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Mustard vesicants alter expression of the endocannabinoid system in mouse skin

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Abstract

Vesicants including sulfur mustard (SM) and nitrogen mustard (NM) are bifunctional alkylating agents that cause skin inflammation, edema and blistering. This is associated with alterations in keratinocyte growth and differentiation. Endogenous cannabinoids, including *N*-arachidonylethanolamine (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG), are important in regulating inflammation, keratinocyte proliferation and wound healing. Their activity is mediated by binding to cannabinoid receptors 1 and 2 (CB1 and CB2), as well as peroxisome proliferator-activated receptor alpha (PPAR α). Levels of endocannabinoids are regulated by fatty acid amide hydrolase (FAAH). We found that CB1, CB2, PPAR α and FAAH were all constitutively expressed in mouse epidermis and dermal appendages. Topical administration of NM or SM, at concentrations that induce tissue injury, resulted in upregulation of FAAH, CB1, CB2 and PPAR α , a response that persisted throughout the wound healing process. Inhibitors of FAAH including a novel class of vanillyl alcohol carbamates were found to be highly effective in suppressing vesicant-induced inflammation in mouse skin. Taken together, these data indicate that the endocannabinoid system is important in regulating skin homeostasis and that inhibitors of FAAH may be useful as medical countermeasures against vesicants.

Graphical Abstract

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Keywords

endocannabinoids; FAAH; sulfur mustard; epidermis; vesicants; inflammation

Introduction

The endocannabinoids *N*-arachidonylethanolamine (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG), and various *N*-acylethanolamines including palmitoylethanolamine (PEA) and oleoylethanolamine (OEA), are endogenous fatty acid signaling molecules involved in regulating inflammation (Biro et al., 2009; Kupczyk et al., 2009). In the skin, they also control keratinocyte proliferation, differentiation, and wound healing (Maccarrone et al., 2003; Ramot et al., 2013; Toth, Dobrosi, et al., 2011). Endocannabinoids function by binding to cannabinoid receptors and lipid signaling molecules such as the nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR α) (Di Marzo et al., 2001; Dubrac et al., 2011; Kendall et al., 2013; O'Sullivan et al., 2010). Two major G protein-coupled endocannabinoid receptors have been identified in the skin, CB1 and CB2 (Galiege et al., 1995; Kupczyk et al., 2009; Pertwee, 2014; Stander et al., 2005; Zheng et al., 2012). Whereas binding of AEA to CB1 is involved in controlling keratinocyte growth, differentiation and apoptosis (Maccarrone et al., 2003; Paradisi et al., 2008), endocannabinoid signaling through CB2 regulates genes mediating lipid biosynthesis, immune cell signaling, cell migration and inflammation (Dobrosi et al., 2008; Kishimoto et al., 2005; Oka et al., 2006; Zheng et al., 2012). PPAR α has also been identified in the skin, specifically in keratinocytes, sebaceous glands and T-cells, and it is thought to play a role in wound re-epithelialization, sebocyte differentiation, and the resolution of inflammation (Di-Poi et al., 2004; Dubrac et al., 2011; Michalik et al., 2001).

AEA and various congeners including PEA and OEA are metabolized by the membrane-bound serine hydrolase, fatty acid amide hydrolase (FAAH) (Bisogno et al., 1997; Cravatt et al., 1996; De Filippis et al., 2011). In the skin, FAAH is localized in keratinocytes, melanocytes and fibroblasts (Maccarrone et al., 2003; McPartland, 2008; Pucci et al., 2012). Inhibition of FAAH increases levels of endocannabinoids (Pertwee, 2014) resulting in reduced inflammation and pruritis (Jhaveri et al., 2008; Wise et al., 2008). Alterations in expression and/or activity of FAAH, as well as CB1, CB2 and PPAR α have been linked to a number of skin diseases in animal models including allergic contact dermatitis, acute and chronic contact dermatitis, dermal fibrosis, and skin tumor induction (Biro et al., 2009; Kendall et al., 2013). In human skin, endocannabinoid therapy has shown promise in treating histamine-induced dermatitis, allergic contact dermatitis and pruritis (Dvorak et al., 2003; Lambert, 2007; Paus et al., 2006).

