Cannabinoid receptor types 1 and 2 and perixosome proliferator-activated receptor-α: distribution in the skin of clinically healthy cats and cats with hypersensitivity dermatitis.

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Short running title: Endocannabinoid feline hypersensitivity dermatitis

Abstract

Background - Cannabinoid receptors and PPAR-α are gaining recognition as a promising therapeutic target for the treatment of skin disorders.

Hypothesis/Objectives – The aim of this study was to investigate the distribution of cannabinoid type 1 and 2 receptors (CBR1 and CBR2) and peroxisome proliferator-activated receptor alpha (PPAR-α) in feline skin and verify whether changes occur in the course of hypersensitivity dermatitis.

Animals – Twelve privately owned cats were included in the study. Skin samples were obtained from five dermatologically healthy cats and seven cats clinically diagnosed with hypersensitivity dermatitis.

Methods – Hematoxylin and eosin stained skin sections were investigated for histopathological changes. Indirect immunofluorescence for CBR1, CBR2 and PPAR-α was performed on paraffin-embedded sections, and antibody specificity tested by Western blot analysis.

Results – Skin samples from cats with hypersensitivity dermatitis were all histopathologically diagnosed with eosinophilic dermatitis. CB receptors and PPAR-α were distributed throughout the skin in both healthy and allergic cats. In normal feline skin, these receptors were mainly distributed in the epithelial compartment. Receptor expression increased in hypersensitivity compared to healthy skin, with the main distribution changes being in suprabasal CBR1, dermal CBR2 immunolocalization and the marked expression of PPAR-α in hyperplastic epidermis and perivascular infiltrate.

Conclusions and clinical importance –Increased expression of the investigated receptors in the skin of cats with hypersensitivity dermatitis suggests an endogenous protective strategy and paves the way for the use of natural cannabinoid receptor or PPAR-α agonists as a valuable approach to feline hypersensitivity dermatitis.

Introduction

Feline hypersensitivity dermatitis (HD) is often encountered in veterinary clinical practice, and results from cutaneous allergic reactions to environmental, food and/or flea allergens[1,2]. Excluding flea-bite and food-induced HD on the basis of clinical assessment, along with testing for parasiticides and food provocation, non–flea, non food-induced hypersensitive cats are likely to be allergic to environmental allergens[1,2]. Although sometimes referred to as feline atopic dermatitis[3], this latter condition does not always present increased serum levels of IgE[4]. Similarity with either human and canine counterparts is controversial, and feline HD is thus the preferred terminology[1]. Feline HD usually manifests with one or more of the following cutaneous reaction patterns: head and neck pruritus with excoriations, self-induced alopecia, miliary dermatitis, and/or eosinophilic lesions (including eosinophilic plaques, eosinophilic granulomas, and indolent ulcers)[2]. These conditions are currently treated by allergen-specific immunotherapy, antihistamines, essential fatty acids, glucocorticoids and cyclosporine[5]. Some cats with non-flea, and/or non-food-induced hypersensitivity dermatitis also benefited from oclacitinib treatment[5].

Alternative non-pharmacological approaches have also been tested and proposed. For example, a micronized formulation of palmitoylethanolamide (PEA)[6] improved signs (pruritus, erythema and alopecia) and skin lesions, while concurrently decreasing skin mast cell degranulation in cats with eosinophilic lesions[7]. PEA is an endogenous fatty acid amide and an endocannabinoid-related compound, as it shares both synthetic and degradative pathways with prototypic endocannabinoids[8]. Notably, PEA indirectly interacts with the so called cannabinoid receptors type 1 and 2 (CBR1, CBR2) and has anti-inflammatory properties mediated by peroxisome proliferator-activated receptor-alpha (PPAR-α)[8,9]. Both CBR1 and CBR2 have been described in human keratinocytes[10], and several other human skin cell types [11]. CBR1 and CBR2 are also over-expressed in the skin of dogs affected with atopic dermatitis[12]. Implications for skin protective action of cannabinoid receptor agonists have been repeatedly suggested[11,13-18] and CB receptors are considered to play a crucial role in epidermal differentiation and recovery of the epidermal permeability barrier[19]. Currently, several lines of evidence suggest that agonism at CB and CB-related receptors may represent a novel treatment approach to common inflammatory /allergic skin diseases[20,21].

