Mini-Review

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Measuring Endocannabinoid Hydrolysis: Refining our Tools and Understanding

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Abstract. Endocannabinoids (eCBs) are lipid transmitters that are released from membrane precursors in response to specific stimuli, activate cannabinoid receptors—the molecular targets of compounds produced by *Cannabis sativa*—and are then rapidly inactivated by uptake and enzymatic hydrolysis. This signaling system is implicated in a wide range of biological processes, including pain sensation, immunomodulation, appetite regulation, development, and cognitive and emotional states. The balance between eCB release and inactivation determines the extent of eCB accumulation, with enzymatic hydrolysis functioning as an important limiting step. Pharmacological inhibition of eCB-hydrolyzing enzymes offers great therapeutic and experimental promise for enhancing this ubiquitous signaling system only where and when these transmitters are naturally produced. The following mini-review summarizes the latest developments concerning eCB-hydrolyzing enzymes, with an emphasis on the techniques used to measure their activities and how these have helped increase our understanding of the role that eCBs play in regulating fundamental biological functions.

KEYWORDS: ABHD6; ABHD12; endocannabinoid; FAAH; MGL.

N-arachidonoyl ethanolamine (AEA, also known as anandamide) was the first endogenous ligand found to interact with cannabinoid (CB) receptors (1,2). The principal enzyme responsible for its degradation is fatty acid amide hydrolase (FAAH), an integral membrane protein that inactivates AEA by hydrolysis, producing arachidonic acid and ethanolamine (3). In 1996, Benjamin Cravatt and colleagues affinity-purified FAAH from rat liver, identified its coding sequence and, over the course of the subsequent decade, thoroughly characterized its biological function, biochemical characteristics, and pharmacology (3). Significant advances were made with the development of highly selective compounds that inhibit FAAH at nanomolar concentrations *in vivo* (such as the carbamate URB597) and through the generation of FAAH knockout mice, both of which result in an approximate ten-fold elevation in endogenous levels of brain AEA (4,5). These achievements have revealed the fundamental role that FAAH plays in controlling AEA signaling *in vivo*. Both the pharmacological and genetic inactivation of FAAH produce a subset of CB1-dependent biological responses, including analgesic, anxiolytic, antidepressant, sleep-enhancing, and anti-inflammatory

effects in rodents, all without the undesirable side effects on motility, body mass, and body temperature that typically accompany direct CB1 agonism (6). Thus, FAAH inhibition constitutes an exciting area of research and a promising therapeutic target.

FAAH is a member of the serine hydrolase family of enzymes. However, instead of the Ser-His-Asp catalytic triad utilized by the majority of serine hydrolases, FAAH's active site is composed of the Ser-Ser-Lys catalytic triad that is characteristic of the amidase-signature class of enzymes (6). Structural studies suggest that AEA reaches the FAAH active site by first entering into the membrane and then moving through the "acyl chain-binding" channel of FAAH, which is composed of hydrophobic residues and is thought to participate in substrate recognition (7,8). Meanwhile, a water molecule likely also gains access to the FAAH active site *via* a second channel known as the "cytoplasmic port." With Ser241 acting as the nucleophile, the FAAH catalytic triad hydrolyzes AEA, and then the resulting arachidonic acid and ethanolamine molecules exit through the membrane-access and cytosol-access channels, respectively (6). The Ser241 nucleophile is the target of many different pharmacological inhibitors of FAAH, including URB597. As the FAAH inhibitor of choice for many studies, URB597 has demonstrated an impressive array of potentially therapeutic effects in rodents, but it suffers from a rather short duration of action *in vivo* (2–3 h) (9), and from some off-target interactions with enzymes in peripheral tissues (10).