Sulfur mustard (SM, bis[2-chloroethyl] sulfide) and nitrogen mustard (NM, methylbis(2-chloroethyl)amine) are bifunctional alkylating agents first synthesized for chemical warfare and they remain high priority chemical threats (DeVita et al., 2008; Wattana et al., 2009). Depending on the dose and timing of exposure, mustards induce epidermal and dermal injury, inflammation, blistering and scarring (Graham et al., 2009; Shakarjian et al., 2010). This is associated with delayed wound healing (Ghabili et al., 2010; Graham et al., 2005). Long-term effects of mustards in human skin include dermatitis, pruritis, and psoriasis (Balali-Mood et al., 2005; Shohrati et al., 2007). Since endocannabinoids can modulate keratinocyte growth and differentiation and inflammatory responses, we speculate that they may play a role in vesicant-induced skin injury. In earlier studies in mice, our laboratories characterized the progression of tissue injury following exposure of skin to sulfur mustard (Chang et al., 2014; Joseph et al., 2011; Joseph et al., 2014). SM was found to cause skin inflammation as well as distinct structural changes in the epidermis including damage to the stratum corneum, altered cellular differentiation, basal cell apoptosis and loss of dermal appendages. Wound healing was associated with extensive epidermal hyperplasia, hyperkeratosis and parakeratosis. In the present studies, we characterized changes in expression of receptors for endocannabinoids as well as FAAH in mouse skin following exposure to NM and SM. Our findings that mustards cause marked changes in the endocannabinoid system in the skin and that an FAAH inhibitor was effective in reducing skin injury suggests a novel mechanistic pathway for targeting the development of countermeasures.

Materials and methods

Animals and treatments

All animals received humane care in compliance with institutional guidelines as outlined in the National Institute of Health's *Guide for Care and Use of Laboratory Animals*. For NM experiments, female CD-1 mice, 8-10 weeks of age (Charles River Laboratories) were used. Mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg, Ketathesia, Henry Schein Animal Health, Dublin, OH) and xylazine (12 mg/kg, Anased, Henry Schein Animal Health) and the hair on the dorsal lumbar region shaved. Two 6-millimeter diameter glass microfiber filters (GE Healthcare Life Sciences, Buckinghamshire, UK) were placed on the shaved dorsal lumbar skin, on either side of the spine. Twenty μ moles of freshly prepared NM (20 μ l of a 1 M solution prepared in 20% deionized water and 80% acetone (v/v) (Sigma-Aldrich, St. Louis, MO) or control solvent was applied directly on the glass microfiber filters which were then covered with Parafilm® M (Sigma-Aldrich). The filters were removed from the skin after 6 min. Mice were euthanized 1, 2, 3, 4 and 5 days post exposure and 12 mm full thickness skin punch biopsies of exposed areas immediately collected, trimmed, and stored at 4°C in ice cold phosphate buffered saline (PBS) containing 2% paraformaldehyde/3% sucrose. After 24 h, skin samples were rinsed in ice cold PBS containing 3% sucrose, transferred to ethanol (50%), and paraffin embedded. Skin sections (6 μ m) were stained with hematoxylin and eosin (H&E) (Goode Histolabs, New Brunswick, NJ). For SM experiments, male CRL: SKH1-Hr hairless mice, 5 weeks of age (Charles River Laboratories, Wilmington, MA) were used. Animals were exposed to SM on the dorsal skin using a vapor cup model as previously described (Joseph et al., 2014). Mice were

euthanized 1, 3, 7, 14 and 21 days post-exposure and full thickness skin punch biopsies of exposed areas prepared for immunohistochemistry as described below. All experiments with SM were performed at MRIGlobal (Kansas City, MO). The efficacy of candidate FAAH inhibitors was evaluated using a mouse ear vesicant model (MEVM) as previously described (Babin et al., 2000; Casillas et al., 2000; Young et al., 2012) using the sulfur mustard analog 2-chloroethyl ethyl sulfide to induce inflammation. For NM dorsal skin and MEVM experiments CD1 mice were used as they were less costly than SKH1-Hr mice. To evaluate test compounds, ears (3–4 mice per group) were treated with 20 μ L of vehicle control (methylene chloride or acetone) or the test compound (1.5 μ mol) in 20 μ L of the appropriate vehicle. After 5 h, mice were euthanized and ear punches (6 mm in diameter) were collected and weighed. Ear punch masses were averaged and the percentage reduction in vesicant-induced edema and inflammation calculated as previously described (Casillas et al., 2000). In control experiments in mice not exposed to the vesicant, compounds applied to skin in the MEVM did not show signs of toxicity including erythema and/or edema (not shown).

