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MINIREVIEW

Inactivation and Biotransformation of the Endogenous Cannabinoids Anandamide and 2-Arachidonoylglycerol

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ABSTRACT

The cannabinoid field is currently an active research area. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the most characterized endogenous cannabinoids (also known as endocannabinoids). These neuromodulators have been implicated in various physiologically relevant phenomena, including mood (Witkin et al., 2005), the immune response (Ashton, 2007), appetite (Kirkham and Tucci, 2006), reproduction (Wang et al., 2006), spasticity (Pertwee, 2002), and pain (Hohmann and Suplita, 2006). Pharmacological manipulation of AEA and 2-AG signaling should prove to have significant therapeutic applications in disorders linked to endocannabinoid signaling. One way to alter endocannabinoid signaling is to regulate the events responsible for termination of the endocannabinoid signal-cellular uptake and metabolism. However, to pharmacologically exploit AEA and/or 2-AG signaling in this way, we must first gain a better understanding of the proteins and mechanisms governing these processes. This review serves as an introduction to the endocannabinoid system with an emphasis on the proteins and events responsible for the termination of AEA and 2-AG signaling.

Endocannabinoid Signaling

Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the two most characterized members of the endocannabinoid family. AEA and 2-AG act as agonists for both intracellularly and extracellularly localized receptors. After on-demand biosynthesis, AEA and 2-AG serve as agonists for the G protein-coupled cannabinoid receptors CB1 and CB2 and the nuclear peroxisome proliferator-activated receptor (PPAR) family members PPARα and PPARγ (Felder et al., 1993; Munro et al., 1993; O'Sullivan, 2007). AEA is also an endogenous agonist for the vanilloid receptor channel TRPV1 (Zygmunt et al., 1999; Smart et al., 2000) and the gastrin-releasing peptide 55 receptor (Pertwee, 2002; Ryberg et al., 2007; Lauckner et al., 2008). Cessation of AEA and 2-AG signaling occurs via a two-step process: 1) transport of endocannabinoids from the extracellular to the intracellular space; and 2) intracellular degradation by hydrolysis or oxidation.

Cellular Accumulation as a Mechanism for the Termination of Extracellular Endocannabinoid Signaling

Like typical neurotransmitters, endocannabinoids are translocated across the plasma membrane to cease their signaling at the extracellular cannabinoid receptors. However, the mechanism and proteins responsible for AEA and/or 2-AG transport remain elusive and hotly debated. Although some researchers have proposed that these lipophilic endocannabinoids cross the cell plasma membrane via simple diffusion through the lipid bilayer (Glaser et al., 2003, 2005; Kaczocha et al., 2006), other data indicate that the uptake process is a protein-facilitated event (Hillard et al., 1997;

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ABBREVIATIONS: AEA, anandamide; 2-AG, 2-arachidonoylglycerol; CB, cannabinoid receptor; PPAR, peroxisome proliferator-activated receptor; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; COX, cyclooxygenase; LOX, lipoxygenase; HETE-EA, hydroperoxyeicosatetraenoylethanolamide; HETE-GE, hydroperoxyeicosatetraenoic acid glycerol ester; TRPV1, transient receptor potential vanilloid receptor 1; LY2318912, 5-((4-azido-3-iodo-benzoylamino)methyl)tetrazole-1-carboxylic acid dimethylamide; AM404, *N*-(4-hydroxyphenyl)-arachidonoylamide; VDM11, *N*-(4-hydroxy-2-methylphenyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide; JZL184, 4-nitrophenyl 4-(dibenzo[*d*][1,3]dioxol-5-yl(hydroxy)-methyl)piperidine-1-carboxylate.

Beltramo and Piomelli, 2000; Hillard and Jarrahian, 2000; Rakhshan et al., 2000). Numerous studies conducted in various cell types, both of neuronal and non-neuronal origin, have characterized AEA and 2-AG uptake as being temperature-dependent, saturable, and independent of energy in the form of ion gradients or ATP hydrolysis (Hillard et al., 1997; Beltramo and Piomelli, 2000; Maccarrone et al., 2000; Rakhshan et al., 2000; Bisogno et al., 2001; Day et al., 2001; Deutsch et al., 2001; Hillard and Jarrahian, 2003; Hermann et al., 2006). However, even among those in agreement with a protein-facilitated model for endocannabinoid uptake, there remains debate concerning the precise type of protein-facilitated event responsible.

