REVIEW

The Multiple Pathways of Endocannabinoid Metabolism: A Zoom Out

by **Séverine Vandevoorde***[1]) and **Didier M. Lambert**

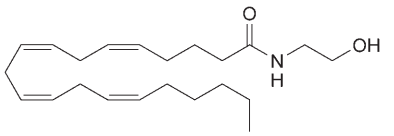
Unité de chimie pharmaceutique et radiopharmacie, UCL/CMFA 7340, Avenue E. Mounier, B-1200 Brussels

Abbreviations. – AA, Arachidonic acid; AEA, arachidonoylethanolamide (anandamide); 2-AG, 2-arachidonoylglycerol; CNS, central nervous system; COX, cyclooxygenase; CRC, colorectal carcinoma cells; DMP, discovery metabolite profiling; ERK, extracellular-signal-regulated kinase; FAAH, fatty acid amide hydrolase; HCA-7, human colon adenocarcinoma; 11-HETE-G, 11-hydroxyeicosatetraenoic acid glycerol ester; 15-HETE-G, 15-hydroxyeicosatetraenoic acid glycerol ester; HFF, human foreskin fibroblasts; HHT, 12-hydroxyheptadecatrienoic acid; HIV, human immunodeficiency virus; HPLC/MS, high-pressure liquid chromatography coupled to mass-spectrometric analysis; HPETE, hydroperoxyeicosatetraenoic acid; HPETEA, hydroxyperoxyeicosa-5,8,12,14-tetraenoylethanolamide; HPETE-G, hydroxyperoxyeicosa-5,8,10,14-tetraenoic acid glycerol ester; IFN-γ, interferon-γ; i.v., intravenous; LOX, lipoxygenase; LPS, lipopolysaccharide; MAGL, monoacylglycerol lipase; MAGLcy, cytosolic monoacylglycerol lipase; MAGLm, membrane-bound enzyme responsible for monoacylglycerol hydrolysis; mIPSCs, miniature inhibitory postsynaptic currents; MS, mass spectrometry; NAAA, *N*-acylethanolamine-hydrolyzing acid amidase; NADA, *N*-arachidonoyldopamine; NPAA, *N*-palmitoylethanolamine-hydrolyzing acid amidase; OEA, oleoylethanolamide; 2-OG, 2-oleoylglycerol; PC, phosphorylcholine; PEA, palmitoylethanolamide; PGD_2-G, prostaglandin D_2 glycerol ester; PGE_2-EA, prostaglandin E_2 ethanolamide; PGE_2-G, prostaglandin E_2 glycerol ester; PGG_2, endoperoxide-containing prostaglandin G_2; PGH_2, endoperoxide-containing prostaglandin H_2; PGH_2-G, prostaglandin H_2 glycerol ester; PGI, prostacyclin; PGIS, prostacyclin synthase; PPAR-α, peroxisome proliferator-activated receptor-alpha; PPAR-γ, peroxisome proliferator-activated receptor-gamma; RBL, rat basophilic leukemia; THC, tetrahydrocannabinol; TRPV-1, transient receptor potential vanilloid; TXA, thromboxane; TXAS, thromboxane synthase; URB597, cyclohexylcarbamic acid 3′-carbamoyl biphenyl-3-yl ester.

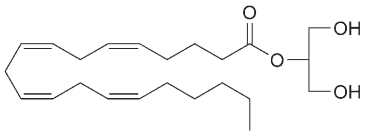
1. Introduction. – In the early 1990s, the cloning of two cannabinoid receptors mediating the pharmacological effects of Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive component of cannabis, launched the quest for the endogenous ligands of these receptors. Seventeen years later, several 'endocannabinoids' (*i.e.*, endogenous ligands of the cannabinoid receptors) have been identified, members of a family of fatty acid amides or esters derived from arachidonic acid (AA). Arachidonoylethanolamide (anandamide, AEA; **1**) was the first endocannabinoid isolated from brain lipid extracts by *Devane* and co-workers [1]. AEA and Δ^9-THC have pharmacological properties in common, including antinociception [2–5], anxiolytic action [6][7], effects on cell

[1]) Present address: National Institute for Radioelements, Department of Research and Development, Avenue de l'Espérance, B-6220 Fleurus (phone: +32 71 82 93 23; fax: +32 71 81 38 12; e-mail: generalmail@ire.be).