To the best of our knowledge, no information is available on CB receptors and PPAR-α in feline skin. Here we investigated the distribution of CBR1, CBR2 and PPAR-α in the skin of dermatologically healthy cats and verified whether changes occur during feline HD.

Methods

Animals and samples: Twelve animals were included in the study. Five were dermatologically healthy and were referred for necropsy at the local veterinary hospital. Normal skin samples were obtained from them, specifically from areas reported as common sites of HD lesions[1]: 1) *regio auricularis; 2) regio nasalis; 3) regio maxillaris; 4) regio colli ventralis; 5) regio umbilicalis; 6) regio sacralis*; 7) *regio femoris,* (Figure 1)The remaining 7 cats were diagnosed with HD (according to accepted criteria[1,2]) and skin biopsies collected for histopathological confirmation of the clinical diagnosis. Anamnesis and clinical data are given in Table 1.This research was carried out according to international regulation governing the use of animals for scientific purposes (Directive 2010/63/EU). Institutional ethical committee approval was not required because skin samples were obtained either from necropsies or from animals referred for diagnostic purposes. Written informed consent was obtained from owners for including their cats in the study.

Histological analyses: Tissue biopsies were promptly immersed in 10% buffered formalin solution (pH 7.4) for 24 h and then processed for routine paraffin embedding. Five-µm thick sections were prepared for morphological evaluation (hematoxylin and eosin staining) and investigated for the following parameters: hyperplasia (0-absent, 1-present), ulcer (0-absent, 1-present), type of infiltrate (eosinophils, mast cells, lymphocytes, plasma cells and histiocytes, in order of prevalence). When present, mast cell infiltrate was scored as mild, moderate or severe on the basis of toluidine blue staining. Finally, a morphological diagnosis was provided.

Immunofluorescence: Indirect immunofluorescence was performed on paraffin-embedded sections. The following primary antibodies were used: rabbit polyclonal anti-CBR1 (1:100, rabbit polyclonal anti-CBR1, ab23703, Abcam, Cambridge, UK.), rabbit polyclonal anti-CBR2 (1:100, rabbit polyclonal anti-CBR2, ab45942, Abcam, Cambridge, UK) and rabbit polyclonal anti-PPAR-α (1:100, rabbit polyclonal anti-PPAR-α, NBP1-03288, Novus Biologicals, Littleton, USA). Epitope retrieval was carried out at 120°C in a pressure cooker for 5 min with a Tris/EDTA buffer, pH 9.0. Non-specific sites were blocked by incubation with 5% normal goat serum (s-1000, Vector Laboratories, Burlingame, USA) and 0.05% Triton-X in 0.1 M PBS, 45 min. After overnight incubation at 4°C with the appropriate primary antibody, sections were rinsed in 0.1 M PBS, (3 x 10 min), followed by incubation with a fluorescein-conjugated goat anti-rabbit IgG (goat anti-rabbit IgG, FI-1000, Vector Laboratories, Burlingame, USA), 10 µg/mL for 1 h at room temperature. Sections were again rinsed in 0.1 M PBS, (3 x 10 min) and mounted with mounting medium containing DAPI (H-1500, Vector Laboratories, Burlingame, USA). Photomicrographs were obtained using a Nikon Eclipse 80i microscope equipped with Nis Elements Br Microscope Imaging Software (Nikon Instruments, Calenzano, Italy). The fluorescence signal was abolished when primary antibody was either omitted or substituted with an

unrelated one. As positive controls, archival paraffin-embedded samples of feline hippocampus (CBR1 antibody), lymph node (CBR2 antibody) and human skin (PPAR-α) were used[12,22].

Western Blot: Specificity of the antibodies used was tested by Western blot analysis. Briefly, 30 µg of protein extracts stored from a previous study[23] were resolved by 12% SDS-PAGE gels and transferred onto nitrocellulose membranes (0.2 µm) using a voltage of 25V for 7 min by a commercial transfer apparatus (Trans-Blot®TurboTM Transfer System, Bio-Rad, Milano, Italy). After electrophoresis, the membranes were blocked and then incubated with appropriately diluted primary antibodies. HRP-conjugated goat anti-rabbit (1:10000, HRP-goat anti-rabbit antibody ADI-SAB-300J, Enzo life science, Farmingdale, USA) was used as secondary antibody. The chemiluminescent images were acquired by a gel documentation system (LAS 4010, GE Health Care, Milano, Italy).