Several different models have been proposed for endocannabinoid uptake that fit under the general heading of a protein-facilitated event: 1) transmembrane carrier (Beltramo et al., 1997; Hillard and Jarrahian, 2000); 2) intracellular sequestration (Hillard and Jarrahian, 2003; Hillard et al., 2007); 3) passive diffusion driven by fatty acid amide hydrolase (FAAH) (Glaser et al., 2003); and 4) carrier-mediated caveolae-related endocytosis (Rakhshan et al., 2000; McFarland et al., 2004, 2008). The majority of these models have been suggested as a result of experiments studying AEA transport only. Evidence exists, however, which suggests that 2-AG and AEA are accumulated in cells via a common mechanism(s) (Beltramo and Piomelli, 2000). Both 2-AG and AEA uptake have been characterized as protein-facilitated events (Beltramo and Piomelli, 2000; Bisogno et al., 2001). In addition, 2-AG has been shown to inhibit AEA uptake in cells, indicating a competitive nature of the two endocannabinoids with regard to transport (Beltramo and Piomelli, 2000; Bisogno et al., 2001).

Transmembrane Carrier Protein

AEA diffusion across the lipid bilayer has been proposed to be facilitated by a membrane-localized protein carrier (Fig. 1A) (Beltramo et al., 1997; Hillard et al., 1997; Hillard and Jarrahian, 2000; Deutsch et al., 2001; Ligresti et al., 2004). Much of the evidence for the existence of a membrane-localized endocannabinoid carrier protein stems from the observation that AEA transport in cells is bidirectional (Hillard et al., 1997; Hillard and Jarrahian, 2000; Maccarrone et al., 2002; Ligresti et al., 2004). Studies conducted in both neuronal and non-neuronal cells demonstrate AEA efflux and uptake (Hillard et al., 1997; Maccarrone et al., 2002). In addition, experiments performed by Hillard and colleagues (Hillard and Jarrahian, 2000) indicate that the elusive membrane-localized AEA carrier is capable of the *trans*-flux coupling effect, a phenomenon whereby in response to extracellular AEA, the membrane-localized carrier protein accumulates at the cell surface in the extracellular-facing direction.

Intracellular Sequestration Model

The intracellular sequestration of endocannabinoids by a fatty-acid binding protein(s) is another proposed mechanism for endocannabinoid uptake suggested by Hillard and colleagues (Hillard and Jarrahian, 2003; Hillard et al., 2007) (Fig. 1B). It is noteworthy that this model simultaneously supports the proposition that AEA passively diffuses across the lipid bilayer and explains the characteristics of AEA uptake consistent with a protein-facilitated process. After the unassisted translocation of AEA across the plasma membrane, the fatty acid-derived AEA may interact with fatty acid-binding proteins (Hillard and Jarrahian, 2003). The intracellular sequestration of AEA by these binding proteins would remove AEA from the intracellular pool of "free" AEA, thus promoting the inward concentration gradient and AEA uptake (Hillard and Jarrahian, 2003).

FAAH-Driven Passive Diffusion

FAAH-mediated hydrolysis of intracellular AEA does, to some extent, drive AEA uptake (Fig. 1C). Our laboratory and others have shown that FAAH activity promotes AEA transport most likely by driving the concentration gradient along which AEA uptake occurs (Cravatt et al., 2001; Day et al., 2001; Deutsch et al., 2001). Cells devoid of FAAH show diminished AEA accumulation compared with those that basally express or overexpress FAAH protein (Day et al., 2001; Deutsch et al., 2001). In addition, recent evidence suggests that most "selective" AEA uptake inhibitors also inhibit FAAH activity (Dickason-Chesterfield et al., 2006). This revelation subsequently begged the question as to whether a specific "AEA transport protein" exists.

Intracellular enzymatic degradation is probably not solely responsible for the movement of endocannabinoids across the plasma membrane. The most compelling data arguing that FAAH alone is not responsible for endocannabinoid uptake come from work with FAAH knockout mice, in which cells and tissues devoid of FAAH are still capable of accumulating AEA in a saturable and pharmacologically manipulated man-

