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Microsomal epoxide hydrolase is not a 2-arachidonyl glycerol hydrolase

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Disciplines
Physiology

Keywords
Endocannabinoids
Metabolism

Type of Observation
Follow-up

Type of Link
Contradictory data

Submitted Apr 5, 2016
Published Jun 21, 2016

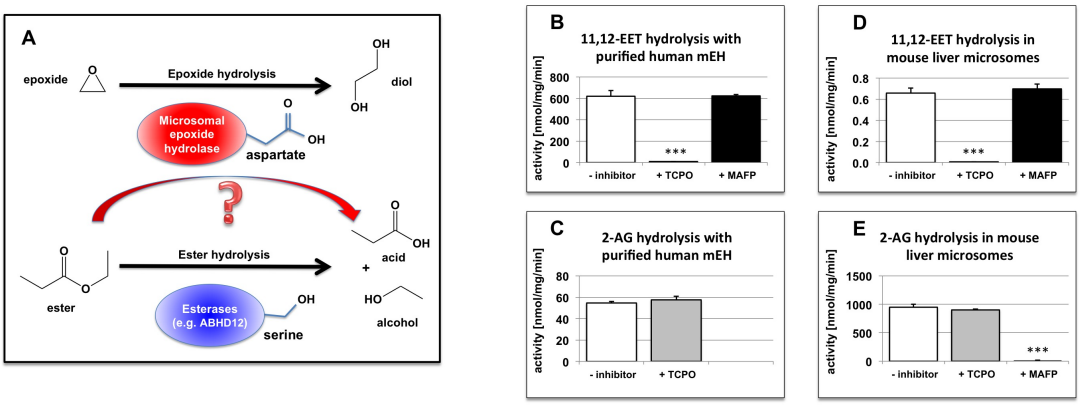
Abstract
The endocannabinoid 2-arachidonyl glycerol (2-AG) is substantially hydrolysed by at least two enzymes, fatty acid amide hydrolase (FAAH) and monoarachidonyl glycerol lipase (MAGL), which thereby terminate its biological activity. In a recent report it has been claimed that microsomal epoxide hydrolase (mEH), hitherto known as a xenobiotic detoxifying enzyme, also rapidly catalyses the breakdown of 2-AG. However, the catalytic site architecture of mEH argues against an esterase activity. We therefore analysed the capacity of recombinant purified human, mouse and rat mEH to hydrolyse 2-AG. In contrast to the previous finding, we find only marginal 2-AG esterase activity (≤50 nmol/mg protein/min) associated with the purified enzymes that was resistant to inhibition by the potent mechanism-based mEH inhibitor 1,1,1-trichloropropene 2,3-oxide (TCPO). Likewise, 2-AG hydrolysis in mouse liver microsomes was resistant to TCPO inhibition while being efficiently blocked by methyl arachidonyl fluorophosphonate (MAFP). MAFP, on the other hand, failed to inhibit epoxide hydrolase activity of both, purified mEH and mouse liver microsomes. We therefore conclude that mEH lacks any appreciable 2-AG hydrolase activity.