Results

Histopathology

Normal skin samples did not show inflammatory infiltrates or other pathological changes. All HD skin samples were provided with a histopathological diagnosis of eosinophilic dermatitis, most (6/7) presenting focal or multifocal ulcers. Epidermal hyperplasia was a constant morphological alteration in all cases except one that showed diffuse ulcerative changes. Mast cells and eosinophils were present as part of the mixed inflammatory infiltrate in all cases. Details on type of infiltrate and scoring of mast cells are reported in table 2.

Immunofluorescence

Skin of healthy cats - CBR1 showed keratinocyte cytoplasmic immunoreactivity throughout the epidermal layers and hair follicle sheaths, in all areas examined. Differentiated sebocytes and hair bulb matrical cells showed CBR1 membrane staining (Figure 2a, 2b). CBR2 localization paralleled CBR1 (Figure 2c, 2d) and was also present in the cytoplasm of apocrine glands. PPAR-α was expressed in the cytoplasm of basal keratinocytes of epidermis, outer epithelial root sheath and isolated dermal papillae (Figure 2e, 2f).

Skin of cats with HD - CBR1 showed a cytoplasmic epidermal immunoreactivity, preferentially located in the superficial layers of the hyperplastic epidermis. This staining was markedly increased next to ulcerative lesions . No changes in spatial distribution were observed in adnexa compared to normal skin; the inflammatory infiltrate did not express CBR1 (Figure 3a, 3b).

CBR2 immunoreactivity was cytoplasmic in the hyperplastic epidermis and preferentially localized to the upper epidermis. A membrane staining pattern was evident in areas adjacent to ulcerative lesions. The subepidermal inflammatory infiltrate showed immunoreactivity in cells morphologically resembling mast cells; endotheliocytes were also immunopositive for CBR2 (Figure 3c,3d).

PPAR-α was multifocally expressed in the hyperplastic epidermis as cytoplasmic immunoreactivity, preferentially located in the upper keratinocyte layer. Alternating waves of cytoplasmic positivity were also seen throughout the entire thickness of the hyperplastic epidermis. In areas adjacent to ulcerative lesions a strong cytoplasmic immunoreactivity of keratinocytes was detected (Figure 3e, 3f). The subepidermal dermis showed immunoreactivity in endothelial cells and perivascular inflammatory infiltrate: immunoreactivity was seen in cells morphologically resembling mast cells, macrophages and fibroblasts.

Positive controls and Western blot

CBR1 immunostaining was found in the fibers of feline hippocampus (Figure 4a) with two immunoreactive bands at 66 and 81 kDa on Western blot analysis (Figure 4d). CBR2 stained the germinal center of feline secondary lymphatic nodules (Figure 4b) and showed two immunoreactive bands at 30 and 56 kDa (Figure 4d). PPAR-α immunostaining was

observed in basal keratinocytes of human epidermis (Figure 4c) with a single immunoreactive band at 30 kDa (Figure 4d).

Discussion

To the best of our knowledge, this is the first study detailing the expression of CBR1, CBR2 and PPAR-α in the skin of healthy and allergic cats. The histopathological alterations observed in HD cats corresponded to the reaction patterns classically described in literature[1,2,24]. In fact, irrespective of cause, the infiltrate was predominantly composed of eosinophils, mast cells, histiocytes, lymphocytes and plasma cells associated with epidermal hyperplastic changes and ulcers[24].

Our study shows CB receptors and PPAR-α to be distributed throughout the skin of both normal and HD cats. Our data paralleled those reported for cannabinoid receptors in normal canine and human skin [11,12] and for human skin in the case of PPAR-α immunolocalization[22].

Skin of healthy cats - In normal feline skin, the investigated receptors were preferentially distributed in the epithelial compartment, with only PPAR-α showing occasional non-epithelial immunolocalization (i.e. dermal papilla).

The preferential pan-epithelial distribution of CBR1 and CBR2 was previously documented in dog embryos[25] and canine skin[12], while in human skin immunolocalization was reported to be layered, i.e., CBR1 mostly in the upper layers (spinous and granular) and CBR2 in basal keratinocytes[11]. Differences in epidermal thickness between humans and pets (6-7 vs 2-3 nucleated cell layers, respectively) might explain this discrepancy. Moreover, data obtained in human skin are somewhat controversial, as CBR1 and CBR2 were described by other authors to be equally distributed in suprabasal layers of epidermis in normal and neoplastic skin[26]. CBR1 and CBR2 expression in normal skin of cats was not unexpected, since it is well known that endocannabinoids act through these receptors to maintain skin proper function in other species[18].

