100 µM [1,2-¹⁴C]-*N*-palmitoylethanolamine (10,000 counts per minute (cpm)/sample) (Saturnino et al., 2010) in a solution of citrate/sodium phosphate 50 mM (pH 5.29 and 0.1% Triton X-100, containing vehicle or increasing concentrations of adelmidrol. The reaction was terminated by the addition of chloroform/methanol (1:1 by vol.) and the quantification of [1,2-¹⁴C]-ethanolamine was carried out by using Liquid Scintillation Analyser (TRI-carb 2100TR).

Fatty acid amide hydrolase (FAAH) assay

AEA hydrolysis was measured by incubating the 10,000 g membrane fraction of whole rat brain (70 µg/sample) in Tris-HCl 50 mM, at pH 9.0-10.0 at 37 °C for 30 min, with *N*-arachidonoyl-[¹⁴C]-ethanolamine (110 mCi/mmol, ARC) properly diluted with unlabelled AEA (Tocris Bioscience), containing vehicle or increasing concentrations of adelmidrol.

Real-time PCR

For all samples tested, the RNA integrity number (Bionalyzer 2100, Agilent) was greater than 8 relative to a 0–10 scale. Quantitative Real-time PCR was performed by an iCycler-iQ5 in a 20 µL reaction mixture as described using 20 ng of cDNA. Assays were performed in quadruplicate (maximum ΔCt of replicate samples < 0.5) and a standard curve from consecutive five-fold dilutions (100 to 0.16 ng) of a cDNA pool representative of all samples was included for PCR efficiency determination. Optimised primers for SYBR-green analysis and optimum annealing temperatures were designed by the Allele-Id software version 7.0 (Biosoft International) and were synthesised (HPLC-purification grade) by MWG-Biotech.

74 Relative expression calculation was corrected for PCR efficiency, normalised with respect to
75 the reference genes RNA polymerase II subunit and β -2-microglobulin and performed by the iQ5
76 software. Results are expressed as fold expression compared with a reference condition (=1).

77

78 *Cell viability*

79 Cell viability was measured using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium
80 bromide (MTT) colorimetric assay. Briefly, after treatment all cells were incubated with MTT (5
81 mg/mL) at 37 °C in 5% CO₂ for 3 h. After 3 h, all cells were lysed with dimethyl sulfoxide (1 mL)
82 and incubated at 37 °C in 5% CO₂ for 6 h. After 6 h, the absorbance was read at 630 nm. Results are
83 expressed as % of cell viability, where optical density values from vehicle-treated cells were
84 defined as 100% of cell viability.