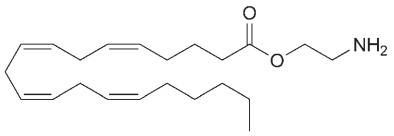
proliferation [8–13], reproduction [14–16], memory processes [17–20], and modulation of feeding [21–23]. Like Δ^9-THC, AEA exhibits *in vitro* partial agonist activity on both the central cannabinoid receptors CB_1 and the peripheral cannabinoid receptors CB_2 [24][25]. *In vivo*, AEA was later extracted and purified from a variety of biological tissues, like the eye [26], spleen, heart, skin [27], and blood plasma [28]. In addition to its cannabinoid action, AEA also acts as a full agonist of the transient receptor potential vanilloid 1 (TRPV-1) [29][30], which mediates the pharmacological effects of capsaicin, the main pungent ingredient of hot chili peppers [31][32]. TRPV-1 Receptors have been described as the mediators of some pharmacological effects of AEA, like its hypotensive effect [33][34].



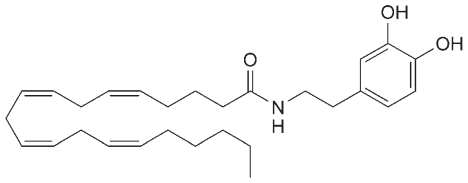
Anandamide (AEA; **1**)



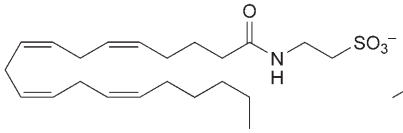
2-Arachidonoylglycerol (2-AG; **2**)



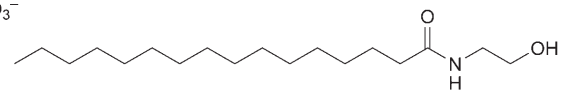
Virodhamine (**3**)



N-Arachidonoyldopamine (NADA; **4**)



N-Arachidonoyltaurine (**5**)



Palmitoylethanolamide (PEA; **6**)

