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Letter to the Editor

Inhibition of fatty acid amide hydrolase exerts cutaneous anti-inflammatory effects both *in vitro* and *in vivo*

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Background

Numerous studies introduced epidermal keratinocytes as 'non-classical' immune-competent cells, hence potent primary regulators and active participants of cutaneous immune functions (1), (s1–6). Therefore, targeting them might provide a novel, highly specific anti-inflammatory therapeutic possibility. The endocannabinoid system (ECS) is an emerging signalling network which regulates multiple cutaneous functions (2,3). The loss of homoeostatic endocannabinoid (eCB) signalling of epidermal keratinocytes was shown to dramatically enhance inflammatory processes, arguing for that the cutaneous eCB tone plays a 'gatekeeper' role in the initiation phase of skin inflammation (4); for further details see Supplementary Background section). Moreover, elevation of the eCB tone, for example by the inhibition of fatty acid amide hydrolase (FAAH), the most important enzyme engaged with the degradation of the eCB anandamide (AEA); (5), exerts ECS-mediated anti-inflammatory actions in multiple organs (5).

Questions addressed

Based on these data, we hypothesized that upregulation of expression/activity of FAAH [thereby decreasing the eCB tone, and increasing the level of the pro-inflammatory 'eCB degradation product' arachidonic acid (AA)] might contribute to the development of the inflammatory processes. Therefore, we aimed at investigating (i) mRNA and protein expressions and activity of FAAH in human keratinocytes in Toll-like receptor (TLR)-induced inflammation models and (ii) the suggested anti-inflammatory effects of two newly developed, potent and selective N-alkylcarbamate FAAH inhibitors WOBE440 (IC_{50} = 25 ± 8 nM) and WOBE479 (IC_{50} = 78 ± 13 nM) (Fig. S1) which show high specificity over other known targets within the ECS [IC_{50} > 10 μM for cannabinoid receptor (CNR)-1, CNR2, monoacylglycerol lipase and the putative endocannabinoid membrane transporter] (6) on primary (NHEK) and immortalized (HPV-KER) (7,8) human epidermal keratinocytes as well as in NC/Tnd mice, a widely used animal model of atopic dermatitis (AD) (9).

Experimental design

Detailed description of the methods can be found in the Supplementary Experimental design section.

Cell culture

Human skin samples were obtained after obtaining written informed consent from healthy individuals, adhering to Helsinki guidelines, and after obtaining permissions from respective institutional and government bodies (protocol No.: DE OEC RKEB/IKEB 3724-2012; document No.: IX-R-052/01396-2/2012).

Determination of cellular viability, apoptosis and necrosis

Viability and cell death were determined by MTT and $DilC_1(5)$-SYTOX Green assays as described previously (s11).

Expression analysis

Molecular expression was monitored by Q-PCR and Western blot as described previously with slight modifications (s11). The released amount of IL6 and IL8 was determined using OptEIA kits (BD Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer's protocol.

Determination of the FAAH activity

The enzymatic activity of FAAH in NHEK and HPV-KER homogenates was assessed by determination of the hydrolysis of [ethanolamine-1-^{3}H]AEA as previously described (s12–16).

Experiments on NC/Tnd mice

The study was conducted at BioTox Sciences (BTS; San Diego, CA, USA). The study design and animal usage were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC; No. 1109-05). Clinical score was determined from observations of the upper back/lower neck with a scale of 0–3 (0: absent; 1: mild; 2: moderate; 3: severe) for erythema, oedema or papulations, and for oozing, crusts or haemorrhages. Each mouse received a single daily topical dose that was applied to the upper back/lower neck area.

Statistical analysis

Data were analysed and graphs were plotted by using Origin Pro Plus 6.0 software (Microcal, Northampton, MA, USA), using Student's two-tailed two samples *t*-test and $P < 0.05$ values were regarded as significant differences.