A challenging requirement for clinical drug discovery is high target selectivity. The pharmaceutical industry has historically resisted designing irreversible enzyme inhibitors as drug candidates because of concerns about increasing off-target effects, which may lead to adverse side effects. Fortunately, newly

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developed technology now allows researchers to address this concern experimentally. In large part, this is due to the development of activity-based protein profiling (ABPP) by the Cravatt laboratory (11), a technology that allows for the concomitant measurement of multiple protein activities, rather than focusing on the activity of one enzyme or merely quantifying the abundance of proteins. This functional proteomics approach utilizes promiscuous active site targeting to screen drugs against entire classes of enzymes within complex tissue samples (12). Indeed, determining the selectivity of serine hydrolase inhibitors (including those that target eCB-hydrolyzing enzymes) by conventional substrate-based assays would be a difficult task considering both the tremendous size of the superfamily of serine hydrolase enzymes (>200 members in humans) and the fact that many of these enzymes are uncharacterized and thus lack known substrates. As such, ABPP promises to significantly accelerate this important endeavor. In brief, this technology involves two main steps: a rapid initial assay of total enzymatic activity, followed by a more detailed analysis of specific enzymes of interest. First, partially purified tissue fractions are incubated with a fluorophosphonate-based probe tagged with rhodamine, which covalently binds to the active site of all serine hydrolases present in the fraction and labels them fluorescently. Tagged enzymes are then separated by gel electrophoresis and identified by their molecular weight, with the intensity of the emitted fluorescence reflecting their relative abundance. When this assay is performed on the same sample in both the presence and absence of an inhibitor, a decrease in fluorescent intensity in the presence of inhibitor indicates that this target is sensitive to the inhibitor. The second step also uses a fluorophosphonate-based probe, but this time tagged with biotin. The incubation of partially purified fractions with this second probe allows for the efficient purification of tagged serine hydrolases by “pulling them down” with streptavidin beads. This is followed by enzymatic digestion and sequencing of specific enzymes by LC-MS-MS, leading to their molecular identification and thorough quantification.

A series of recent reports clearly illustrate the power of ABPP. Various FAAH inhibitors were assayed for their specificity in different tissues, and despite being selective for FAAH in brain, many were found to possess additional targets in peripheral tissues such as liver and kidney (6). Notably, the piperidine urea-based inhibitor PF-750 demonstrated remarkable selectivity for FAAH in multiple human and mouse tissue proteomes, showing no discernible activity against other serine hydrolases *in vitro* or *in vivo* even at relatively high concentrations (13). One thing to keep in mind is that the fluorophosphonate probes used in ABPP exhibit slightly different pharmacological and biochemical properties at an enzyme's active site compared to a given substrate, and thus it is important to verify key results with more conventional methods that directly assess substrate hydrolysis. Nevertheless, ABPP constitutes a powerful new technology that is waiting to be adopted by many laboratories.

Another major hurdle in moving new drugs from bench to bedside stems from the well-known pharmacological differences between orthologous enzymes, and the fact that pre-clinical trials and *in vivo* efficacy data are based on rodent studies (and thus might not automatically translate into humans). Indeed, much of the progress made in developing FAAH inhibitors is based on structural and kinetic data obtained with the rat ortholog of this enzyme (rFAAH), and key differences are present in the human ortholog (hFAAH) (14). Unfortunately, efforts to obtain structural

information on hFAAH have been hampered by low expression yields in recombinant systems and challenging biochemical properties. Mileni *et al.* recently addressed this concern by using site-directed mutagenesis to engineer a “humanized” rat FAAH (h/rFAAH) that contains the human active site within the parent rat protein (14). This mutated enzyme exhibits the pharmacological profile of hFAAH while maintaining the high recombinant expression yields and biochemical properties of rFAAH. In addition to validating h/rFAAH as a useful assay surrogate for hFAAH, the authors also solved its tridimensional structure at a 2.75-Å resolution and elucidated the structural basis for the increased potency (7.6-fold) of PF-750 at hFAAH *versus* rFAAH. To the extent that the availability of h/rFAAH accelerates the process of clinical drug development for inhibitors of AEA hydrolysis, it will serve as an example to translational medicine more broadly.

