Inhibition of microglial fatty acid amide hydrolase modulates LPS stimulated release of inflammatory mediators

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Abstract Anandamide and other fatty acid amides are metabolised by the enzyme fatty acid amide hydrolase (FAAH), which thereby regulates their endogenous levels. Here we demonstrate that cultured rat cortical microglia express FAAH at low levels. The potent FAAH inhibitor URB597 reduced the LPS stimulated microglial expression of cyclo-oxygenase 2 and inducible nitric oxide, with concomitant attenuation of the release of PGE2 and NO. Additional of supplemental exogenous anandamide did not increase the magnitude of attenuation of mediator release. The effect of URB597 on LPS stimulated PGE2 release was not blocked by selective CB1 or CB2 receptor antagonists.
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1. Introduction

Fatty acid amide hydrolase (FAAH) rapidly degrades endogenous fatty acid amides including anandamide (arachidonyl ethanolamine; AEA) and palmitoylethanolamine (PEA), both of which have analgesic and anti-inflammatory properties [4,1]. Comparisons of the content of anandamide and palmitoyl ethanolamine in the brains of wild type and FAAH knockout mice identify FAAH as a major regulator of the in vivo concentrations of these fatty acid amides [4,3].

Microglia express several of the known targets of AEA and PEA [17,11,19], and respond to anandamide by suppression of various inflammatory responses [15,5]. In addition, cultured microglia have been shown to synthesis anandamide, albeit at low levels compared with the more abundant endocannabinoid 2-arachidonylglycerol [6]. A recent study [12] showed expression of both FAAH activity and activity of another endocannabinoid-metabolising enzyme, monoacyl glycerol lipase (MGL), by the BV2 microglial cell line and by primary mouse microglia.

Here we extend these observations to show not only that cultured rat microglia also express functional FAAH, but also that inhibition of the enzyme by the FAAH inhibitor URB597 [14] resulted in a significant down regulation of the expression of the LPS-induced enzymes cyclo-oxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS; NOS2). These effects were accompanied by a concomitant reduction in the release of the inflammatory mediators prostaglandin E2 (PGE2) and (NO) nitric oxide. Selective CB1 or CB2 receptor antagonists did not block these effects, suggesting that they are mediated via other targets of the active mediator whose concentration is elevated by FAAH inhibition.

2. Methods

2.1. Microglial cultures and experimental treatments

Purified cultures of microglial were prepared using a method modified from that of Giulian and Baker [7], as described in [16]. Four hours after plating at 37 °C they were treated with either 10 or 20 μM URB597 (Cayman Chemical) in the presence or absence of the selective CB1 and CB2 antagonists SR141716A and SR144528, or vehicle (0.05% DMSO/0.06% ethanol). Cells were treated with compounds for 120 min prior to addition of 0.03 μg/ml of lipopolysaccharide (LPS) from Sigma–Aldrich (L3129). After 16 h at 37 °C supernatants were collected for biochemical assays. The microglia remaining attached to the floor of the wells were used immediately to assay for nitric oxide production.

2.2. FAAH and MGL enzyme assay

FAAH activity of microglial cells growing in 96 well plates was determined by the method of Wilson et al. [18] performed directly in the culture wells using a final [³H]AEA concentration of 160 nM and a final concentration of 10 μM Cay 10400 [1]; (Cayman Chemical Co, Ann Arbor, Michigan) to set blank values. MGL was assayed using [³H] 2-mono-oleoyl glycerol (80 nM) as substrate in the presence of 10 μM Cay 10400 to block FAAH activity.

2.3. RNA preparation and PCR

Total RNA was purified and reverse transcribed, after which PCR was carried out with the Expand High Fidelity PCR system (Cat# 1759078, Roche, Indianapolis, IN, USA). The FAAH forward primer was CTATGGTGTCCCTGTGAGCCT, and the reverse primer was GAGGGGTCATCAGCTGTTCCAC (predicted band size 1301 bp). The amplification was carried out for 40 cycles at 62 °C as the annealing temperature. Rat FAAH cDNA from recombinant cells was used as the positive control, no reverse transcription and water as negative controls.

2.4. Immunofluorescence

Immunofluoresence procedures were as described in [16]. Primary antibodies and dilutions were: Cayman rabbit anti-FAAH, 1/250; Serotec mouse anti-CD11b, 1/50; Wako rabbit anti-Iba, 1/250; Secondary antibodies were Alexa-fluor anti-mouse or anti-rabbit Ig as appropriate.