Immunohistochemistry

Tissue sections were deparaffinized and blocked at room temperature with 1% BSA for 1 h for FAAH, 25% normal goat serum for 2 h for CB2 and PPAR α , or 25% normal goat serum in 1% BSA for 2 h for CB1. Tissue sections were then incubated overnight at 4°C with rabbit affinity purified polyclonal antibodies against FAAH (1:250; Cayman Chemical, Ann Arbor, MI), CB1 receptor (1:250; Cayman Chemical), CB2 receptor (1:250; Cayman Chemical), PPAR α (1:250; Cayman Chemical), IgG control antibody or blocking peptide controls (Cayman Chemical, 1:1 FAAH amino acids 561-579, CLRFMREVEQLMTPQKQPS, 1:10 CB1 receptor amino acids 461-472 MSVSTDTSAEAL, 1:1 CB2 receptor amino acids 20-33 NPMKDYMILSGPQK, 1:1 PPAR α : amino acids 22-36 PLSEEFLLQEMGNIQE). This was followed by incubation with biotinylated goat anti-rabbit secondary antibody (1:200; Vector Laboratories, Burlingame, CA) for 30 min at room temperature; binding was visualized using 3,3'-diaminobenzidine (Vector Laboratories).

FAAH inhibitors and enzyme assays

The preparation of the vanillyl alcohol carbamates (4452: N-(2-phenoxyethyl)-, [4-(acetyloxy)-3-methoxyphenyl]methyl ester carbamic acid; 4453: N-(2-phenylethyl)-, [4-(acetyloxy)-3-methoxyphenyl]methyl ester carbamic acid; 4455: N-(cyclohexylmethyl)-, [4-(acetyloxy)-3-methoxyphenyl]methyl ester carbamic acid; 4464: N-[2-(4-morpholinyl)ethyl]-, [4-(acetyloxy)-3-methoxyphenyl]methyl ester carbamic acid has been described elsewhere (Laskin et al., 2013). The calculated log n-octanol/water partition coefficient (cLogP), $P = (\text{amount of compound dissolved in octanol} / \text{amount of compound dissolved in water})$ was used as a measure of hydrophobicity; the greater the cLogP, the more hydrophobic the compound. cLogP values were determined using ChemBioDraw Ultra 12.0 (CambridgeSoft, Perkin Elmer Informatics, Waltham, MA). A fluorescent FAAH Inhibitor Screening Assay Kit (Cayman Chemical) was used to evaluate the ability of the vanillyl alcohol carbamates to inhibit FAAH activity (Ramaraio et al., 2005; Wang et al., 2006). Assays were run in triplicate according to the manufacturer's instructions. Fluorescence was monitored using a SpectraMax M5 microplate reader (Molecular Devices,

Sunnyvale, CA) with excitation and emission wavelengths set at 340 nm and 450 nm, respectively. The IC₅₀, or half maximal inhibitory concentration of each chemical was calculated GraphPad Prism 6.0 (GraphPad Software, San Diego, CA).

Results

Effects of NM on expression of cannabinoid receptors, PPAR α and FAAH in mouse skin

Skin from control CD-1 mice expressed low constitutive levels of CB1 and CB2 throughout the epidermis and dermal appendages (Figs. 1 and 2). Expression of these receptors was noted in sebaceous glands and outer root sheaths of hair follicles. Following NM exposure, CB1 was upregulated in the epidermis, pilosebaceous units and inflammatory cells within the dermis, a response noted after 1-3 days (Fig. 1). Increased CB2 was evident 2-3 days post NM in the hyperplastic epidermis and degenerating pilosebaceous units (Fig. 2). After 4-5 days, there was an overall decrease in expression of CB1 and CB2 in the hyperplastic epidermis (Figs. 1 and 2 and not shown). CB1 was primarily expressed in basal keratinocytes while CB2 was expressed in both basal and suprabasal keratinocytes. Of note, increased CB2 was evident in the uppermost layers of the stratum granulosum directly below the stratum corneum. A marked increase in CB1 was evident in keratinocytes in the basal and spinous layers. Anti-CB1 and anti-CB2 antibody binding was inhibited by their respective blocking peptides indicating that antibody binding was specific for these receptors (Fig. 2 and not shown).

Low constitutive levels of PPAR α were expressed throughout the epidermis, the outer root sheath of hair follicles, and in sebaceous glands in control skin (Fig. 4 and not shown). One to three days post NM exposure, increased PPAR α expression was evident in the epidermis and sebaceous glands, as well as in inflammatory cells within the dermis (Fig. 4). Nuclear localization of PPAR α was evident in sebocytes one day post-NM, while 2-5 days post-NM increased PPAR α was largely cytoplasmic. PPAR α expression was upregulated throughout the hyperplastic epidermis after 3 days post NM. Four days post NM, marked increases in PPAR α expression were observed within the stratum granulosum, stratum spinosum and in the stratum basale (Fig. 4). Five days post NM, PPAR α was upregulated in the stratum basale and stratum spinosum with decreased PPAR α expression in the stratum granulosum (Fig. 4). PPAR α was also localized in remnants hair follicle outer root sheaths and within dystrophic sebaceous glands. PPAR α was also highly expressed in the stratum corneum and within areas of parakeratosis. Anti-PPAR α antibody binding was inhibited using a PPAR α -specific blocking peptide demonstrating that antibody binding was specific for PPAR α (Fig. 3).