PPAR-α plays an increasingly recognized role in skin homeostasis[20,21], yet to the best of our knowledge there is just one study showing the cutaneous distribution of this receptor[22]. The latter study was performed in normal human skin and showed a clear cytoplasmic pattern in the basal layer of the epidermis[22], consistent with our findings. Ligand-dependent nucleo-cytoplasmic shuttling[27] might explain the cytoplasmic staining pattern of a nuclear receptor like PPAR-α is. Interestingly, isolated dermal papillae were immunoreactive for PPAR-α. Conceivably, this could relate to hair follicle cycling. Based on morphological parameters, the vast majority of hair follicles were in the late anagen – early catagen phase[28]. In order to determine if PPAR-α immunoreactivity is linked to a particular hair cycling stage, however, a comparison of serial sections stained by hematoxylin-eosin and immunohistochemistry is needed.

Skin of cats with HD – Irrespective of cause, we observed a mixed inflammatory infiltrate, epidermal hyperplasia and ulcerative lesions, as reported by others in feline HD[24].

Spatial distribution of the investigated receptors changed in the skin of cats with HD compared to normal skin: CBR1 was still confined in epidermis but its distribution, although cytoplasmic, was generally suprabasal and more evident in areas surrounding ulcers. CBR2

immunoreactivity paralleled that of CBR1 in the hyperplastic epidermis, and showed a membrane expression in areas surrounding ulcers; moreover, CBR2 was present in endothelium and infiltrating cells in the dermis. The pattern observed might thus correspond to an upregulation of both CBR1 and CBR2.

The endocannabinoid system is involved in attenuation of skin allergic response: in mice with experimentally-induced skin allergy, genetic ablation of CBR1 and CBR2 resulted in a more severe dermatitis[29] and higher skin levels of PEA compared with wild-type counterparts[30]. Furthermore, CBR1 inhibited epidermal keratinocyte growth[17] and different CBR2 agonists were reduced skin inflammation in several experimental models of allergy[15,31,32]. As such, the increased CBR1 and CBR2 expression in feline HD skin, as well as CBR2 immunoreactivity of dermal resident and infiltrating cells might be regarded as a skin response to inflammation, aimed at restoring homeostasis. A delicate balance between CBR1 and CBR2 signaling is essential for skin health, with CBR1 being involved in increasing inflammation while CBR2 overactivation in a decreased immune response, as recently reported[18]. Why CBR1 should be over-expressed in the hyperplastic epidermis and not found in the dermal infiltrate, while CBR2 is over-expressed in both skin compartments is unclear, but might reflect different pathways regulating inflammation.

The present study documents, for the first time, distribution of PPAR-α in the skin of HD cats. Immunostaining was markedly increased compared to healthy skin, with a ”wave” of PPAR-α expression in hyperplastic epidermis. Particularly high expression was seen next to ulcerative lesions, dermal blood vessels and perivascular infiltrate (mast cells and dermal endotheliocytes/fibroblasts). This finding is in line with previous data showing over-expression of PPAR-α in epidermal keratinocytes under allergic conditions[30]. Interestingly, recent findings increasingly point to activation of PPAR-α playing an essential role in the inhibition of skin inflammation in the course of dermatitis. In fact, PPAR-α agonists inhibit inflammatory infiltrates in a murine model of atopic dermatitis[33] and improve clinical signs[34]. During wound healing, PPAR-α is mainly involved in the early inflammatory phase, mediating rapid re-epithelialization[35]. The above effects are consistent with the dual role of PPAR-α in skin, acting to restore the epidermal barrier and provide anti-inflammatory activity[20]. Our results suggest that the observed increased PPAR-α expression corresponds to an improved availability of the target to perform its protective action.

PEA, an endocannabinoid congener, exhibits increased skin levels in experimentally-induced and naturally-occurring hypersensitive dermatitis[30,36]. PEA can act via a so-called “entourage effect” by indirectly operating through cannabinoid receptors and as an endogenous agonist for PPAR-α[8]. The increased expression of CB receptors and PPAR-α detected in the skin of HD cats might thus be part of a broader protective strategy involving their respective ligands and biosynthetic/degradative pathways. Further studies are warranted to address this question.