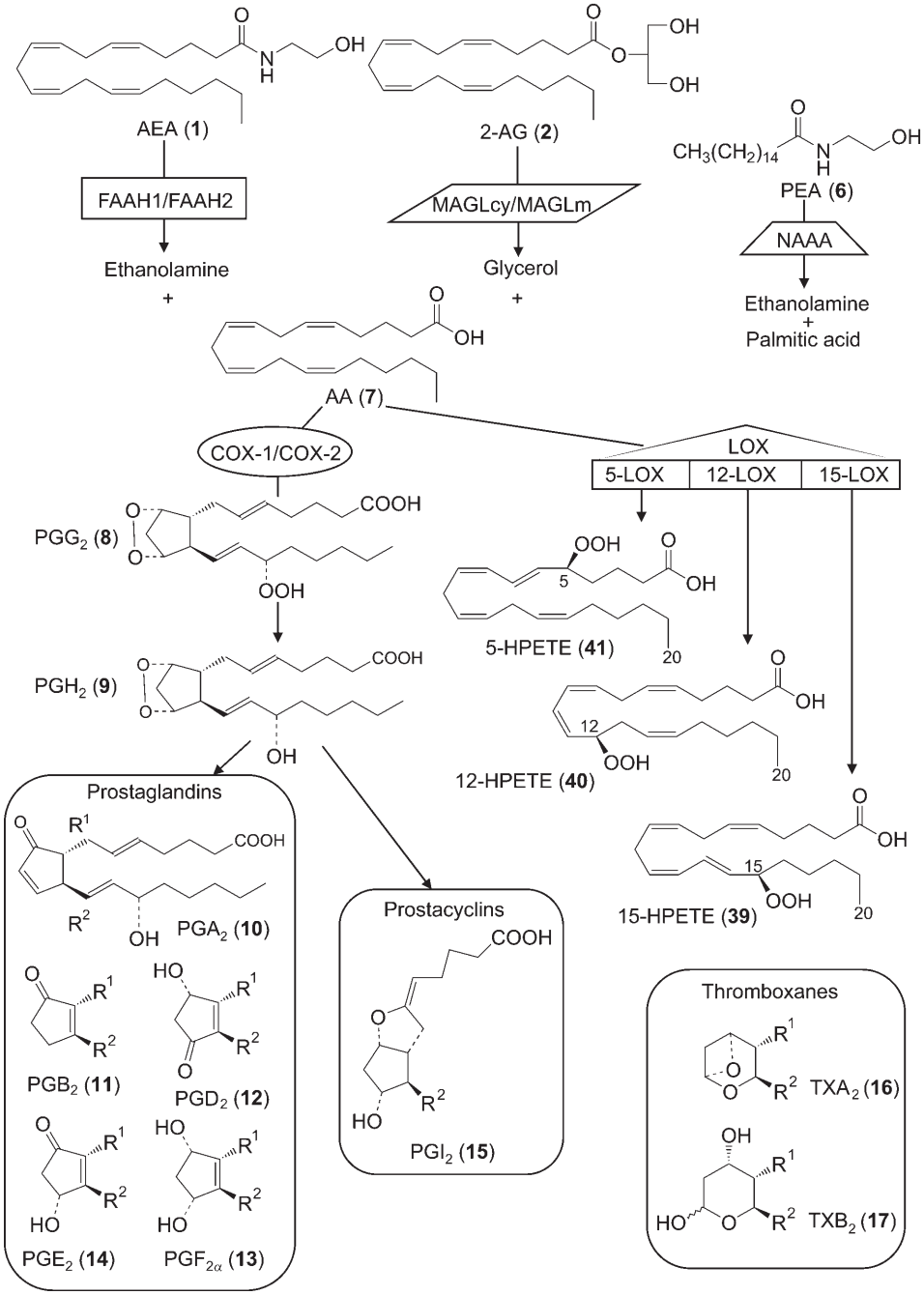
2-Arachidonoylglycerol (2-AG; **2**) is the second-most-studied endocannabinoid. 2-AG was first isolated from canine gut [35] and rat brain [36], where it was reported to be present in amounts greater by a factor of 170 than AEA [37]. 2-AG is an agonist of both CB_1 [38] and CB_2 [39][40] cannabinoid receptors, and it exerts pharmacological properties including neuroprotection [41][42], cardioprotection [43][44], and antiproliferation [45][46]. During the last years, new members of the endocannabinoid family have been identified. Virodhamine (*O*-arachidonoylethanolamine; **3**) was isolated from rat brain and identified as a CB_2 agonist [47]. *N*-Arachidonoyldopamine (NADA; **4**) was extracted from rat brain and bovine dorsal root ganglions, and reported to be a CB_1 agonist and an agonist of TRPV-1 receptors [48–50]. NADA is a potent vasorelaxant [51][52], but it also induces TRPV-1-dependent thermal hyperalgesia [53]. Moreover, NADA was reported to inhibit HIV-1 infection in several human cell lines, through a mechanism independent of the CB_1 and TRPV-1 pathways

[54]. The *N*-acyl taurines are the most-recent endocannabinoids identified by a novel method christened 'discovery metabolite profiling' (DMP), which consists in standard-free mass-spectrometry (MS) analysis of biological extracts. Several saturated *N*-acyl taurines were first isolated from mice brains and spinal-cord extracts [55]. Polyunsaturated *N*-acyl taurines, including *N*-arachidonoyltaurine (**5**), were later extracted from mice livers and kidneys, and were reported to be activators of the TRPV-1 and TRPV-2 receptors [56]. In addition to these endocannabinoids, other endogenous fatty acid amide ligands have been characterized. They exhibit cannabimimetic effects without activating the well-known cannabinoid receptors. Palmitoylethanolamide (PEA; **6**) is a saturated analogue of AEA, which was isolated from rat brain, liver, skeletal muscle [57], testis [58], paw skin [59], mouse spinal cord [60], and canine-heart extracts [61]. PEA is a potent anticonvulsant [62], and it exhibits antiproliferative effects on human breast cancer cells [63]. PEA has antinociceptive [59] and anti-inflammatory [64] properties, which are reversed by the CB_2 antagonist SR144528 (for a review on the pharmacological actions of PEA, see reference [65]). Several hypotheses were proposed to rationalize the actions of PEA, including the existence of a CB_2-like cannabinoid receptor activated by PEA, and a possible 'entourage effect', which would suggest that PEA interferes with the degradation of the endocannabinoids and, consecutively, enhances their endogenous levels and strengthens their CB_1- or CB_2-mediated biological actions. However, the peroxisome proliferator-activated receptor-alpha (PPAR-α) was recently identified as the mediator of the anti-inflammatory actions of PEA [66][67]. This list of endocannabinoids is not exhaustive, and sustained improvements in methods allowing separation, purification, and detection of the components of biological samples, including chromatographic and MS methods, will make easier the identification of new family members that are still under the limits of detection of the currently available technologies [68].