In contrast to the impressive progress made in the characterization and manipulation of AEA hydrolysis, this process has proceeded much more slowly with regard to the second well-known eCB, 2-arachidonoyl glycerol (2-AG) (15,16). Despite being discovered after AEA, 2-AG has emerged as the prototypical eCB in many ways: It is much more abundant and efficacious at CB receptors than AEA (17); the biological machinery for the production and inactivation of 2-AG is logically organized around CB receptors (18); and 2-AG has proven to be a central player in the ubiquitous phenomenon of eCB-dependent synaptic plasticity (19) and in the regulation of neuroinflammation (20). As such, selective manipulation of 2-AG hydrolysis *in vivo* is an exciting goal with considerable therapeutic potential.

Several lines of evidence implicate monoacylglycerol lipase (MGL) as the primary enzyme mediating 2-AG hydrolysis in the nervous system: Recombinant expression of MGL reduces receptor-dependent 2-AG accumulation in cortical neurons (21); immunodepletion of MGL significantly decreases 2-AG hydrolysis activity in rat brain tissue (22); and ABPP of serine hydrolases in mouse brain ascribed ~85% of the total 2-AG hydrolysis activity to MGL (23). MGL is a ~33-kDa protein that functions most efficiently at a pH near 8.0 (24). It hydrolyzes monoglycerides, but not di- or triglycerides (24). It hydrolyzes medium- and long-chain monoacylglycerols at variable rates, with the highest rate of hydrolysis being observed for arachidonoylglycerol (regardless of whether the substrate contains a 1(3)- or 2-ester bond) (25). MGL has not yet been crystallized, but site-directed mutagenesis studies have confirmed that it utilizes the Ser-His-Asp catalytic triad that is typical for the active sites of most serine hydrolases (26). MGL is thought to contain a movable lid domain covering its active site, which opens when interacting with a lipid–water interface (26,27). This could be a reason why MGL has proven to be a challenging target for selective pharmacological inhibition, since hydrophilic compounds would not be expected to gain easy access to the active site.

One of the most common approaches used to quantify MGL activity *in vitro* is the use of radioactive substrate. The most obvious substrate for MGL is 2-AG, but the closely related molecule 2-oleoylglycerol (2-OG) is also commonly used experimentally (likely due to the efficiency with which it is hydrolyzed by MGL (21)). However, despite their similarity, 2-AG and 2-OG exhibit small, but significant, differences in their kinetics and affinities for MGL (personal communication), so caution should be used when substituting one for the other. Another point of flexibility in the radiolabeled substrate assay concerns the moiety

that is labeled. For example, when using 2-AG as substrate, either the arachidonoyl moiety or the glycerol moiety could be radiolabeled. In a typical MGL enzymatic assay, the labeled 2-AG will react with the enzyme-containing sample for a set period of time, resulting in variable rates of hydrolysis depending on the sample (and the pharmacological agents added to it). This reaction is then stopped by the addition of an aqueous/organic mixture, such as in the classic Folch extraction procedure which is based on set volume ratios of methanol and chloroform to separate the hydrophilic and lipophilic fractions (28). Thus, when 2-AG is hydrolyzed, the hydrophilic glycerol moiety is released and partitions in the aqueous phase, whereas the hydrophobic arachidonic acid (AA) moiety remains in the organic phase along with any unhydrolyzed hydrophobic 2-AG. If the glycerol moiety had been radiolabeled, then the aqueous phase can be directly recovered and amounts of hydrolyzed 2-AG quantified by simple liquid scintillation spectroscopy (for example, see (29)). On the other hand, if the AA moiety had been labeled, then the organic phase must be further fractionated by thin-layer chromatography to separate the liberated AA from the unhydrolyzed 2-AG (for example, see (17)). While more labor intensive, the latter approach can be a useful control to, for example, ensure that a hydrophobic inhibitor is indeed reducing enzymatic hydrolysis, rather than interacting directly with the radiolabeled substrate and preventing its partition into the hydrophilic phase. When labeling a substrate, one must pay special attention not to alter its integrity; thus, ^3H constitutes the radiolabel of choice. The complete absence of an artificial label is obviously ideal, and in this case measuring the released AA can be achieved by gas or liquid chromatography coupled to mass spectrometry. However, these direct analytical techniques can sometimes be challenging and time consuming (30), and often lack the quantitative precision associated with radioactive techniques.