Introduction
In a recent publication, Nithipatikom and colleagues [1] claimed that the xenobiotic-metabolising enzyme microsomal epoxide hydrolase (mEH) [2] efficiently hydrolyses the endocannabinoid 2-arachidonyl glycerol (2-AG) [3]. Endocannabinoid signalling is important for a variety of physiological processes such as pain sensation [4], cognition and emotion [5] and appetite regulation [6]. Thus the capability of mEH to control 2-AG levels would have substantial implications, also because mEH is almost ubiquitously expressed throughout the human body [7].
The study by [1] was inspired by the fact that mEH belongs to the structural superfamily of α/β hydrolase fold enzymes [8] and therefore shares structural similarity to a wide range of esterases and lipases [9]. Two of these, ABHD6 and ABHD12, have recently been shown to possess 2-AG hydrolase activity [10].
In their experiments, the authors used microsomes of cell lines (over)expressing mEH. Microsomes are membrane preparations obtained by differential centrifugation of cell or tissue homogenates and contain essentially all the membrane-associated proteins that are not confined to large organelles such as nuclei and mitochondria. Therefore, the contribution of mEH to the total protein content of these microsomes probably does not exceed 1%. Unfortunately, the authors do not give quantitative measures for this, but the fact that they use 30 µg microsomal protein for their western blot analyses is in agreement with the above estimate. In their turnover experiments, the authors observed 2-AG hydrolysis in microsomes that, after subtraction of the significant background reaction observed in non-transfected material, correlated with the degree of mEH expression and was responsive to siRNA mediated mEH down-regulation and inhibition by small molecules, the most potent of these being methyl arachidonyl fluorophosphonate (MAFP). From their results, they conclude that mEH is a novel 2-AG hydrolase.
Some facts cast doubt on the interpretation of the study: 1) Although mEH is indeed a member of the α/β hydrolase fold enzyme superfamily, it belongs to a sub-class that differs in the most important component of its catalytic machinery from esterases and lipases: While the active site catalytic nucleophile of lipases/esterases is a serine that forms a covalent intermediate with the acid component of its ester substrates [11], epoxide hydrolases carry an aspartic acid at this position, forming an ester intermediate with the alcoholic component of its substrates [12] (Fig. 1A; Fig. S1 for a more detailed explanation). 2) The control membrane preparations used in the study possess already a high background activity for 2-AG hydrolysis that is not attributable to mEH, FAAH or

MAGL, and the increase in activity, where it can be taken from the authors' presentation, is only a two- to three fold gain, 3) the strongest inhibitor identified in the study, MAFP, is known as a powerful inhibitor of PLA2-type lipases [13], yet no potency to inhibit mEH has so far been demonstrated, and finally 4) neither 2-AG hydrolytic activity nor sensitivity to MAFP inhibition of purified mEH has been tested by the author, which would be a definitive proof for their claim.

Objective

We therefore set out to re-assess the potential activity of mEH as a 2-AG hydrolase by directly testing this activity with purified mEH.



a

Figure Legend

Figure 1.

(A) The separate types of α/β hydrolase fold enzymes use different catalytic residues to hydrolyse different types of substrates: while epoxide hydrolases cleave the ether bond in epoxides with an aspartic acid side chain, esterases hydrolyse esters using a serine side chain (for further details see Fig. S1). Epoxide hydrolase activity of purified human mEH **(B)** and of mouse liver microsomes **(D)** is sensitive to TCPO inhibition and resistant to MAFP treatment. In contrast, 2-AG hydrolase activity of purified human mEH **(C)** and of mouse liver microsomes **(E)** is resistant to TCPO treatment, while this activity in liver microsomes is strongly inhibited by MAFP. The column heights give the mean of three separate measurements. Error bars indicate the standard deviation. ***p <0.001.

NB: MAFP sensitivity of 2-AG hydrolase activity associated with the human mEH purified from bacteria was not tested because of the lack of relevance: the bacterial enzyme obviously contributing to this activity (see our collective results) may or may not be inhibited by the compound. Either result would not have any impact on our conclusions.

Enzyme preparation

Microsomal epoxide hydrolase from rat, mouse and human was expressed from pET20b(+)-based expression constructs in *E. coli* BL21AI as C-terminally His-tagged proteins and purified essentially as described earlier [16] [17] [14]. Microsomes were prepared by differential centrifugation of homogenates from the livers of 6 week old male sEH -/- mice [18] according to standard protocols [19].

