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# 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.**


*Keywords:* FAAH; Fatty acid amide hydrolase; Fatty acid amide; Endocannabinoid; Microglia; Cannabinoid receptor; iNOS; Nitric oxide; Cyclo-oxygenase 2; Prostaglandin E2; TNF; URB597

## 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 [$^3$H]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 [$^3$H] 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

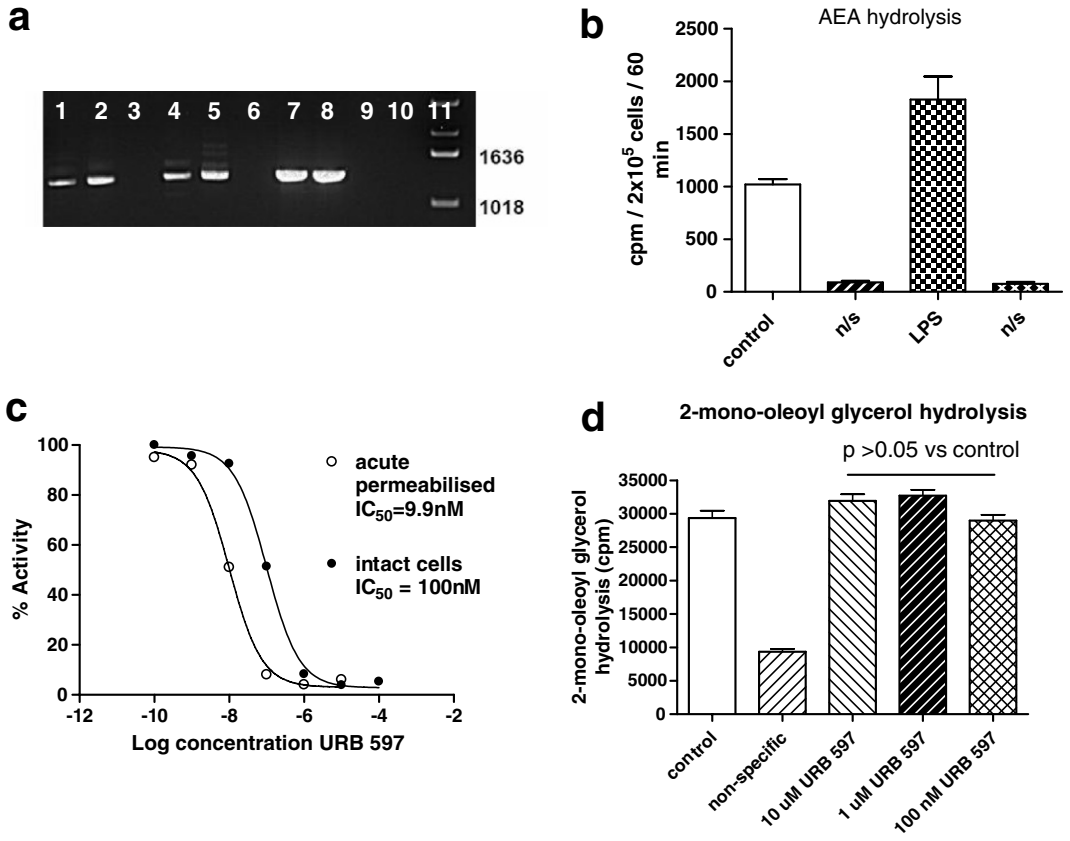


Fig. 1. FAAH expression by microglia. (a) rtPCR detection of FAAH mRNA. Samples were run as duplicate reactions: 1, 2 – untreated microglial RNA; 3 – Microglia control omitting reverse transcriptase; 4, 5 – LPS treated microglial RNA; 6 – LPS treated microglial RNA omitting reverse transcriptase; 7, 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 $2 \times 10^5$ 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.