Although performed in a small number of animals our findings, together

293 with data on the benefits of micronized PEA in eosinophilic cats[7] proposes
294 the endocannabinoid system as a potential therapeutic target for feline
295 HD.

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385 Figure Legends
386
387 Figure 1: image showing areas where skin samples were collected: 1)
388 *regio auricularis; 2) regio nasalis; 3) regio maxillaris; 4) regio colli*
389 *ventralis; 5) regio umbilicalis; 6) regio sacralis*; 7) *regio femoris.*
390
391 Figure 2: Photomicrographs showing CBR1, CBR2 and PPAR-α
392 immunolocalization in normal feline skin. CBR1 was expressed by
393 epidermis (a) and sebaceous glands (b). CBR2 immunolocalized to
394 epidermis, hair follicle walls and sebaceous glands (c) as well as inner
395 epithelial root sheath cells in the hair bulb (d). Basal keratinocytes (e) and
396 hair papilla cells of one of two hair bulbs (f) expressed PPAR-α. Scale bar:
397 50 μm.
398
399 Figure 3: Photomicrographs showing CBR1, CBR2 and PPAR-α
400 immunolocalization in feline HD skin. CBR1 immunolocalized in the
401 epidermis (a) and markedly increased next to an ulcer (b; asterisk).
402 Epidermis, endothelial (full arrowhead) and subepidermal perivascular
403 cells (empty arrowhead) expressed CBR2 (c), keratinocytes displayed a
404 membrane pattern next to an ulcer (d; asterisk). PPAR-α immunolocalized
405 in basal and upper cells of hyperplastic epidermis (e) as well as in
406 keratinocytes adjacent to ulcer (f; asterisk). Scale bar: 50 μm.
407
408 Figure 4: Photomicrographs showing CBR1, CBR2 and PPAR-α
409 immunolocalization in samples used as positive control (a, b, c) and
410 Western blot analysis (d). a) CBR1 localizes to fibers of cat hippocampus;
411 b) CBR2 immunostaining of the germinal center of a reactive cat lymph
412 node; c) human epidermal basal keratinocytes show immunoreactivity
413 against PPAR-α antibody; d) immunoreactive bands obtained for the used
414 antibodies on feline protein extracts. Scale bars: 50 μm.
415

416 Tables

417

418 Table 1: Anamnestic data of the HD cats

	Breed	Age (months)	Sex	Anatomical location	Clinical diagnosis
1	European	72	F	Abdomen, Thigh	NFNFIHD
2	European	12	M	Neck	FBH
3	European	8	M	Neck	NFNFIHD
4	European	9	F	Neck	FIHD
5	European	48	M	Interscapular area	FIHD
6	European	72	F	Abdomen, Lip	NFNFIHD
7	European	48	M	Neck	NFNFIHD

419 NFNFIHD = non-flea non-food-induced hypersensitivity dermatitis; FIHD =
420 food-induced hypersensitivity dermatitis; and FBH = flea bite
421 hypersensitivity.

422

423 Table 2: Histological findings in HD cats

	Hyperplasia	Ulcer	Type of infiltrate	Mast cell infiltrate
1	nd	1	M,P,N,L,H,E	severe
2	1	1	E,L,M,H	moderate
3	1	0	E,M,P,L,H	moderate
4	1	1	M,E,N,H	moderate
5	1	1	E,M,H	severe
6	1	1	M,P,E,L,H	severe
7	1	1	M,E,N	moderate

424 0-absent, 1-present, M=Mast cells, P= Plasma cells, N= Neutrophils; L=
425 Lymphocytes; H= Histiocytes; E= Eosinophils