Substrate turnover

For substrate turnover, 10–50 ng of purified mEH, 8 µg microsomal protein (epoxide hydrolase activity) or 400 ng microsomal protein (2-AG hydrolase activity) were preincubated in a 1.5 ml eppendorf cup with or without 5 mM TCPO or 50 µM MAFP in 10 µl reaction buffer (Tris-HCl, 20 mM, NaCl, 100 mM, gelatine, 0.1%, pH 7.4) for 5 min at room temperature. Thereafter, the reaction was initiated by the addition of substrate, either 11,12-EET (final concentration 4 µM) or 2-AG (final concentration 100 µM), in 40

µl reaction buffer and the incubation was continued for 10 min at 37°C. Thereafter, the reaction was terminated by the addition of 50 µl acetonitrile. After centrifugation at 13'000 rpm for 5 min at room temperature, 80 µl of the supernatant were transferred to a crimp top vial, sealed and subjected to LC-MS/MS analysis that was performed on a 4000 QTRAP mass spectrometer (AB Sciex) connected to an Agilent 1100 HPLC system.

HPLC separation of analytes

20 µl of sample were injected per run. Analytes were separated on a reverse phase column (Gemini 5 µM NX-C18, 110 Å, 150×2 mm), using HPLC grade water + 0.0125% NH_3 (solvent A) and acetonitrile + 5% methanol + 0.0125% NH_3 (solvent B) as the eluents at a flow rate of 350 µl/min, with one of the following protocols.

Procedure 1 (11,12-EET and 11,12-DHET quantification after turnover experiments with pure enzymes). 1) isocratic elution at 84% solvent A for 0.1 min; 2) linear gradient to 74% solvent A within 1.9 min; 3) linear gradient to 64% solvent A within 7 min; 4) linear gradient to 5% solvent A within 0.7 min; 5) isocratic flow at 5% solvent A for 1.6 min; 6) linear gradient to 84% solvent A within 0.1 min; 7) isocratic flow at 84% solvent A for 3.6 min. Under these conditions, the product 11,12-DHET eluted at around 7 min and the substrate 11,12-EET eluted at around 10 min.

Procedure 2 (11,12-EET, 11,12-DHET and arachidonic acid quantification after turnover experiments with microsomes) 1) isocratic elution at 84% solvent A for 0.1 min; 2) linear gradient to 74% solvent A within 1.9 min; 3) linear gradient to 64% solvent A within 16 min; 4) linear gradient to 5% solvent A within 0.7 min; 5) isocratic flow at 5% solvent A for 1.6 min; 6) linear gradient to 84% solvent A within 0.1 min; 7) isocratic flow at 84% solvent A for 4.6 min. Under these conditions, 11,12-DHET eluted at around 7 min, 11,12-EET eluted at around 10 min and arachidonic acid eluted at 15–16 min.

Procedure 3 (arachidonic acid and 2-AG quantification after turnover experiments with purified human mEH; produces higher signal intensities with arachidonic acid using pure enzyme but cannot be used for microsomal turnover experiments, due to a pronounced matrix effect obscuring the arachidonic acid signal). 1) isocratic elution at 30% solvent A for 0.1 min; 2) linear gradient to 20% solvent A within 1.9 min; 3) linear gradient to 1% solvent A within 8 min; 4) isocratic flow at 1% solvent A for 3 min; 5) linear gradient to 30% solvent A within 2 min; 6) isocratic flow at 30% solvent A for 2 min. Under these conditions, the substrate 2-AG eluted at around 6.4 min and the hydrolytic product arachidonic acid eluted at around 1.5 min. Under these conditions, a strong matrix effect was observed after 2-AG hydrolysis with microsomes that heavily masked the arachidonic acid signal while leaving the 2-AG signal unaffected. The respective samples were therefore in addition analysed with Procedure 2.