After the identification of the first endocannabinoids, special interest was dedicated to the elucidation of the mechanisms able to terminate their signaling activity. So far, two major pathways have been identified: the 'hydrolase' and the 'oxygenase' pathways. The first one includes the enzymes fatty acid amide hydrolase (FAAH) [69–71], monoacylglycerol lipase (MAGL) [72], and *N*-palmitoylethanolamine-selective acid amidase [73][74], which was recently renamed as *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) [75]. All three catalyze the hydrolytic cleavage of the amide or ester bond of the endocannabinoid, which either releases the corresponding fatty acid and ethanolamine (in the case of AEA and PEA) or glycerol (in the case of 2-AG), as shown in *Scheme 1*. FAAH, MAGL, and NAAA are now fully characterized and cloned, and without being completely selective for a specific substrate, they catalyze preferably the hydrolysis of the endocannabinoids AEA, 2-AG, and PEA, respectively. The second pathway involves the well-known cyclooxygenase (COX) [76][77] and lipoxygenase (LOX) enzymes [78][79], which are not specific of the endocannabinoid metabolism (*Scheme 1*). Both induce oxidation of the arachidonic moiety of the endocannabinoids. The purpose of this review is to present the main features of these enzymes responsible of the fate of endocannabinoids.

2. Hydrolase Enzymes. – 2.1. *Fatty Acid Amide Hydrolase* (FAAH). 2.1.1. *Molecular Structure and Catalytic Mechanism.* In 1993, *Deutsch* and *Chin* published

Scheme 1. *Graphical Representation of the Hydrolase Pathway of Endocannabinoid Metabolism, Followed by the Metabolism of Arachidonic Acid* (AA) *by Cyclooxygenase* (COX) *and Lipoxygenase* (LOX) *Enzymes*



the first report on the enzymatic degradation of AEA in neuroblastoma and glioma cells, by action of an 'anandamide amidase' enzyme [80]. This enzyme, which was later identified as 'anandamide amidohydrolase' in rat [81] and porcine [82] brains, was finally renamed as FAAH in 1996, when *Cravatt* and co-workers succeeded to purify and clone this enzyme from rat liver plasma membranes, confirming that FAAH is capable of recognizing a plurality of fatty acid amides [69]. Mouse [70], porcine [83], and human [70] FAAH were subsequently cloned and found to exhibit 73% identity at the overall amino acid level, with 90% identity at the amidase signature, which is a highly conserved region rich in Ser, Gly, and Ala residues common to more than 80 members of the family of amidase enzymes (for details on the molecular structure of FAAH, see references [84][85]). The mammalian FAAH proteins are 579 amino acids in length, and the amidase signature is localized between residues 215 and 257.

The major progress in the understanding of the catalytic mechanism of FAAH was unquestionably achieved by *Bracey* and co-workers, who reported its 2.8 Å X-ray crystal structure in 2002 [71]. The study confirmed the first results reported by *Patricelli* and *Cravatt* two years earlier, by mutagenesis of all potentially catalytic residues of FAAH [86]. The residues Lys^{142}, Ser^{217}, Ser^{218}, Ser^{241}, and Arg^{243} were pointed out to be critical for amidase activity. Moreover, the study from *Bracey* brought to light that FAAH is a dimeric enzyme composed of a core involving a Ser-Ser-Lys triad. In 2003, *McKinney* and *Cravatt* highlighted the importance of Ser^{241}, Ser^{217}, and Lys^{142} in the formation of a tetrahedral intermediate during the catalytic hydrolysis of an amide by FAAH [87]. Nowadays, these studies greatly facilitate the understanding of the inhibitory mechanism of compounds interfering with the metabolism of endocannabinoids by FAAH, such as carbamates [88][89], but they also whet the discovery of new potent FAAH inhibitors with pharmacological properties [90] (for reviews on the pharmacological properties of drugs that interfere with endocannabinoid metabolism, see the works of *Pertwee* [91], and *Fowler* and co-workers [92–95]).