Low levels of FAAH were expressed in the epidermis, the outer root sheath of hair follicles, and in sebaceous glands (Fig. 5). Within 1-3 days after NM, an increase in FAAH expression was evident throughout the epidermis and hyperplastic neoepidermis at the wound edge, in the isthmus and outer root sheath of hair follicles, and in inflammatory cells in the dermis and hypodermis (Fig. 5 and not shown). After 4-5 days post NM, FAAH was differentially expressed in the hyperplastic epidermis. FAAH was primarily localized in the granular layer of the epidermis directly below the stratum corneum. Anti-FAAH antibody binding was

inhibited by a blocking peptide, indicating that antibody binding was specific for FAAH (Fig. 4).

Effects of SM on expression of cannabinoid receptors, PPAR α and FAAH in mouse skin

As observed in CD-1 mice, skin from control SKH-1 mice expressed low constitutive levels of CB1 and CB2 throughout the epidermis and dermal appendages (Figs. 6 and 7). One day post SM, an increase in the cannabinoid receptors was evident in hair follicles, while 3-7 days post SM, increases in CB1 and CB2 were evident in neoepidermis (Figs. 6 and 7). CB1 and CB2 were also expressed in hyperplastic epidermis 14-21 days post SM; increased expression of CB2 was noted in suprabasal layers of the epidermis. In control SKH-1 mouse skin, low constitutive levels of PPAR α were expressed throughout the epidermis, the outer root sheath of hair follicles, and in sebaceous glands (Fig. 8). One to three days post SM, increased PPAR α was evident in the epidermis and remnant sebaceous glands (Fig. 8). PPAR α expression was upregulated throughout the hyperplastic epidermis by 7 days post SM. At later times, 14-21 days post SM, PPAR α was upregulated in the stratum basale and stratum spinosum with decreased PPAR α expression in the stratum granulosum (Fig. 8). PPAR α was also localized in remnants of the hair follicle outer root sheaths and follicular cysts, and was also highly expressed in the stratum corneum and within areas of parakeratosis.

In skin from control SKH-1 mice, low level FAAH expression was noted in suprabasal layers of the epidermis, the outer root sheath of hair follicles, and in sebaceous glands (Fig. 9). Within 1 day of SM exposure, an increase in FAAH was evident in the epidermis and sebaceous glands. By three days, FAAH was expressed in the hyperplastic neoepidermis at the wound edge (Fig. 9). FAAH expression was also upregulated in the hyperplastic epidermis 7 days post SM. Increased FAAH was expressed in basal cells and proliferating cells above the basal cell layer. A decrease in FAAH expression was noted in the hyperplastic epidermis during wound healing, 14-21 days post SM. At this time, FAAH was expressed in differentiated layers of the epidermis; low levels of FAAH were expressed in proliferating basal cells (Fig. 9).

Effects of FAAH inhibitors on skin inflammation

We have designed several lipophilic vanillyl alcohol carbamates as inhibitors of FAAH with cLogP values ranging from 1.04-3.35 that can be applied directly to skin (Laskin et al., 2013). Three of these compounds 4452, 4453 and 4455 are active inhibitors of FAAH with IC₅₀ values ranging from 14-93 μ M. Each of these compounds was found to be active in mitigating mustard-induced skin injury in the MEVM; 4453 (80%) > 4452 (51%) > 4455 (40%). Interestingly, a more hydrophilic vanilloid carbamate derivative (4456, cLogP = 1.04) was not active as either an FAAH inhibitor or inflammation suppressant (Fig. 10).