Results

We found that FAAH is expressed in HPV-KERs both at the mRNA and protein levels (Fig. S2; Fig. 1a–b). Importantly, we also found that upon administration of TLR2 or -4 activators [lipoteichoic acid (LTA) and lipopolysaccharide (LPS), respectively], expression of FAAH at the protein level tended to be increased. Interestingly, at the mRNA level, only 3-hr LPS treatment induced significant increase (Figs. 1a–b, S2), and in NHEKs, only LTA was able to enhance the expression (Fig. 1c–d). Importantly, both HPV-KERs and NHEKs exhibited significant elevations in FAAH activity upon TLR2 activation (Fig. 1e–f). The above findings raised the possibility that the decreased eCB tone mediated by FAAH upregulation might theoretically contribute to the development of the TLR-activation-induced pro-inflammatory responses in the human skin. To challenge this hypothesis, next, we investigated the putative anti-inflammatory effects of selective FAAH inhibitors, by monitoring the production of key pro-inflammatory cytokines (*IL1A*, *IL1B*, *IL6* and *IL8*; s17–19). As expected, both the commercially available FAAH inhibitor URB597 (s20), as well as novel inhibitors (WOBE440 and WOBE479) almost completely prevented the LTA-induced upregulation of the aforementioned cytokines (Fig. 2a–b). Importantly, they also significantly suppressed the LTA-induced release of IL6 and IL8 (Fig. 2c–d). FAAH inhibitors exert their effects via the elevation of the local AEA tone, which subsequently activates CNR1 and CNR2 (5). In a good agreement with the literature data (4), combined antagonism of these receptors markedly abolished the anti-inflammatory actions of the FAAH inhibitors (Fig. S3). Importantly, concentrations of FAAH inhibitors having been proven to exert anti-inflammatory actions (i.e. 100 and 200 nM for WOBE440 and WOBE479, respectively) did not induce any measurable cytotoxicity when applied either in short-term (8 and 24 h; Fig. S4a–d) or in long-term experiments (72 h; Fig. S5a–b), indicating that they can most probably be administered without the risk of significant cutaneous cytotoxicity. Finally, we aimed at investigating the putative *in vivo* efficiency of the FAAH inhibitors, employing a widely accepted animal model of AD, that is the NC/Tnd mice (9). After appropriate antigen exposure and the development of AD-like cutaneous symptoms, mice were treated with the novel FAAH inhibitors or vehicle, as described in the Supplementary Experimental Design section. As a 'positive' control, tacrolimus ointment (Protopic®; commonly administered in the treatment of AD; s21) was used. Of great importance, FAAH inhibitors significantly reduced both the total disease score (for details, see Supplementary Experimental design section) and ear thickness. It is also important to note that beneficial effects of the FAAH inhibitors were comparable to those exerted by tacrolimus (Figs. 2e–f; S6a–d) and that no (obvious macroscopic or behavioural) side effects developed during the 1-month-long drug administration. These data strongly suggest that inhibition of FAAH results in substantial anti-inflammatory actions *in vivo* as well.

Conclusions

For detailed discussion, see Supplementary Conclusion section 1. Taken together, our results introduce FAAH as an important, TLR2-dependent regulator of cutaneous inflammatory processes (Fig. S7a–b). Thus, according to our translationally relevant, complementary *in vitro* and *in vivo* data, local inhibition of FAAH could provide a highly targeted and hence most probably side effect-free tool for mitigating cutaneous inflammation (further discussion of the expected beneficial effects can be found in the Supplementary Conclusion section 2). Therefore, these data should encourage one to test, next in appropriate clinical trials the *in vivo* efficiency of the FAAH inhibitors in the management of inflammatory skin diseases, such as AD.

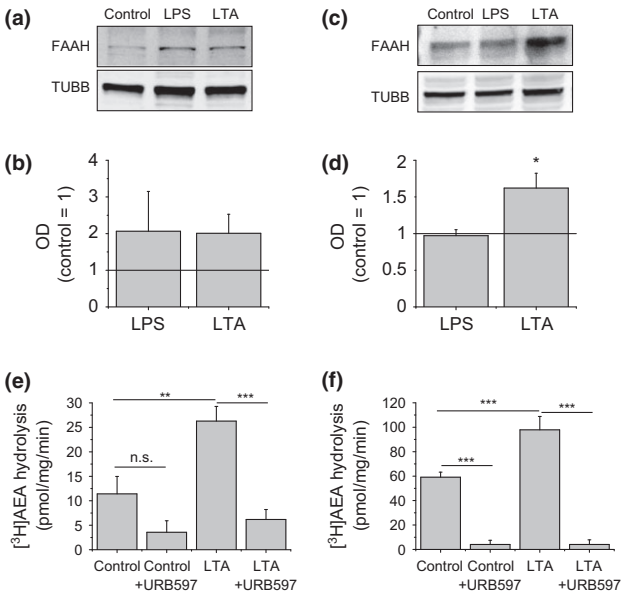

Figure 1. Protein expression and activity of FAAH is upregulated upon Toll-like receptor activation in immortalized (HPV-KER) and primary (NHEK) human epidermal keratinocytes. (a–d) Western blot analysis of lysates of HPV-KERs (a) or NHEKs (c) treated with LPS, LTA (5 and 10 μg/ml, respectively) or vehicle (24 h). Two additional experiments yielded similar results. (b, d) Statistical analyses of the above Western blot experiments (c: HPV-KER; d: NHEK). OD: optical density of the FAAH bands normalized to the corresponding β-tubulin (TUBB) signals. Data are presented as mean ± SEM of 3 independent experiments regarding vehicle control as 1 (solid line). *$P < 0.05$ vs. control group. (e-f) Measurement of FAAH activity on HPV-KERs (e) and on NHEKs (f) following 24-h treatments with LTA (10 μg/ml) or vehicle. Specific inhibition of FAAH activity ([^{3}H]AEA hydrolysis) was achieved with URB597 (100 nM). Data are expressed as mean ± SEM of three independent experiments each of them performed in triplicates. **, ***$P < 0.01$ and 0.001, respectively; n.s.: non-significant difference.