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2.5. Biochemical assays

Nitric oxide release from cultured microglia was assessed on live cells using 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate ;Molecular Probes, USA) in the procedure developed by Kojima et al. [8] using excitation and emission wavelengths of 490 nm and 520 nm. Prostaglandin E_2 (PGE_2) and TNF levels were analyzed using ELISA kits from Cayman and R&D Systems, respectively.

2.6. Immunoblotting

Microglia growing in six well plates were pretreated with 20 μM URB597 followed by 0.03 μg/ml LPS. After 16 h 20–30 μg of solubilised protein from each sample was analyzed by immunoblotting for the expression of iNOS and COX-2 using GAPDH as a loading control. Primary antibodies were: iNOS (BD Biosciences 1:1000), COX-2 (Cayman Chemical 1:27,000) and GAPDH (Abcam1:1000).

2.7. Statistics

Unless otherwise specified in the text or figure legends, all values are expressed as mean ± standard error of mean (S.E.M., $n = 3$ individual experiments each with three to six replicates). Data were analyzed by analysis of variance (ANOVA) followed by post hoc analysis (Dunnett's test, Prism 4.0, GraphPad, San Diego, CA) and statistical significance inferred at $P < 0.05$.

3. Results

3.1. Expression of FAAH by cultured microglia

By PCR, a diagnostic band of 1301 bp length was seen in RNA samples prepared from either untreated or LPS treated microglia, but was absent if water was substituted for RNA in the cDNA synthesis reaction, or if reverse transcriptase was omitted from the initial reaction (Fig. 1a).

Both control and LPS treated cells hyrolysed tritiated anandamide in a reaction blocked by the selective FAAH inhibitor Cay 10400 (Fig. 1b). The standard assay buffer includes Triton, so the cells are permeabilised during the assay. In permeabilised cells, this hydrolysis was sensitive to inhibition by URB597 with an IC_{50} of about 10 nM (Fig. 1c). Under conditions mimicking the physiological experiments (incubation in tissue culture medium followed by assay of residual enzyme activity) this IC_{50} was shifted to 100 nM, with concentrations in the low micromolar range being required to achieve inhibitions greater than 90%. We detected MGL activity as reported by Muccioli et al. [12], but unlike these
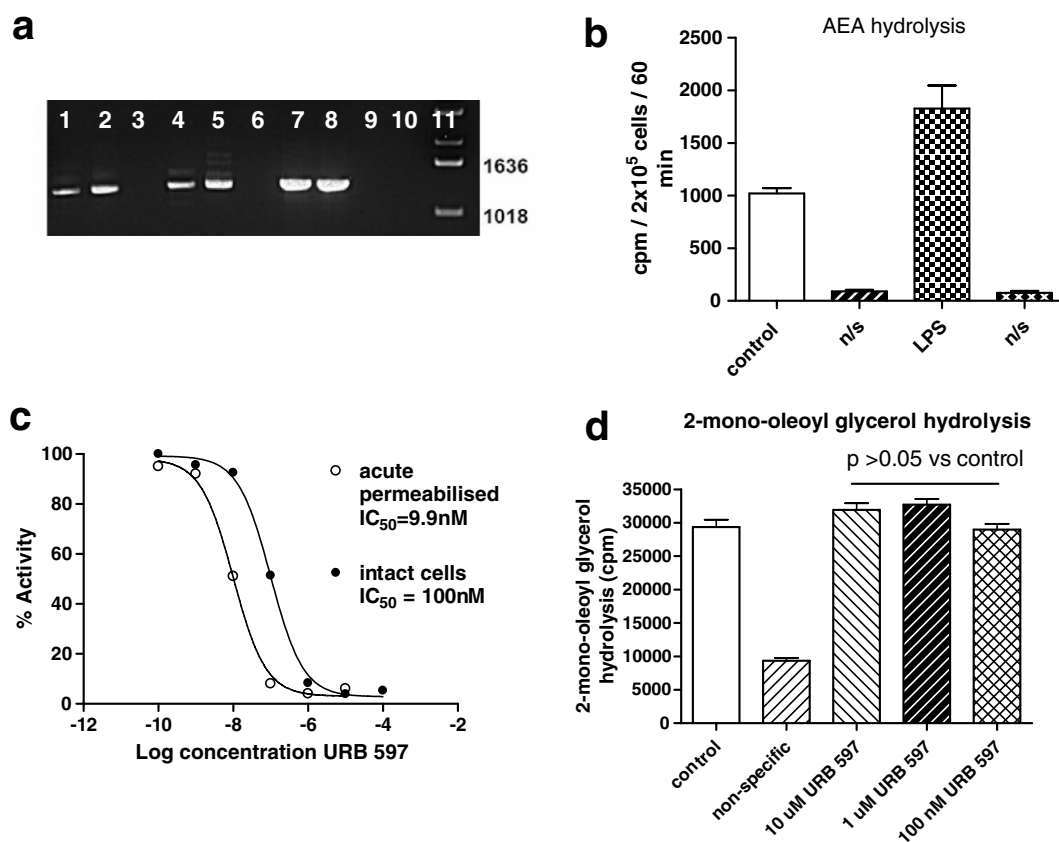