Discussion

Endocannabinoids including AEA and 2-AG, as well as related *N*-acylethanolamines, have been identified in the epidermis and dermis of human skin (Kendall et al., 2013; Kupczyk et al., 2009). They are thought to be important in regulating cell growth and differentiation, as

well as in controlling skin inflammation (Biro et al., 2009; Kupczyk et al., 2009; Maccarrone et al., 2003). Endocannabinoids have also been detected in human skin suction blister fluid, presumably generated in response to trauma where they likely function to suppress inflammation and promote wound healing (Kendall et al., 2015). As NM and SM are skin blistering agents, endocannabinoids are also likely to be generated in vesicant-induced blisters. *N*-acylethanolamines have been shown to suppress the release of the proinflammatory chemokines in poly-[I:C]-stimulated human keratinocytes (Petrosino et al., 2010; Petrosino et al., 2016). In human skin, endocannabinoid signaling has been shown to lead to suppression of mast cell maturation and activation, an important component of dermal inflammatory responses (Sugawara et al., 2012). Topical application of *N*-acylethanolamines and cannabinoids has also been shown to be effective in reducing skin inflammatory diseases including eczema, allergic contact dermatitis and pruritis (Dvorak et al., 2003; Karsak et al., 2007; Lambert, 2007; Paus et al., 2013; Petrosino et al., 2010). Similarly, *N*-palmitoylethanolamine suppresses ultraviolet light-induced erythema and thymidine dimer formation in human skin, while in mice, it suppresses inflammation in the carrageenan-induced paw edema model (Kemeny et al., 2007; Wise et al., 2008).

Constitutive expression of cannabinoid receptors CB1 and CB2, as well as PPAR α , was evident throughout the epidermis and dermal appendages of normal mouse skin, suggesting a role for these proteins in skin homeostasis. A marked increase in expression of these proteins was evident in epidermis at early times (up to 3-4 days) following exposure to NM or SM. At later times during the wound repair process, increases in CB1, CB2 and PPAR α persisted in the hyperplastic epidermis. CB1 was predominantly localized in basal and suprabasal keratinocytes, while CB2 was largely expressed in suprabasal cells. Differential expression of cannabinoid receptors in basal and suprabasal keratinocytes suggests that they may perform distinct functions in the skin during the wound healing process. For example, basal cell CB1 may be important in regulating endocannabinoid-mediated keratinocyte proliferation, while suprabasal cell CB2 may regulate keratinocyte differentiation. During wound healing PPAR α was localized in basal cells and in areas of suprabasal epidermis above the basal cells, suggesting that it may function not only in regulating proliferation, but also in early stages of the keratinocyte differentiation process (Hanley et al., 1998; Komuves et al., 2000; Sertznig et al., 2008). In addition to promoting keratinocyte growth and differentiation during wound healing, upregulation of cannabinoid receptors and PPAR α and cannabinoid receptor signaling during wound healing is also likely to be important in mediating the anti-inflammatory effects of endocannabinoids (Di-Poi et al., 2004; Lo Verme et al., 2005; Michalik et al., 2007).

We also noted that inflammatory cells accumulating at the wound site following NM- or SM-induced injury upregulate CB1. Innate immune cells in the skin have been reported to express various endocannabinoid proteins (Chiurchiu et al., 2014; Stander et al., 2005; Sugawara et al., 2012). Both pro- and anti-inflammatory effects of endocannabinoids have been described in neutrophils and macrophages (Lenglet et al., 2013; Tomar et al., 2015). It is likely that the early appearance of these cells in the wound site (1-3 days) following injury contributes to inflammation and tissue damage, whereas at later stages they contribute to the resolution of inflammation, tissue remodeling and wound healing (Daley et al., 2010).

Expression of the endocannabinoid system in these cells suggests that endocannabinoid signaling may contribute to the functional activity of these immune cells.

FAAH is key in mediating endocannabinoid metabolism (Ahn et al., 2009; Cravatt et al., 1996; Di Marzo et al., 2007). In normal mouse skin, relatively low levels of FAAH are constitutively expressed throughout the interfollicular and follicular epidermis, suggesting it also functions to maintain epidermal and follicular homeostasis. Marked increases in FAAH expression were noted 1-3 days following exposure to NM or SM and this was coordinate with epidermal cell degradation and eschar formation. Early increases in expression of FAAH likely contribute to mustard-induced inflammation and injury by reducing levels of anti-inflammatory endocannabinoids, while generating arachidonic acid, a precursor to prostaglandins and other proinflammatory mediators (Holt et al., 2005; Olah et al., 2016; Schlosburg et al., 2009). At later times following NM or SM (> 3 days), levels of FAAH decrease, times coordinate with rapid keratinocyte proliferation and wound healing. This is presumably due to restoration of endocannabinoid levels in the skin, as well as decreases in levels of proinflammatory eicosanoids.