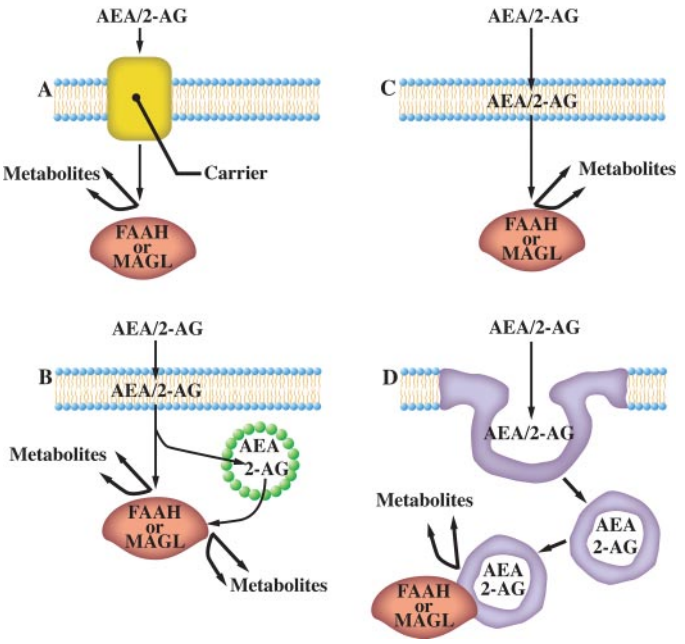

Fig. 1. Various proposed models for endocannabinoid transport. Although the majority of these models were developed based on data from AEA uptake studies, there is some evidence to suggest that AEA and 2-AG uptake occur via a common mechanism. A, a transmembrane carrier protein assists in the translocation of endocannabinoids across the plasma membrane. B, endocannabinoids passively diffuse across the plasma membrane along a catabolism-driven concentration gradient but are sequestered in an intracellular compartment or by binding to an intracellular binding protein before metabolism. C, endocannabinoids passively diffuse across the plasma membrane along a concentration gradient that is driven by their rapid metabolism. D, endocannabinoids are transported into cells via a protein carrier-mediated caveolae-related endocytic event.

ner (Fegley et al., 2004; Ligresti et al., 2004; Ortega-Gutiérrez et al., 2004). Likewise, Fowler and Ghafouri (2008) showed that 2-AG uptake is not prevented by pharmacological inhibition of 2-AG hydrolysis in all cell types, indicating possible cell-specific mechanisms for 2-AG uptake but most importantly that hydrolysis is not the sole factor mediating transport.

Perhaps some of the most convincing evidence against FAAH being solely responsible for AEA uptake comes from the development of selective AEA uptake inhibitors. Ortar and colleagues (2008) announced their development of a series of tetrazole-based selective anandamide uptake inhibitors that do not inhibit FAAH or other metabolizing enzymes, thus supporting the existence of a distinct protein target responsible for mediating endocannabinoid uptake. All of these data taken together suggest that, in addition to FAAH activity, a distinct protein-facilitated transport process is responsible for promoting the cellular accumulation of AEA.

Carrier-Mediated Caveolae-Related Endocytosis

Our laboratory has proposed that AEA uptake occurs via a protein carrier-mediated caveolae-related endocytic event (Fig. 1D) (Rakhshan et al., 2000; McFarland et al., 2004, 2008). We demonstrated that inhibition of caveolae-related endocytosis or prevention of caveolae formation led to a significant decrease in cellular AEA accumulation, thus implicating a role for these membrane microdomains in the AEA uptake process (McFarland et al., 2004, 2008). We propose that extracellular AEA binds a carrier protein located within caveolae, and that subsequently, caveolae-derived vesicle formation and endocytosis of the membrane-packaged endocannabinoid are induced (McFarland and Barker, 2004; McFarland et al., 2004, 2008). The subsequent delivery of internalized AEA to FAAH may be a critical step in freeing the carrier protein for additional AEA transport events. As described, carrier-mediated endocytosis could be used to reconcile most of the other models for endocannabinoid uptake discussed above, including the FAAH-mediated maintenance of the AEA concentration gradient, the existence of a membrane-localized AEA binding protein, and the possible sequestration of intracellular AEA.

Catabolic Degradation as a Mechanism for Terminating Endocannabinoid Signaling

After cellular uptake, AEA and 2-AG are subject to metabolism by the serine hydrolases FAAH and monoacylglycerol lipase (MAGL), respectively. In addition, AEA and 2-AG have been shown to undergo oxidation by cyclooxygenase-2 (COX-2) and the 12- and 15-lipoxygenases (12-LOX and 15-LOX) (Di Marzo, 2006).