MS-based quantification of analytes

All analytes were detected and quantified by multiple reaction monitoring (MRM) in the negative mode on a 4000 QTRAP equipped with an ESI source. For 11,12-EET, the parental ion had an M/Z of 319.2. The fragments with an M/Z of 301.1 and 167.1 were used as qualifier and quantifier, respectively. For 11,12-DHET, parental ion, qualifier and quantifier had an M/Z of 319.2, 168.9 and 166.8, respectively. For arachidonic acid, parental ion, qualifier and quantifier had an M/Z of 303.2, 58.9 and 259.2, respectively. For the identification of 2-AG, the analytical parameters for arachidonic acid were used, because the significant decay of 2-AG to arachidonic acid under the ionisation conditions employed resulted in a much stronger signal for this decay product than for the parent compound itself which is difficult to ionise using ESI. Entrance potential (EP), declustering potential (DP), collision energy (CE) and collision exit potential (CXP) for the different analyses were the following: 11,12-EET qualifier (EP -10, DP -70, CE -16, CXP -7); 11,12-EET quantifier (EP -10, DP -65, CE -18, CXP -13); 11,12-DHET qualifier (EP -10, DP -70, CE -26, CXP -11); 11,12-DHET quantifier (EP -10, DP -70, CE -26, CXP -11); arachidonic acid qualifier (EP -10, DP -80, CE -28, CXP -13); arachidonic acid quantifier (EP -10, DP -80, CE -22, CXP -11).

Quantification was achieved by relating the signal intensities to those obtained with external standards of known concentrations.

Data documentation

Examples of all types of analyses are provided as supplementary material to demonstrate the sensitivity of the employed analytical procedures. Figure S2 shows the purity of re-

combinant rat mouse and human mEH, as well as representative ion chromatograms for the 2-AG turnover to arachidonic acid. Figure S3 shows representative chromatograms of purified human EH 2-AG and 11,12-EET turnover in the presence and absence of inhibitors while figure S4 shows the same type of analyses with mouse liver microsomes.

Statistical evaluation

The effect of inhibitor treatment on the respective enzyme activities in the individual preparations was statistically evaluated by running student's t-tests (unpaired, two-tailed). All measurements were done in triplicates, resulting in a degree of freedom of 4 for each comparison. Because 7 individual comparisons were made (see below), the threshold for statistical significance was set to $p < 0.007$ after Bonferroni correction.

Figure 1B, 11,12-EET hydrolysis with purified human mEH:

solvent <-> TCPO: $t(4) = 19.23$, $p < 0.0001$

solvent <-> MAFP: $t(4) = 0.093$, $p = 0.9306$

Figure 1C, 2-AG hydrolysis with purified human mEH:

solvent <-> TCPO: $t(4) = 1.353$, $p = 0.2475$

Figure 1D, 11,12-EET hydrolysis in mouse liver microsomes:

solvent <-> TCPO: $t(4) = 24.53$, $p < 0.0001$

solvent <-> MAFP: $t(4) = 1.061$, $p = 0.3848$

Results & Discussion

Recombinant expression and His-tag-based metal chelate chromatography purification yielded purified mouse, rat and human mEH protein that displayed turnover rates with the prototypic substrate 11,12-epoxyeicosatrienoic acid (11,12-EET) of 0.1, 0.3 and 0.65 µmol per mg protein per minute, respectively. On SDS polyacrylamide gel electrophoresis and subsequent Coomassie staining the recombinant proteins displayed the expected dominant signal at 52 kDa (Fig. S2A), with some minor impurities amounting to 14, 18 and 27% for the rat, mouse and human enzyme, respectively, as determined by densitometric quantification.