The idea that increases in FAAH can contribute to skin toxicity suggests that FAAH inhibitors may be effective in suppressing mustard-induced skin injury. Indeed, a number of studies have demonstrated that FAAH inhibitors including carbamates, α -ketoheterocycles, alkylsufonylfluorides and alkyl ureas increase endocannabinoids and *N*-acylethanolamines resulting in reduced pain and inflammation (Ahn et al., 2009; Blankman et al., 2013; Cravatt et al., 2004; Holt et al., 2005; Schlosburg et al., 2009; Wise et al., 2008). Our laboratory has developed a series of vanillyl alcohol carbamate derivatives that are effective FAAH inhibitors (Fig. 10). The vanilloid moiety, both as an amine (*i.e.*, 4-hydroxy-3-methoxybenzylamine or vanillyl amine) and as an alcohol (*i.e.*, 4-hydroxy-3-methoxybenzyl alcohol or vanillyl alcohol), is a well-known construct in many candidate anti-inflammatories (Pal et al., 2009; Tomohiro et al., 2013). Vanillyl alcohol itself is a natural anti-inflammatory found in ginger and in a wide variety of other botanicals utilized as an additive in foods, pharmaceuticals, and cosmetics (Jung et al., 2008; Raffai et al., 2015). Our compounds were all synthesized from vanillyl alcohol with the lipophilic (fatty amine) side chain derived from either phenethyl, phenoxyethyl, cyclohexylmethyl, or morpholinylethyl (Laskin et al., 2013). Varying the hydrocarbon amine side chain allows considerable manipulation of overall molecular lipophilicity. In addition, acetylating the para-phenol hydroxyl on the vanilloid greatly increases shelf life of these inhibitors while having no effect on their biological activity.

Compounds 4453, 4452 and 4455 (Fig. 10) were all effective inhibitors of FAAH activity. These relatively lipophilic compounds (cLogP = 2.72-3.03) also inhibited inflammation in the MEVM. 4464, a more hydrophilic carbamate (cLogP = 1.04), was inactive in both the FAAH assay and the MEVM. These data demonstrate the importance of hydrophobic-hydrophilic balance in FAAH inhibition. The reduced activity against FAAH with our non-arylated compounds (4455 and 4464) may reflect the absence of an essential planar phenyl ring in their molecular architectures, reported by others to contribute to FAAH inhibitor activity (Keith et al., 2012; Keith et al., 2014). The fact that the FAAH inhibitors suppress

mustard-induced inflammation is consistent with the idea that increases in FAAH contribute to skin inflammation and injury.

Sebocytes from control and mustard-treated mouse skin were found to express FAAH, cannabinoid receptors and PPAR α . These data are consistent with earlier studies showing constitutive endocannabinoid protein expression in sebaceous glands of dogs, mice and humans (Campora et al., 2012; Stander et al., 2005; Zheng et al., 2012). These findings indicate that, as in other skin cell types, endocannabinoid proteins function in maintaining homeostasis (Dobrosi et al., 2008; Toth, Olah, et al., 2011). Mature, differentiated sebocytes produce sebum, while proliferating cells replenish terminally differentiated cells that have undergone apoptosis (Schneider et al., 2010; Zouboulis, 2004). Following NM- or SM-induced injury, FAAH and CB2 were homogeneously distributed in the sebaceous glands, while CB1 and PPAR α were most upregulated in flattened, proliferating cells near the distal end of the sebaceous gland and in nucleated sebocytes. These data suggest that FAAH and CB2 are important in controlling sebocyte growth and differentiation, while CB1 and PPAR α signaling regulates proliferation. As observed in keratinocytes, 1-3 days post NM or SM, there was a marked increase in expression of these proteins. As endocannabinoids control sebocyte function, regulating growth, differentiation and sebum biosynthesis, these changes may be important in protecting the skin following injury (Dobrosi et al., 2008). Conversely, excessive sebum production may contribute to cytotoxicity. Sebocyte lipids and lipid-derived products can undergo peroxidation reactions which generate cytotoxic mediators (Tochio et al., 2009; Zouboulis, 2004). These lipid peroxides can also stimulate keratinocytes to produce pro-inflammatory mediators including prostaglandins, IL-1 α and IL-6, as well as antioxidants such as heme oxygenase-1, catalase and glutathione S-transferase (Ottaviani et al., 2006; Zhou et al., 2013; Zouboulis et al., 2014). PPAR α ligands have been reported to inhibit sebaceous gland lipogenesis (Downie et al., 2004) and this may be important in regulating sebocyte function following injury.