Catalytic degradation/modification of AEA and 2-AG not only serves as a mechanism for the augmentation of cellular uptake and cessation of extracellular signaling as mentioned above, but also regulates the intracellular signaling events of these two endocannabinoids. Below, we briefly review the roles of the aforementioned enzymes in AEA and 2-AG metabolism.

Endocannabinoid Hydrolysis: FAAH1, FAAH2, and MAGL

FAAH1 and FAAH2. FAAH-mediated hydrolysis of AEA yields arachidonic acid and ethanolamine (Fig. 2A) (Deutsch and Chin, 1993). Two FAAH isoforms (FAAH1 and FAAH2) have been identified (Cravatt et al., 1996; Wei et al., 2006). The intracellularly localized FAAH1 and FAAH2 are both members of the amidase signature protein family and share approximately 20% sequence identity (Giang and Cravatt, 1997; McFarland et al., 2004; McKinney and Cravatt, 2005; Wei et al., 2006). Both isoforms are integral membrane proteins because of a single transmembrane domain on their respective N termini, although their orientation in the membrane differs (Cravatt et al., 1996; Wei et al., 2006). FAAH1 has been proposed to contain a cytoplasmic-facing C terminus, whereas the C terminus of FAAH2 faces the lumen (Wei et al., 2006). The two FAAH isoforms also vary in their expression patterns (Wei et al., 2006). FAAH1 has been cloned from several different species, including mice, rats, and humans, and is preferentially expressed in the brain, testis, and small intestine (McKinney and Cravatt, 2005; Wei et al., 2006). FAAH2 is not expressed in rodents and is the predominant isoform found in cardiac tissue (Wei et al., 2006). In addition, FAAH1 has been reported to have greater activity with regard to fatty acid ethanolamides such as AEA (Wei et al., 2006).

MAGL. Although some reports suggest that FAAH may also play a role in 2-AG degradation (Di Marzo, 2006), the major enzyme responsible for 2-AG metabolism seems to be the serine hydrolase MAGL (Dinh et al., 2002). MAGL has no sequence similarity with any member of the amidase signature protein family, including either FAAH isoform, or any other mammalian protein (Saario and Laitinen, 2007). However, MAGL does contain the α/β-hydrolase-fold common to many lipases (Saario and Laitinen, 2007). As a proposed

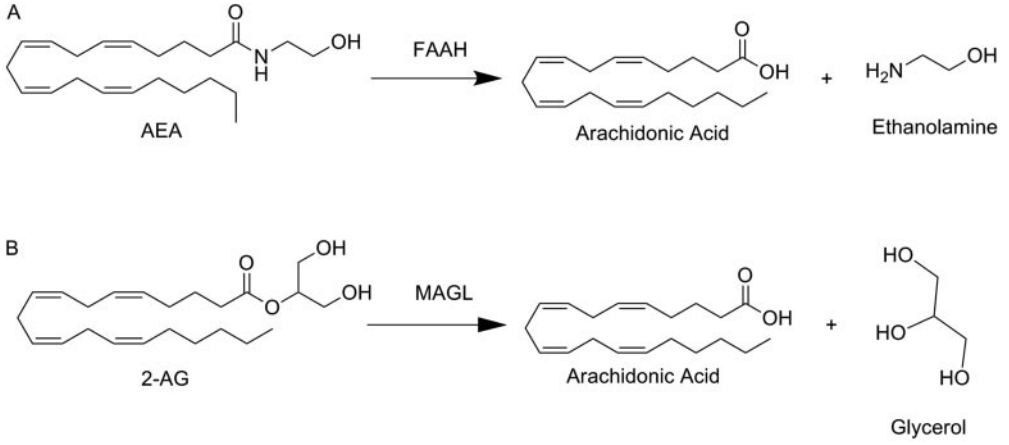

Fig. 2. Hydrolysis of the endocannabinoids AEA and 2-AG. A, FAAH catalyzes the hydrolysis of AEA into arachidonic acid and ethanolamine. B, MAGL catalyzes the hydrolysis of 2-AG into arachidonic acid and glycerol.

serine hydrolase, MAGL is capable of hydrolyzing both medium- and long-chain fatty acids (Saario and Laitinen, 2007). MAGL-mediated hydrolysis of 2-AG yields arachidonic acid and glycerol (Fig. 2B) (Karlsson et al., 1997). Overexpression and small interfering RNA-mediated knockdown of MAGL results in increased and decreased 2-AG inactivation, respectively (Dinh et al., 2002, 2004). MAGL protein is expressed in a variety of human, rat, and mouse tissues (Saario and Laitinen, 2007; Long et al., 2009).