A recent paper by Muccioli, Labar, and Lambert offers yet another approach to measuring eCB hydrolysis (31). These authors speculate that the lack of potent and specific inhibitors of MGL could be due in part to the inefficiency of the assays that are commonly used to characterize MGL activity. They therefore developed a low-cost/high-throughput colorimetric assay to screen compounds against purified recombinant human MGL (32). Using this assay, the authors identify a potent new carbamate-based inhibitor of MGL, CAY10499, which seems to utilize a novel (non-carbamate-dependent) mechanism of action (31). The availability of such an efficient method for evaluating inhibitors against human MGL is a welcome development, and CAY10499's potentially unique mechanism of inhibition certainly warrants further attention. However, while this method shows great promise for assessing potency and kinetics of drugs acting on MGL, it does not address selectivity, and thus CAY10499 will have to be tested against many other serine hydrolases. One drawback of the colorimetric approach is that the chromogenic substrate (4-NPA) likely exhibits different structural and kinetic interactions with MGL compared to 2-AG or other substrates, so it is possible that inhibitors of 4-NPA hydrolysis might not inhibit 2-AG hydrolysis in exactly the same fashion. Thus, the series of methods that we have outlined are complimentary, and ought to be utilized in parallel to maximize our understanding of MGL inhibition: some for throughput screening, some for confirmation. Regarding high-throughput screening, another very similar fluorescence-based assay for screening compounds against human MGL was also published last year (33). This assay utilizes the fluorogenic

substrate 7-HCA, and it carries the same advantages and disadvantages discussed above.

Despite having cloned MGL more than a decade ago, important tools are still lacking. Knockout mice for this gene have not yet been generated, a crystal structure of the protein has not been obtained, and only recently have promising compounds been reported to selectively inhibit MGL at nanomolar concentrations. One inhibitor, *N*-arachidonoyl maleimide (NAM), appears quite selective for MGL (23,34), but its mode of action—covalent modification of exposed cysteinyl sulfhydryl groups—is likely to preclude its therapeutic use (25). There has been a conspicuous absence of data on the role that MGL plays in hydrolyzing 2-AG *in vivo*, and new pharmacological tools would clearly help elucidate the importance of this enzyme in many of the pathophysiological processes that are modulated by eCBs. Along these lines, a recent report described a potent and selective MGL inhibitor with impressive *in vivo* efficacy (35). Using a combination of rational design and competitive ABPP, the Cravatt laboratory developed the carbamate-based inhibitor JZL184. Upon administration to mice, JZL184 produced a rapid and sustained blockade of 2-AG hydrolysis in brain, resulting in an eight-fold increase in brain 2-AG levels without altering AEA levels (35). Furthermore, JZL184-treated mice exhibited an assortment of CB1-dependent behavioral effects, including analgesia, hypomotility, and hypothermia (35). These results support the notion that 2-AG is centrally involved in a broad range of eCB-dependent signaling pathways throughout the nervous system. Taken together with previous studies, the emerging picture suggests that AEA and 2-AG have distinct but overlapping functions *in vivo*. With selective inhibitors now available for two of the principal eCB-hydrolyzing enzymes, FAAH and MGL, investigators are in a prime position to experimentally tease apart the specific functions of AEA and 2-AG in a variety of biological processes.