Fig. 1. FAAH expression by microglia. (a) rtPCR detection of FAAH mRNA. Samples were run as duplicate reactions: 1, 2 – untreated microglial RNA; 3 – Microglia control omitting reverse transcriptase; 4, 5 – LPS treated microglial RNA; 6 – LPS treated microglial RNA omitting reverse transcriptase; 7, 8 – SH-K-NM recombinant cells transfected with rat FAAH mRNA; 9 – recombinant FAAH RNA omitting reverse transcriptase; 10 – PCR reaction with water substituted for RNA; 11.1Kb marker ladder. (b) Expression of FAAH enzyme activity by cultured microglia. Data expressed as c.p.m. per 2×10^5 cells for a 60 min reaction. NS – non-specific activity (10 μM of the potent selective FAAH inhibitor CAY10400). (c) Determination of apparent IC_{50} of URB597 in FAAH activity assay on acutely permeabilised microglial cultures or intact cells preincubated with compound in tissue culture medium. IC_{50} values were 9.9 and 100 nM, respectively. (d) Monoacyl glycerol lipase activity was assayed using [^{3}H] 2-mono-oleoyl glycerol (80 nM) as substrate in the presence of 10 μM Cay 10400 to block FAAH activity. Since no selective inhibitors for this enzyme yet exist, non-specific activity was determined by assaying hydrolysis of cells briefly fixed in 4% paraformaldehyde.

authors, we found that this activity was insensitive to concentrations of URB597 up to 10 μM (Fig. 1d).

More than 95% of the cells stained with the microglial markers CD11b or Iba (Fig. 2a,b). As shown in Fig. 2c, a low but specific staining was seen with the Cayman anti-rat FAAH antibody in essentially all cells. This staining was not seen if first antibody was omitted (Fig. 2d), and co-localised with CD11b expression (Fig. 2e,f). Since almost all of the cells are microglia, and almost all stain with FAAH antibody, and co-localisation

with a microglial marker was observed, we conclude that FAAH is indeed expressed by microglia in these cultures.

3.2. URB597 inhibition of LPS stimulated COX2 expression and PGE2 release

For all the experiments reported here, LPS was used at a concentration of 0.03 μg/ml.

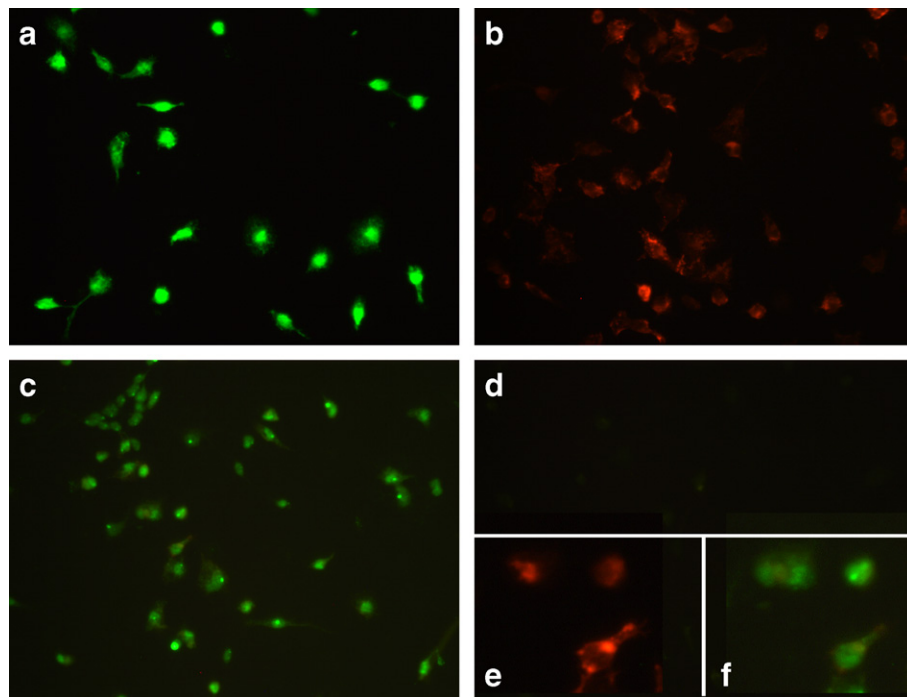The vehicle used for addition of URB597 and the selective CB1 and CB2 antagonists was DMSO (final concentration