Endocannabinoid Oxidation: COX-2 and the 12- and 15-Lipoxygenases

AEA and 2-AG are not only subject to hydrolysis, but because of their structure, they can also be metabolized by several of the same enzymes that are responsible for arachidonic acid oxidation, including COX-2 and the 12- and 15-lipoxygenases (Di Marzo, 2006).

COX-2. COX-2 is responsible for catalyzing the oxidation of AEA and 2-AG into various prostaglandin-ethanolamides (or prostamides) and prostaglandin-glycerol esters, respectively (Fig. 3, A and B) (Woodward et al., 2008). Until recently, whether such metabolites existed in vivo was unknown. However, Hu and colleagues (2008) have provided evidence to suggest that, indeed, at least some such in vivo reactions do occur. It is noteworthy that the endocannabinoid-derived prostaglandins have unique pharmacological profiles that seem to involve as-of-yet-unidentified receptors (Di Marzo, 2006; Hu et al., 2008; Woodward et al., 2008).

Lipoxygenases. AEA and 2-AG have also been identified as substrates for both 12-LOX and 15-LOX in intact cells. Oxidative metabolism of AEA by 12-LOX and 15-LOX results in the formation of 12- and 15-hydroperoxyeicosatetraenoylethanolamide (12-HETE-EA and 15-HETE-EA), respectively (Fig. 4A) (Edgemond et al., 1998; Veldhuis et al., 2003). Likewise, 12-LOX- and 15-LOX-mediated oxidation of 2-AG results in the formation of 12- and 15-hydroperoxyeicosatetraenoic acid glycerol ester (12-HETE-GE and 15-HETE-GE), respectively (Fig. 4B) (Moody et al., 2001; Kozak et al., 2002). Unlike the endocannabinoid-derived prostaglandins, the lipoxygenase derivatives of AEA and 2-AG seem to mediate their biological activities via established receptors, including the cannabinoid receptors, PPAR-α, and TRPV1 (Edgemond et al., 1998; Craib et al., 2001; Kozak et al., 2002; Di Marzo, 2006).

Pharmacological Manipulation of Endocannabinoid Uptake and Metabolism

The cannabinoid system is currently an active research area because of the many physiological and pathophysiological implications associated with AEA and 2-AG signaling, such as appetite (Kirkham and Tucci, 2006), pain (Hohmann and Suplita, 2006), anxiety (Witkin et al., 2005), fertility (Wang et al., 2006), neurodegeneration (Battista et al., 2006), the immune response (Ashton, 2007), and cardiac health (Ashton and Smith, 2007). Pharmacological manipulation of endogenous AEA and 2-AG levels is one way to selectively regulate their associated signaling events for therapeutic purposes. Thus, the proteins involved in endocannabinoid uptake and metabolism and the events responsible for termination of endocannabinoid signaling are attractive targets for pharmacological exploitation aimed at modulating AEA and 2-AG signaling.

The Search for Selective Endocannabinoid Reuptake Inhibitors

Unfortunately, the combination of the elusiveness of the protein(s) responsible for AEA and/or 2-AG uptake and the unresolved relationship that exists between endocannabinoid uptake and FAAH/MAGL activity has hindered the development of selective endocannabinoid reuptake inhibitors. In fact, one 2006 study showed that nearly all "selective" AEA uptake inhibitors also block FAAH activity to one extent or another (Dickason-Chesterfield et al., 2006).

Yet recent developments indicate that the identities of the endocannabinoid transporter(s) may soon be determined. Moore and colleagues (2005) announced their development of the potent tetrazole-based specific AEA uptake inhibitor LY2318912. This compound seems to bind a protein target distinct from FAAH and does not cross the cell membrane, supporting the hypothesis of a plasma membrane-localized or -associated AEA carrier (Moore et al., 2005). In addition, several new molecules designed to isolate and identify the putative transporter protein(s) have recently been developed, including several photoaffinity radioligands and a biotin-tagged AEA (Balas et al., 2005, 2006; Moore et al., 2005; Schiano Moriello et al., 2006; Fezza et al., 2008).