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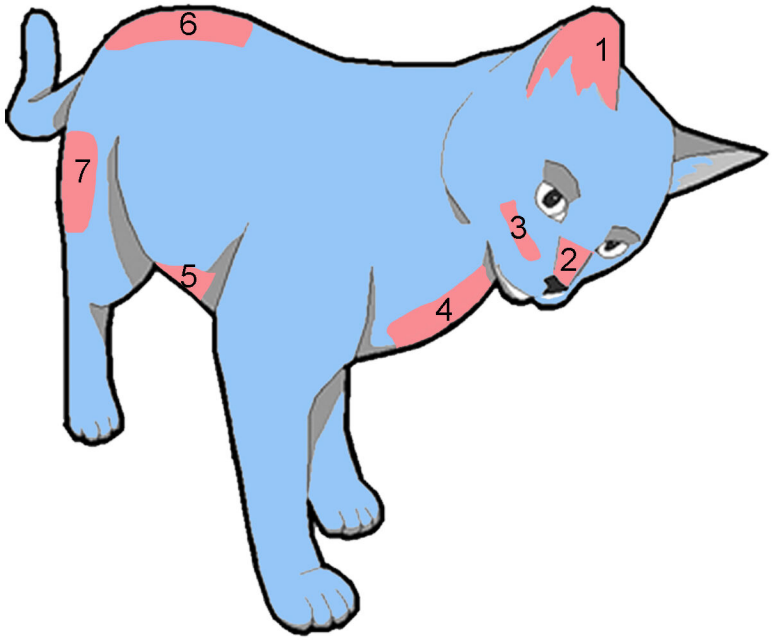
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