Fig. 2. Immunofluorescent detection of FAAH in single microglia. Greater than ninty-eight percent of the cells in the enriched cultures were stained with the selective microglial markers CD11b (a) or iba (b). Fewer than two percent of the cells in the cultures were small GFAP$^+$ astrocytes at times less than 24 h after plating. Low but distinct and specific FAAH activity was detected in essentially all the cells in the culture (c). Omitting the first antibody abolished this fluorescence (d, same exposure as c). (e, f) Three cells in which FAAH staining is co-localised with CD11b staining.
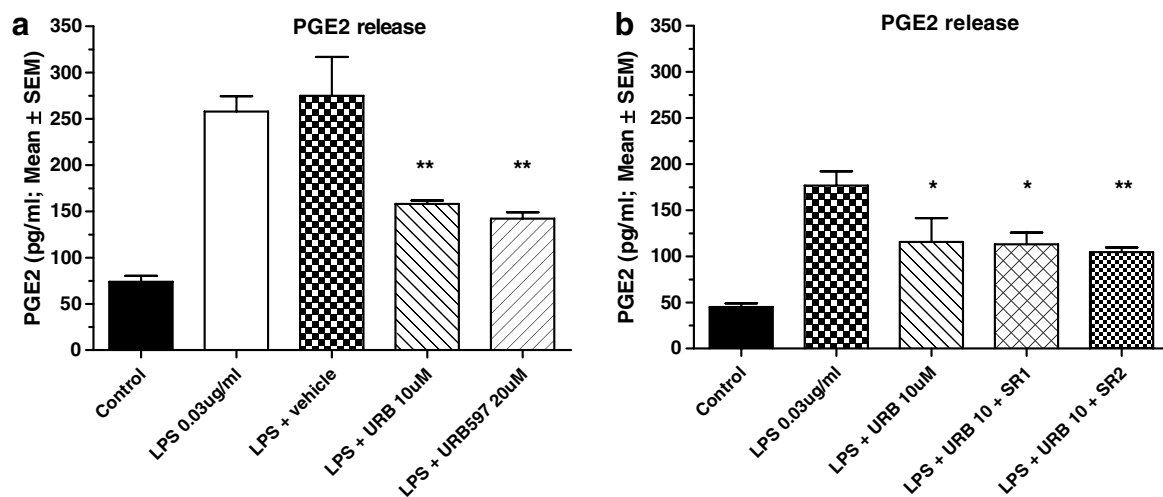

Fig. 3. Effect of URB597 on LPS stimulated PGE2 release. (a) Inhibition of LPS stimulated PGE2 release by URB 597 (10 or 20 μM). (b) URB 597 mediated attenuation of LPS stimulated PGE2 release was not blocked by either SR141716A (SR1) or SR144528 (SR2). **$P < 0.01\%$ vs. LPS; *$P < 0.05\%$ vs. LPS.
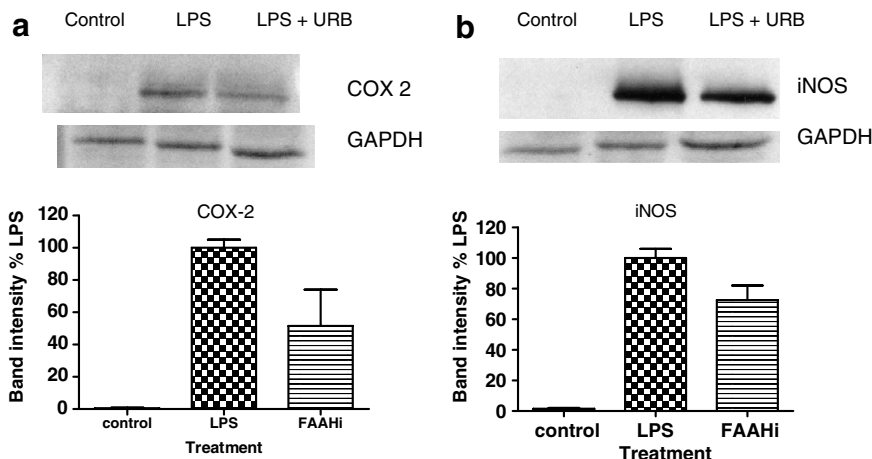
Fig. 4. Effect of URB 597 on LPS induced COX-2 and iNOS expression. Upper panels: immunoblots of samples from control, LPS treated, and LPS + URB597 (20 μM: FAAHi) treated microglia. GAPDH was used as a loading control. Lower panels; blot quantitation from $n = 2$ independent experiments with mean and range.