The increased specificity for endocannabinoid uptake inhibition exhibited by some of the tetrazole-based compounds may prove to be useful not only in the identification of the

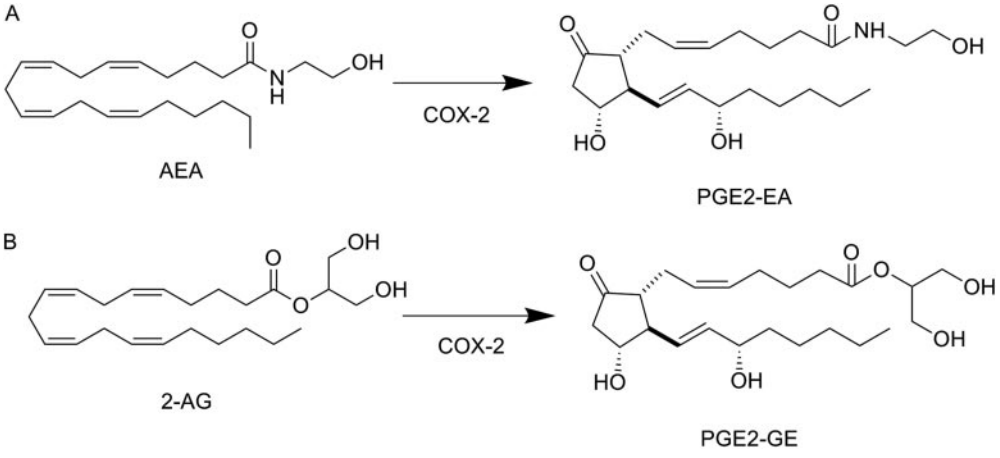

Fig. 3. The major metabolites generated via COX-2-mediated oxidation of the endocannabinoids AEA (A) and 2-AG (B). PGE_2-EA, prostaglandin E2 ethanolamide; PGE_2-GE, prostaglandin E2 glycerol esther.

elusive protein(s) involved in endocannabinoid transport but also in dissecting the role of endocannabinoid signaling in certain physiological and behavioral phenomena. Trezza and Vanderschuren (2009) suggest that the results of in vivo studies using endocannabinoid reuptake inhibitors may be affected by the specificity of the inhibitor used. They found that the nonspecific uptake inhibitor AM404 and the more specific uptake inhibitor VDM11 had contradicting effects on the social play of rats possibly as a result of off-target effects elicited by AM404 that are unassociated with CB1, CB2, or TRPV1 receptors (Trezza and Vanderschuren, 2009). Thus, compounds that specifically inhibit endocannabinoid uptake will better elucidate the true behavioral and physiological consequences of augmented endocannabinoid signaling.

Although many compounds initially believed to be specific for the endocannabinoid transporter have been shown to also inhibit FAAH and/or MAGL, these nonspecific AEA uptake inhibitors do have use in endocannabinoid research. For instance, in vivo studies using AM404 have implicated the endocannabinoid system in the neuropathic and inflammatory pain pathways (Mitchell et al., 2007; La Rana et al., 2008) and in the mediation of antidepressant-like effects (Adamczyk et al., 2008). AM404 has also been shown to reduce ethanol administration in rats, suggesting use for the compound in the treatment of alcoholism, although the exact signaling pathway responsible for this effect is unknown (Cippitelli et al., 2007).