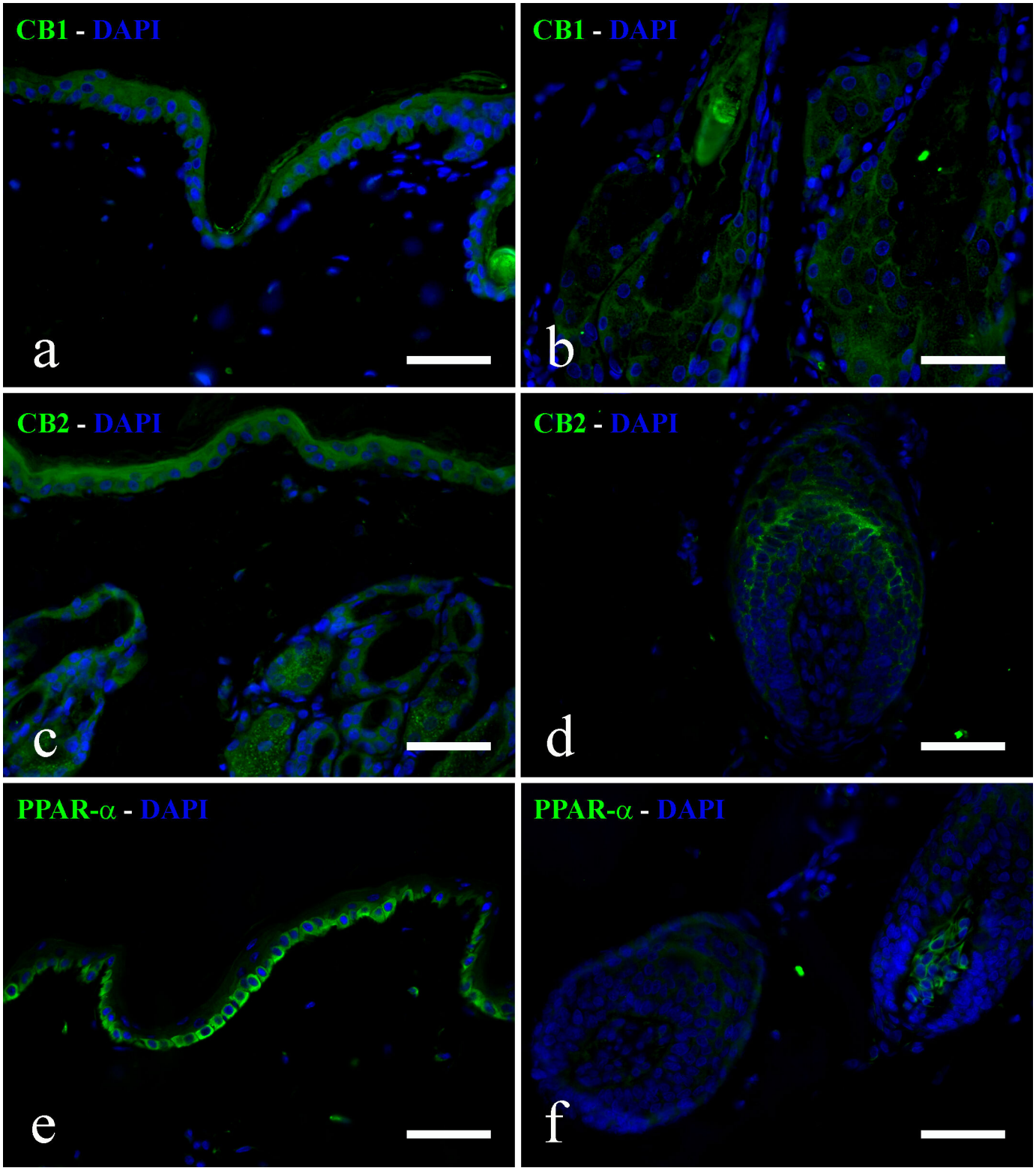
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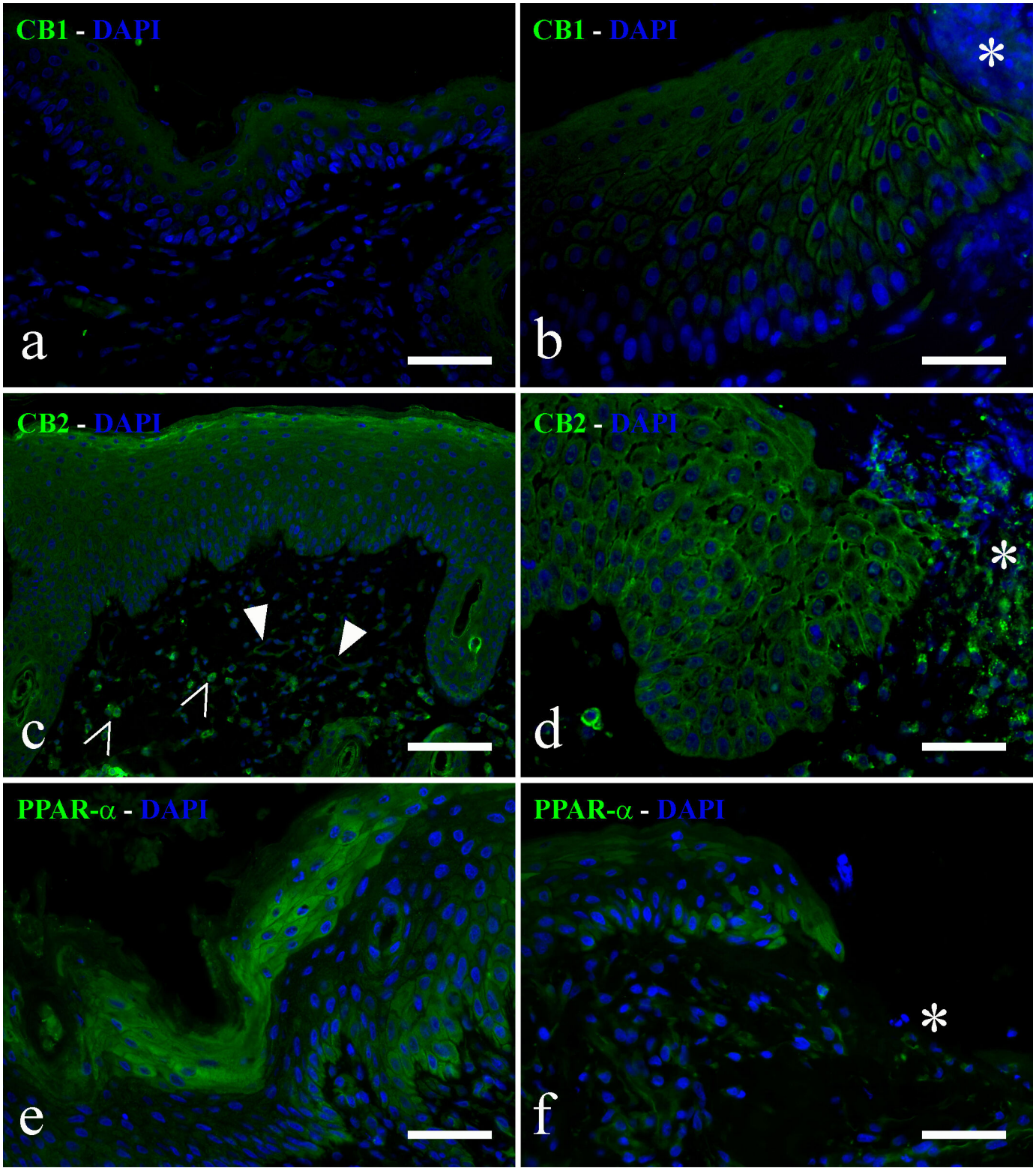