In summary, our findings indicate that FAAH, a key catabolic enzyme important in regulating levels of various fatty acid amides including AEA and many *N*-acylethanolamines, as well as receptors for these mediators including CB1, CB2 and PPAR α , are present in mouse skin, particularly in the interfollicular epidermis and dermal appendages. Importantly, these proteins were markedly upregulated in the skin following treatment with NM or SM, indicating that the endocannabinoid system plays a role in mustard-induced skin injury and/or wound repair. These results, together with our findings that FAAH inhibitors suppress mustard-induced skin inflammation, further support the idea that the endocannabinoids function in regulating skin homeostasis, as well as vesicant-induced inflammation and toxicity. Further studies are needed to better understand the role of the endocannabinoid system in mediating skin injury as this will be important in identifying therapeutic targets that may prevent or reduce skin damage following exposure to vesicants.

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Abbreviations

AEA	anandamide
AG	2-arachidonoyl glycerol
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
CB receptor	cannabinoid receptor
FAAH	fatty acid amide hydrolase
NM	nitrogen mustard
OEA	oleylethanolamide
PEA	palmitoylethanolamide
PPARα	peroxisome proliferator activated receptor alpha
SM	sulfur mustard

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Highlights

- Sulfur mustard and nitrogen mustard are potent skin vesicants
- The endocannabinoid system regulates keratinocyte growth and differentiation
- Vesicants are potent inducers of the endocannabinoid system in mouse skin
- Endocannabinoid proteins upregulated include FAAH, CB1, CB2 and PPAR α
- FAAH inhibitors suppress vesicant-induced inflammation in mouse skin

Figure 1. Effects of nitrogen mustard on CB1 receptor expression

Histological sections, prepared after exposure to control (CTL) or 1, 2, 3, 4, and 5 days after exposure to NM, were stained with an antibody to CB1 receptor. One representative section from 3 mice/treatment group is shown. Antibody binding was visualized using a Vectastain Elite ABC kit (original magnification, $\times 400$; Bar, 50 μm). Epidermis (E), dermis (D), hair follicles (H), sebaceous glands (S), eschar (ES). Solid arrow indicates epidermal expression of CB1 receptors; open arrow indicates CB1 expression in hair follicles; arrowhead indicates

CB1 expression in sebaceous glands; asterisk indicates inflammatory infiltrate within the eschar.

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Figure 2. Effects of nitrogen mustard on CB2 receptor expression

Histological sections, prepared after exposure of mice to control (CTL) or 1, 2, 3, 4, and 5 days after exposure to NM, were stained with an antibody to CB2 receptor. One representative section from 3 mice/treatment group is shown. Antibody binding was visualized using a Vectastain Elite ABC kit (original magnification, $\times 400$; Bar, 50 μm). Epidermis (E), dermis (D), hair follicles (H), sebaceous glands (S), eschar (ES). Solid arrow indicates epidermal expression of CB2 receptors; open arrow indicates CB2 expression in hair follicles; arrowhead indicates CB2 expression in sebaceous glands.

Figure 3. Effects of blocking peptides on FAAH, CB1, CB2, and PPARα antibody binding to tissue sections

Left panels: histological sections prepared 3 days after exposure to NM were stained with antibodies to CB1 (a), CB2 (c), PPARα (e), or FAAH (g). Right panels: histological sections treated with antibodies and respective blocking peptides to CB1 (b), CB2 (d), PPARα (f), and FAAH (h). One representative section from 3 mice/treatment group is shown. Antibody binding was visualized using a Vectastain Elite ABC kit (original magnification, $\times 400$; Bar, 50 μm).

Nitrogen Mustard

PPARα

Figure 4. Effects of nitrogen mustard on PPARα expression

Histological sections, prepared after exposure of mice to control (CTL) or 1, 2, 3, 4, and 5 days after exposure of mice to NM, were stained with antibody to peroxisome proliferator receptor alpha (PPARα). One representative section from 3 mice/treatment group is shown. Antibody binding was visualized using a Vectastain Elite ABC kit (original magnification, $\times 400$; Bar, 50 μm). Epidermis (E), dermis (D), hair follicles (H), sebaceous glands (S), eschar (ES), parakeratosis (PK). Solid arrow indicates epidermal expression of PPARα; open arrow

indicates PPAR α expression in hair follicles; arrowhead indicates PPAR α expression in sebaceous glands; asterisk indicates inflammatory infiltrate within the eschar.

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Nitrogen Mustard

FAAH

Figure 5. Effects of nitrogen mustard on FAAH expression

Histological sections, prepared after exposure of mice to control (CTL) or 1, 2, 3, 4, and 5 days after exposure of mice to NM, were stained with antibody to fatty acid amide hydrolase (FAAH). Antibody binding was visualized using a Vectastain Elite ABC kit. One representative section from 3 mice/treatment group is shown (original magnification, $\times 400$; Bar, 50 μm). Epidermis (E), dermis (D), hair follicles (H), sebaceous glands (S), eschar (ES). Solid arrow indicates epidermal expression of FAAH; open arrow indicates FAAH expression in hair follicles; arrowhead indicates FAAH expression in sebaceous glands.