A complicating factor for the manipulation of 2-AG signaling *in vivo* is the existence of multiple enzymes found to hydrolyze 2-AG, the relative importance of which seems to vary between cell types (36). For example, our lab found that a microglia cell line, BV-2, efficiently hydrolyzes 2-AG in the absence of MGL, indicating that these cells express a novel enzyme capable of hydrolyzing 2-AG (29). A comprehensive profile of 2-AG-hydrolyzing enzymes in mouse brain led to the identification of two previously uncharacterized serine hydrolases capable of hydrolyzing 2-AG: ABHD12 and ABHD6 (23). These enzymes are likely to exhibit unique pharmacological and kinetic profiles, as well as different expression and localization patterns. The existence of these enzymes has potentially contributed to confusing results obtained with first-generation “MGL” inhibitors, since those compounds might not differentiate between MGL, ABHD12, and ABHD6. Interestingly, a recent study aimed at assessing the selectivity of drugs commonly used to inhibit 2-AG biosynthesis identified tetrahydrolipstatin (THL) as a relatively potent inhibitor of ABHD12 (37). Furthermore, ABPP has been utilized to develop novel inhibitors for many enzymes, including ABHD6 (38). These new drugs are likely to play critical roles in the further clarification of the complex 2-AG signaling network. Indeed, some of the functions previously ascribed to MGL could in fact be mediated by ABHD12 or ABHD6, so it will be interesting to see how significant these enzymes prove to be.

Despite their biochemical similarities, there are important differences between the pathophysiological roles of AEA and 2-AG, and it will be valuable to have the tools to manipulate them

independently *in vivo* to maximize the therapeutic potential of targeting eCB signaling. Many new techniques have been developed to help achieve this goal. ABPP is arguably the most significant addition to the arsenal of enzymatic activity assays now being used to investigate potential new drugs. This technique provides an unprecedented level of unbiased comprehensive assessment, since it can simultaneously interrogate the functionality of each and every member of entire classes of enzymes within native tissue samples. However, the initial development of quality inhibitors remains a pre-requisite, and while ABPP can make significant contributions to this endeavor as well, other assays (both new and old) can provide vital solutions where ABPP falls short. For example, new colorimetric assays allow for the high-throughput screening of large numbers of potential inhibitors of MGL. This should prove to be a valuable first screen to identify new lead compounds, which can then be explored and expanded upon with other methods, such as scintillation counting of radiolabeled 2-AG or mass spectrometry to assess 2-AG levels directly, both of which can help flesh out the details of an inhibitor's ultimate utility.

This is an exciting time for eCB signaling research. CB receptors are abundantly expressed throughout the nervous system (and peripheral tissues) where they mediate a plethora of therapeutic effects. Rather than targeting these receptors directly with exogenous agonists, efforts are underway to activate these receptors indirectly by boosting the levels of their endogenous agonists *via* inhibition of eCB hydrolysis. In addition to utilizing natural ligands, with the benefit of their hard-won evolutionary adaptations, this approach has the added advantage that the increased CB receptor activation only occurs at the subset of receptors where eCBs are being endogenously produced (this is especially important in light of the fact that CB receptors are amongst the most abundant receptors in the brain, and are thus susceptible to various side effects when direct agonists are systemically administered). With AEA hydrolysis *in vivo* being mediated by a single, well-characterized enzyme (FAAH), the development of inhibitors of AEA hydrolysis has progressed smoothly to the point where specific and potent inhibitors with good efficacy at human FAAH are reaching clinical trials. MGL, ABHD12, and ABHD6 are clearly on the same trajectory.

In summary, significant progress has been made with innovative techniques that can measure the activity of eCB-hydrolyzing enzymes, and the development of new pharmacological inhibitors is helping to increase our understanding of the intricacies of this fascinating signaling system. The emerging picture seems to be that there is a certain degree of redundancy with eCB signaling *in vivo* (both between the functionality of the two principal eCBs and with regard to 2-AG hydrolysis), but each new player in this system also brings novel properties and possibilities to the whole. We are all eager to complete the puzzle.

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