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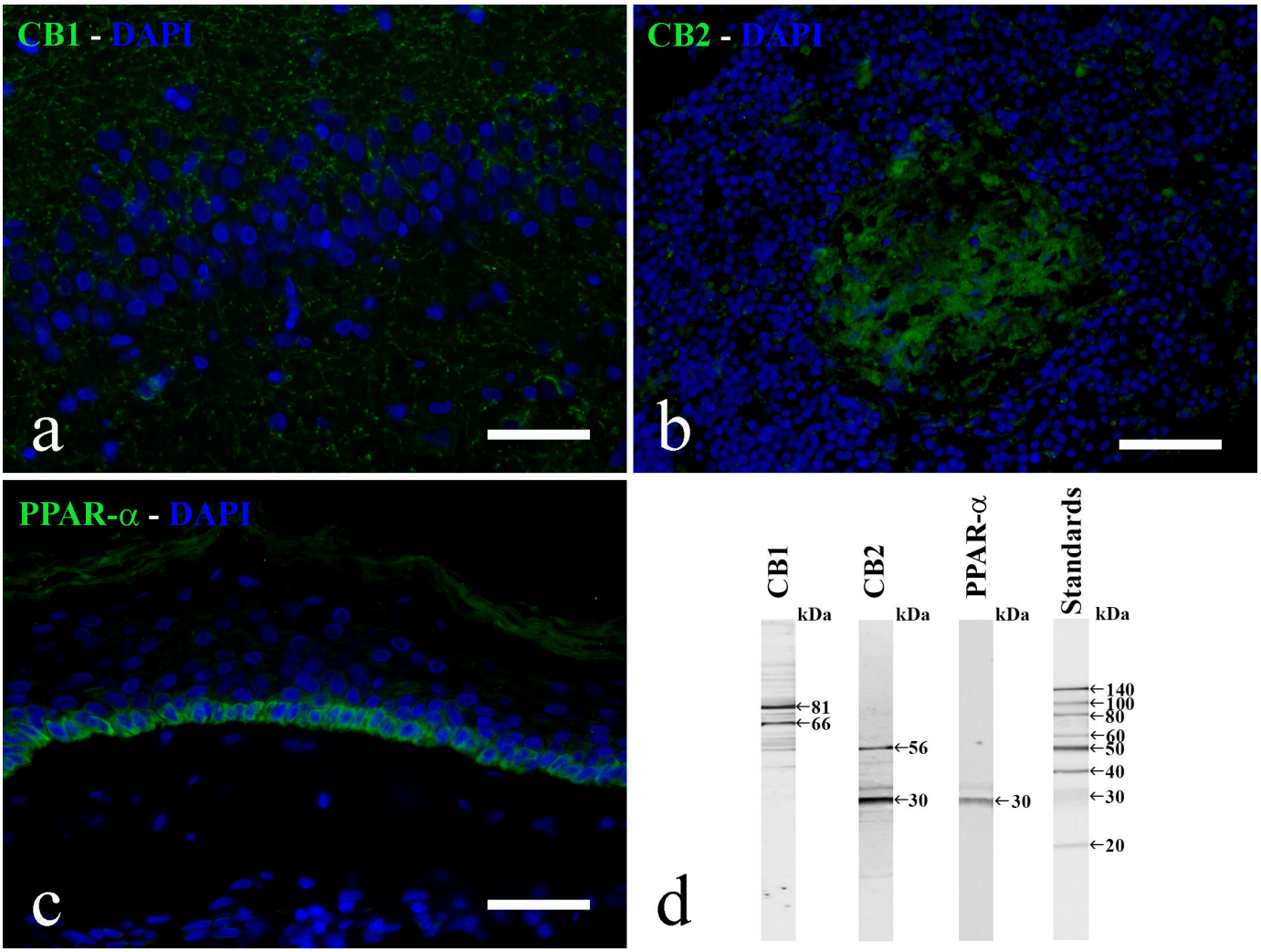
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