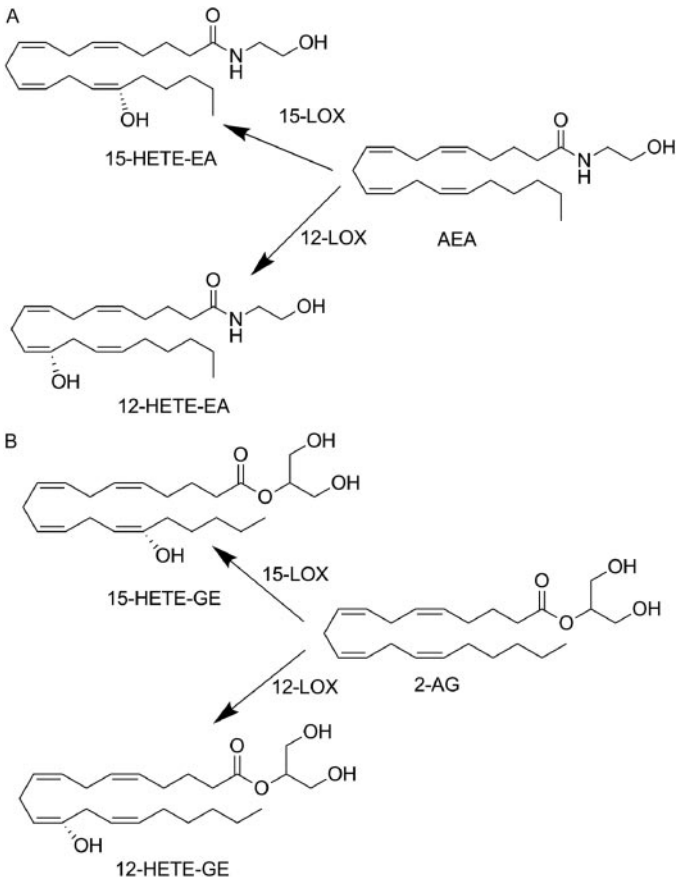

Fig. 4. The major metabolites generated via oxidation of AEA (A) and 2-AG (B) by the 12- and 15-LOXs.

Inhibitors of AEA and 2-AG Hydrolysis

In instances in which increased AEA or 2-AG signaling may have therapeutic benefit such as chronic pain or anxiety, inhibition of AEA and 2-AG enzyme-mediated hydrolysis may be desirable. Specifically, inhibiting FAAH could increase AEA signaling in two ways: 1) by preventing AEA hydrolysis; and 2) by decreasing the rate of AEA uptake into cells by interfering with the inward concentration gradient perpetuated by intracellular AEA hydrolysis.

In addition, the metabolites of AEA and 2-AG hydrolysis may themselves play roles in disease. For instance, 2-AG metabolites have been implicated as stimulatory factors in the pathogenesis of prostate cancer (Endsley et al., 2007). Endsley and colleagues (2007) observed that in the androgen-independent prostate carcinoma (PC-3) cells, exogenous application of 2-AG increased the production of arachidonic acid, which is then oxidized by 12-lipoxygenase. The resulting oxidized product, 12-HETE-GE, stimulates prostate cell invasion. The authors propose that inhibition of 2-AG hydrolysis in such instances may prove to have therapeutic potential.

Over the years, a significant number of FAAH inhibitors have been developed (Seierstad and Breitenbucher, 2008). In addition to the development of FAAH inhibitors, some compounds currently on the market, including several nonsteroidal anti-inflammatory drugs, have been shown to inhibit FAAH activity (Fowler et al., 2001; Seierstad and Breitenbucher, 2008). However, the development of MAGL inhibitors has lagged historically. Evidence suggests that boronic acids potently inhibit FAAH and may serve as good starting compounds for the development of better MAGL inhibitors (Minkkilä et al., 2008). Long and colleagues (2009) announced their development of a selective MAGL inhibitor, JZL184, that produces antinociceptive effects, hypomotility, and hypothermia in mice (Long et al., 2009). This advancement offers many possibilities not only for therapeutic development of MAGL inhibitors but also with regard to research aimed at dissecting the overlapping and distinct effects of AEA and 2-AG signaling.

Endocannabinoid-Derived Oxidative Metabolites as Pharmacological Targets

There have been many pathophysiological implications for the endocannabinoid-derived oxidative metabolites produced by COX-2 and the 12- and 15-LOXs. For example, data exist suggesting that COX-2-mediated oxidation of endocannabinoids plays an important regulatory role in hippocampal neuronal transmission and synaptic plasticity (Sang et al., 2006; Yang et al., 2008). In addition, as mentioned above, 12-LOX-generated oxidative metabolites of AEA may be agonists for TRPV1, a key channel in pain modulation (Craib et al., 2001). These are just two examples of cell-signaling events potentially mediated by the oxidative metabolites of AEA and 2-AG. Many questions still remain regarding the signaling fates of these and other endocannabinoid metabolites.

We have examined the various ways in which the endocannabinoids AEA and 2-AG may be inactivated. Endocannabinoid inactivation can be pharmacologically modulated at the level of cellular accumulation or intracellular metabolism. Metabolism occurs predominantly via the hydrolytic en-

zymes FAAH and MAGL or the oxidative enzymes COX-2, 12-LOX, and 15-LOX. The molecular process responsible for the intracellular accumulation of AEA and 2-AG has yet to be fully elucidated, but the majority of data to date suggest that endocannabinoid uptake is protein-facilitated. Much still remains to be learned regarding these events, including the identity of the putative endocannabinoid transporter(s) and identification of the signaling events mediated by the metabolic derivatives of AEA and 2-AG.

Acknowledgments

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