2-AG turnover with purified rat, mouse and human mEH revealed relatively poor, apparently TCPO-resistant enzymatic activity (Fig. S2B-G) that roughly correlated with the degree of impurity of the enzyme preparation. The most active preparation was that of human mEH with an estimated turnover rate of 50 nmol arachidonic acid formed per milligram protein per minute. Because the activity reported by [1] for human mEH was several orders of magnitudes higher (see last paragraph of this section for a comparison), these results already demonstrate that mEH does not possess substantial 2-AG hydrolase activity. However, to clarify whether the measurable 2-AG hydrolase activity in our recombinant purified enzymes might be attributed to any impurities of the preparation rather than to mEH itself, we refined the analysis of enzymatic turnover in the presence and absence of 1,1,1-trichloropropene 2,3-oxide (TCPO), a potent mEH inhibitor. This compound acts as a suicide substrate, characterised by an extremely slow hydrolysis rate of the covalent intermediate formed in the first step. Therefore, any activity of the enzyme requiring the catalytic nucleophile is efficiently blocked by a sufficient TCPO concentration. In the presence of 1 mM TCPO, 99% of the human mEH enzymatic activity were blocked using 4 µM 11,12-EET as substrate (Fig. 1B; the Km for human mEH with 11,12-EET is reported to be 0.4 µM [14]). By contrast, 2-AG hydrolysis by human mEH remained essentially unchanged with 100 µM 2-AG (Fig. 1C; the Km with 2-AG is 40 µM according to [1]). This shows that 2-AG hydrolytic activity is not associated with the mEH protein itself, but is most probably due to minor impurities of bacterial esterases in our enzyme preparation. In addition, we tested MAFP for its capability to block mEH hydrolase activity. With an IC50 of 7.9 nM this compound was identified as the most potent inhibitor by [1] to block what they proposed to be mEH-mediated 2-AG hydrolysis. However, 11,12-EET hydrolysis by purified human mEH remained unaffected in the presence of 10 µM MAFP (Fig. 1B).

To test whether the native membrane environment in the endoplasmic reticulum provides essential components for any mEH-mediated 2-AG hydrolytic activity which might explain the lack of the respective enzymatic activity with the purified enzyme, we next analysed 2-AG and 11,12-EET hydrolysis in microsomes prepared from the liv-

ers of sEH -/- mice, lacking the mEH sister enzyme soluble epoxide hydrolase (sEH), the presence of which might otherwise interfere with the efficacy of TCPO to inhibit 11,12-EET hydrolysis. In the absence of any inhibitor, the microsomes showed the expected hydrolytic activities with 11,12-EET (0.66 nmol/mg/min; Fig. 1D) and 2-AG (950 nmol/mg/min; Fig. 1E). Again, EET hydrolysis was highly sensitive to TCPO inhibition and resistant to MAFP treatment (Fig. 1D), while the opposite effect was observed with 2-AG hydrolysis (Fig. 1E).

The 2-AG hydrolase activity reported by [1] is very high. From figure 4 of their manuscript, an enzymatic activity of around 300 nmol 2-AG being hydrolysed per minute and milligram microsomal protein can be deduced. Given the above estimate that at best 1% of the microsomal enzyme can be attributed to mEH, this would imply that mEH has a specific 2-AG hydrolase activity of ≥30 µmol per milligram per minute. We do not find activity with purified mEH from rat, mouse or human in excess of 50 nmol per milligram per minute. On top, the inhibitor sensitivity of both the 2-AG hydrolysis by purified mEH enzyme and the mouse liver microsomes is incompatible with its origin from mEH, but suggests that even this minor activity in our preparations originates from bacterial enzymes that have not completely separated from the mammalian proteins during purification.

Conclusions

Our results clearly demonstrate that mEH lacks any appreciable 2-AG hydrolase activity. With respect to the observations made by [1], we suggest as an alternative explanation that mEH might activate a so far uncharacterised MAFP-sensitive 2-AG hydrolase.

The next obvious step is to identify the 2-AG hydrolase that is apparently activated by mEH, as well as the mechanism by which this activation takes place. According to the report by Nithipatikom et al., this hydrolase can neither be FAAH nor MAGL. In our view, the potential candidates are ABHD6, ABHD12, CES1 and CES2 due to their reported significant capacity to hydrolyse 2-AG [10] [15].

Additional Information

Methods

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Supplementary Material

Please see https://sciencematters.io/articles/201605000008.

Funding Statement

This work was funded by the Swiss National Foundation, grant n° 31003A_146635.

Acknowledgements

We are gratefully to Dr. Frank Gonzalez for providing us with his sEH -/- mouse strain.

Ethics Statement

Not applicable.

Citations

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