Figure 6. Effects of sulfur mustard on CB1 receptor expression

Histological sections, prepared after exposure to control (CTL) or 1, 3, 7, 14 and 21 days after exposure of mice SM, were stained with antibody to CB1 receptor. One representative section from 3 mice/treatment group is shown. Antibody binding was visualized using a Vectastain Elite ABC kit (original magnification, $\times 400$; Bar, 50 μm). Epidermis (E), dermis (D), hair follicles (H), sebaceous glands (S), eschar (ES). Solid arrow indicates epidermal expression of CB1 receptors; open arrow indicates CB1 expression in hair follicles;

arrowhead indicates CB1 expression in sebaceous glands; asterisk indicates inflammatory infiltrate within the eschar.

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Sulfur Mustard

CB2 Receptor

Figure 7. Effects of sulfur mustard on CB2 receptor expression

Histological sections, prepared after exposure to control (CTL) or 1, 3, 7, 14 and 21 days after exposure of mice to SM, were stained with an antibody to CB2 receptors. One representative section from 3 mice/treatment group is shown. Antibody binding was visualized using a Vectastain Elite ABC kit (original magnification, $\times 400$; Bar, 50 μm). Epidermis (E), dermis (D), hair follicles (H), sebaceous glands (S), eschar (ES). Solid arrow indicates epidermal expression of CB2 receptors; open arrow indicates CB2 expression in hair follicles; arrowhead indicates CB2 expression in sebaceous glands.

Sulfur Mustard

PPAR α

Figure 8. Effects of sulfur mustard on PPAR α expression

Histological sections, prepared after exposure to control (CTL) or 1, 3, 7, 14 and 21 days after exposure of mice to SM, were stained with antibody to PPAR α . One representative section from 3 mice/treatment group is shown. Antibody binding was visualized using a Vectastain Elite ABC kit (original magnification, $\times 400$; Bar, 50 μ m). Epidermis (E), dermis (D), hair follicles (H), sebaceous glands (S), eschar (ES), parakeratosis (PK). Solid arrow indicates epidermal expression of PPAR α ; open arrow indicates PPAR α expression in hair follicles; arrowhead indicates PPAR α expression in sebaceous glands.

Sulfur Mustard

FAAH

Figure 9. Effects of sulfur mustard on FAAH expression

Histological sections, prepared after exposure to control (CTL) or 1, 3, 7, 14 and 21 days after exposure of mice to SM, were stained with antibody to fatty acid amide hydrolase (FAAH). Antibody binding was visualized using a Vectastain Elite ABC kit. One representative section from 3 mice/treatment group is shown (original magnification, $\times 400$; Bar, 50 μm). Epidermis (E), dermis (D), hair follicles (H), sebaceous glands (S), eschar (ES). Solid arrow indicates epidermal expression of FAAH; open arrow indicates FAAH expression in hair follicles; arrowhead indicates FAAH expression in sebaceous glands.

			FAAH	CEES	
Compound	Name	R	IC ₅₀ (μM) ¹	% Inhib. ²	cLogP ³
4453	Phenethyl		14	80	2.80
4452	2-Phenoxyethyl		31	51	2.64
4455	Cyclohexylmethyl		93	40	3.35
4464	Morpholinethyl		>800	4	1.04

1. Concentration of compound inhibiting FAAH activity by 50%.
2. Percent inhibition of vesicant-induced edema.
3. cLogP, a measure of compound hydrophobicity, was determined using ChemBioDraw Ultra Version 12.0 (Cambridge Soft, PerkinElmer Informatics, Waltham, MA).

Figure 10. Activity of vanillyl alcohol carbamates as inhibitors of inflammation in the mouse ear vesicant model

Left panel, effects of increasing concentrations of vanillyl alcohol carbamates on FAAH activity. Right panel, comparison of the ability of vanillyl alcohol carbamates to inhibit FAAH activity and vesicant-induced inflammation in the mouse ear vesicant model. ¹Concentration of compound inhibiting FAAH activity by 50%. ²Percent inhibition of vesicant-induced edema. ³cLogP, a measure of compound hydrophobicity, was determined using ChemBioDraw Ultra 12.0 software (CambridgeSoft, Perkin Elmer Informatics, Waltham, MA).