0.025% per addition) and that for anandamide was ethanol (final concentration 0.6%). A mix of 0.1% DMSO and 0.6% ethanol had no effect on the levels of release of any of the mediators we examined.

Stimulated levels of PGE2 were elevated approximately 4-fold over unstimulated controls (Fig. 3), corresponding to a strong up regulation of COX2 expression (Fig. 4a). Treatment with 10 μM URB597 reduced the release of PGE2 to approximately 50% of control values, but we saw no reproducible effect at 2.5 or 5 μM. Neither addition of 20 μM anandamide nor increasing the URB597 concentration significantly increased the degree of inhibition. Concomitant treatment with the selective CB1 receptor antagonist SR 141716A or the CB2 antagonist SR144528 (both at 10 μM) did not reverse the effect of URB597 (Fig. 3b) Treatment with 20 μM URB597 during the stimulation period reduced the expression of COX2 to a mean of 51% of LPS treated controls.

3.3. URB597 inhibition of LPS stimulated TNF and NO release and iNOS expression

LPS treatment elevated NO release 15–20-fold over basal levels corresponding to a strong increase in iNOS expression

Table 1
Summary of mediator release experiments

Assay	10 μM URB	10 μM URB + 20 μM AEA	20 μM URB
PGE2	51 (11.3); $n = 3$	47.5; $n = 1$	56; 61 $n = 2$
NO	71 (16.4); $n = 3$	64; 73; $n = 2$	67; $n = 1$
TNF	79.2 (10); $n = 4$	72; $n = 1$	74 (5.3); $n = 3$

Figures are percentage inhibitions of mediator release by URB 597 at 10 μM with or without additional anandamide (20 μM) supplement, or 20 μM URB 597 without additional anandamide compared with LPS stimulated microglia in the same experiment; brackets are standard deviations, n = total number of experiments for specified condition.
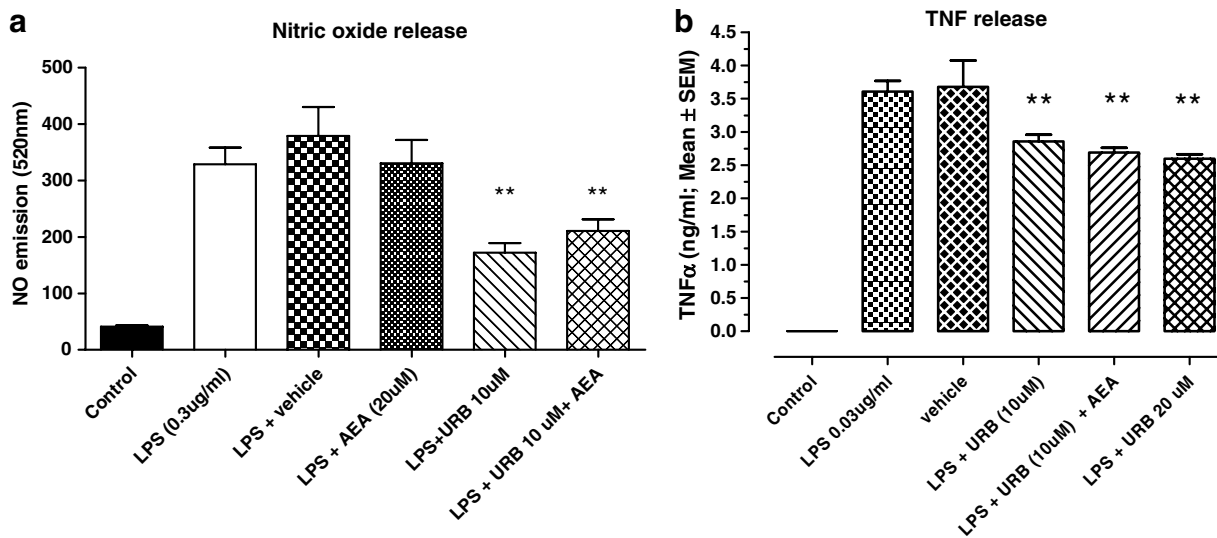

Fig. 5. Effect of URB597 on LPS stimulated NO and TNFα release. URB = URB597; AEA = anandamide. **$P < 0.01\%$ vs. LPS; *$P < 0.05\%$ vs. LPS.