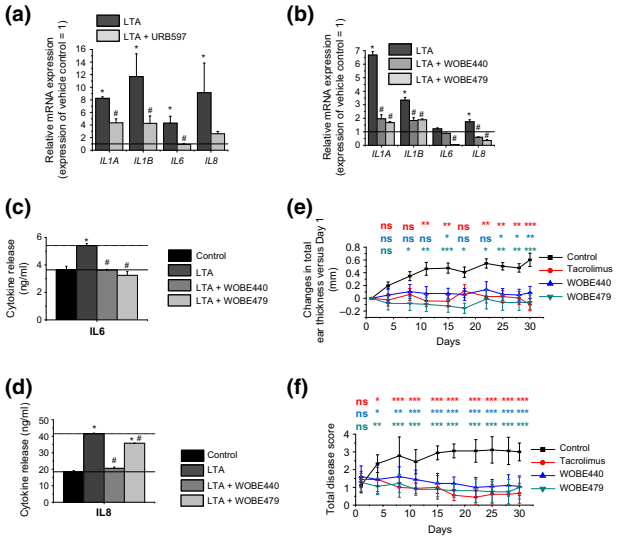

Figure 2. FAAH inhibitors exert significant anti-inflammatory actions both *in vitro* and *in vivo*. (a-b) Q-PCR analyses of HPV-KERs following the indicated 24-h treatments (LTA: 10 μg/ml; URB597: 100 nM; WOBE440: 100 nM; WOBE479: 200 nM). Data are presented using $\Delta\Delta$CT method regarding *PPIA*-normalized mRNA expressions of the vehicle control as 1 (solid line). Data are expressed as mean ± SD of 3 independent determinations. Two additional experiments yielded similar results. (c–d) Determination of the released cytokine concentration following 24-h treatments (LTA: 10 μg/ml; WOBE440: 100 nM; WOBE479: 200 nM). Data are presented as mean ± SEM of three independent determinations. One additional experiment yielded similar results. (a–d) $^{*}P < 0.05$ compared to the vehicle control. $^{\#}P < 0.05$ compared the LTA-treated, FAAH inhibitor-free group. (e–f) Alterations in total ear thickness (e) and in total disease score (f) following the indicated treatments (tacrolimus: 0.1 [w/v]%; WOBE440 and −479: 1 [w/v]%). *, ** and *** mark significant ($P < 0.05$, 0.01 or 0.001, respectively) differences of the graphs with the same colour compared to the daily control group. $N = 8–9$ animals were investigated in each group.

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Authors' contribution

AO and LA performed *in vitro* experiments and analysed the data. SN and JG performed FAAH activity assays. AO wrote the manuscript. AO, MS, CA and TB designed the research study, and all authors reviewed the manuscript. LK and VT provided HPV-KERs; MS and CA provided WOBE440 and WOBE479. All authors read and approved the final version of the manuscript.

Conflict of interest

This study was supported by an industrial research grant (see Acknowledgement), and two of the authors (MS and CA) are employees of the sponsor. AO was employed by the sponsor between 02/01/2014 and 01/31/2015.

Supporting Information

Additional supporting data may be found in the supplementary information of this article.

Data S1. Supplementary Background, design and conclusion.
Data S2. Supplementary References
Figure S1. Chemical structures of the novel FAAH-inhibitors WOBE440 and WOBE479 (6).
Figure S2. FAAH is expressed in HPV-KERs at the mRNA level.
Figure S3. FAAH-inhibitors exert remarkable anti-inflammatory actions via activating CNR1 and CNR2 receptors.
Figure S4. Effective anti-inflammatory concentrations of the FAAH-inhibitors are not cytotoxic.
Figure S5. Effective anti-inflammatory concentrations of the novel FAAH-inhibitors are not cytotoxic even in case of long-term application.
Figure S6. Topically applied FAAH-inhibitors improve symptoms of NC/Tnd mice.
Figure S7. Overview of the role of FAAH in mediating cutaneous inflammation.

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