(Figs. 4b, 5a and Table 1). URB597 at 2.5 or 5 µM added at the time of LPS stimulation produced no reduction in NO release (not shown), while 10 µM URB597 produced somewhat variable inhibition between experiments (71% S.D. = 16.4; n = 3; Table 1). In two separate experiments, supplementing 10 µM URB597 with 20 µM anandamide produced a similar mean level of inhibition but with lower inter-experiment variability (64%; 73%). Increasing the concentration of URB597 to 20 µM did not significantly increase the degree of inhibition observed. URB597 treatment throughout the induction period reduced the expression of the iNOS band to a mean of 70% of LPS treated controls (Fig. 4b) in immunoblotting experiments. TNF release was also attenuated modestly (Fig. 5b and Table 1).

4. Discussion

While this work was in progress, the expression of FAAH by the microglial cell line BV2 and by primary cultured mouse microglia was reported [12]. We demonstrated that cultured rat primary microglia also express FAAH using three approaches, PCR, the detection of pharmacologically characteristic enzyme activity, and immunofluoresence on individual cells. All approaches concurred in suggesting that the enzyme is expressed, albeit at relatively low levels, by both unstimulated and LPS treated microglia. We used immunofluorescence methods to show that essentially all cells in the cultures (∼95% of which are microglia) express the enzyme, and that FAAH staining co-localised with cells expressing microglial markers.

URB597 treatment of the microglia concomitant with LPS treatment reduced the release of PGE2 and NO, and this correlated with a reduced expression of the enzymes COX2 and iNOS, respectively. Preliminary experiments indicated URB597 concentrations of 10 µM were required to see these effects reproducibly, and no significant potentiation of the effect was seen by concomitant addition of exogenous anandamide (20 µM). In acute anandamide hydrolysis assay of permeabilised cells, URB597 had an IC_{50} value of about 10 nM, which was shifted to 100 nM, if we exposed the cells to the compound under conditions mimicking the physiological inhibition experiments. Although we needed concentrations of URB597 of 10 µM to achieve significant and reproducible inhibition of PGE2 and NO release, it should be noted that at this IC50, low micromolar concentrations would be required to inhibit 90% or more of this activity. Such levels of inhibition may be required before significant accumulation of substrate occurs (our unpublished observations). Similar to the report of Muccioli et al. [12], we found that microglia expressed a MGL-like activity, but in our hands this was insensitive to URB 597 concentrations up too 10 µM. We therefore, believe that it is most likely that inhibition of FAAH, rather than microglial MGL, is responsible for the anti-inflammatory effects of URB 597 we observed.

Selective CB1 or CB2 antagonists did not abolish inhibition of PGE2 release by URB 597. Anandamide is known to be an agonist at additional non-CB1 non-CB2 targets some of which are expressed by microglia [2,10], so this observation is not inconsistent with a mechanism by which FAAH inhibition results in accumulation of anandamide (or other endocannabinoid) which then mediates the observed reduction in mediator release. The hypothesis that FAAH-regulated lipids can exert a cannabinoid receptor-independent effect on inflammatory responses is supported by the non-CB receptor mediated anti-oedema effects seen in FAAH knockout mice after formalin injection [9].

We did not test the effect of selective CB receptor antagonists on release of NO or TNF, so it remains a possibility that they may play some role in the release of these mediators.

The maximum levels of inhibition we observed were of the order of 40–50% for both PGE2 and NO, and, more modestly, 25% for TNFα. These values are similar to those reported for microglial iNOS expression and NO release by Ortega-Gutti-erez et al. [13], using the putative anandamide transporter UCM707. Whether such levels of inhibition could translate into a therapeutically useful effect in any given pathological condition can only be evaluated in vivo, where the microglia are functioning in a complex milieu including other cellular sources of fatty acid amide mediators.

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