2.1.2. *Distribution and Substrates.* FAAH Distribution is noticeably different between human and rat. In human, FAAH is mainly present in pancreas, brain, kidney, skeletal muscle [70], and placenta [96]. In rat, FAAH is mainly detected in liver, small intestine, brain, kidney, spleen, testis, and uterus, but is absent from skeletal muscle and heart [80][81][97]. The presence of FAAH was also confirmed in ocular tissues from different species, including rat, pig, fish, and ox [98–101]. Within rat brain, highest FAAH activities were observed in the area of globus pallidus, hippocampus, cerebellum, substantia nigra, and cerebral cortex, and the lowest in brainstem and medulla [102]. Expression of FAAH was also reported in mouse uterus, and confirmed to be regulated during pregnancy [103–105].

Detection of FAAH was also reported in different cell lines, including mouse N18TG2 neuroblastoma [80][106], rat C6 glioma [80], J774 macrophages [107], RBL-2 H3 basophilic leukemia cells [108], bronchioalveolar non-small-cell lung carcinoma [80], human dendritic cells [109], postnatal radial glia (RC2+ cells), adult nestin type I (nestin(+)GFAP+) neural progenitor cells [110], and primary hepatocytes [111]. However, the following cell lines were reported to lack FAAH: human epitheloid carcinoma (HeLa), human larynx epidermoid carcinoma (Hep2), human hepatocellular carcinoma (HepG2) [80], and monkey COS-7 kidney fibroblast-like cells [112].

As mentioned above, FAAH is able to recognize a plurality of fatty acid amides, but substrate specificities differ from enzyme preparations to others. So, enzymes extracted from rat liver [113] and mouse N18TG2 cells [114] quickly hydrolyse PEA, while enzymes from rat [81] and porcine [82] brains hardly metabolize this substrate. FAAH catalyzes the hydrolysis of the following amides: lauroylethanolamide (C12 : 0), myristoylethanolamide (C14 : 0), palmitoylethanolamide (C16 : 0), stearoylethanolamide (C18 : 0), oleoylethanolamide (C18 : 1), the sleep-inducing factor oleamide (C18 : 1), and the different homologues of the recently identified *N*-acyltaurines (C18 : 2, C18 : 1, C20 : 4, C22 : 6), but the preferred substrate is always reported to be AEA (C20 : 4), whatever the source of FAAH used. *In vitro*, FAAH also catalyzes the hydrolysis of the ester bond of 2-AG (C20 : 4), methyl arachidonate, and oleoyl methyl ester. The esterase activity of FAAH was, however, shown to be less important *in vivo* (see *Section 2.2.1* on monoacylglycerol lipase (MAGL)). The role of the Lys^{142} residue was demonstrated to be crucial for amidase activity of FAAH, since its mutation to Ala produces a mutant FAAH that converts esters faster than amides [115]. Similarly, another FAAH mutant was recently generated to underline the identity of the amino acid residue crucial for the recognition of *N*-acyltaurine substrates. In this study of *McKinney* and *Cravatt* and co-workers [116], the role of the Gly^{268} residue was pointed out, since its mutation to Asp results in an FAAH mutant that hardly metabolizes *N*-acyltaurine substrates (*Scheme 1*).

2.1.3. *Generation of FAAH$^{(-/-)}$ Mice and Alternative Endocannabinoid Metabolism.* To clarify the role played by FAAH *in vivo*, the most powerful investigation tool was developed by *Cravatt* and co-workers in the form of mice in which FAAH was genetically disrupted (so-called FAAH$^{(-/-)}$ mice). FAAH$^{(-/-)}$ Mice exhibit reduced pain sensitivity correlated with an increase in the endogenous brain levels of AEA, PEA, and oleoylethanolamide. This phenotypic hypoalgesia in thermal nociceptive tests is, however, completely abolished by the administration of the CB_1-receptor antagonist SR141716A (rimonabant) [117][118]. Afterwards, several studies involving FAAH$^{(-/-)}$ mice were reported to characterize the behavioral differences between the wild-type and FAAH$^{(-/-)}$ mice. The latter were reported to exhibit a proconvulsant activity after AEA administration, which was shown to enhance the severity of bicuculline- or kainite-induced seizures [119]. Most of the comparison studies confirmed the increased responsiveness of FAAH$^{(-/-)}$ mice to exogenous administration of AEA, but revealed similar profiles between wild-type and knock-out mice in the cardiac performance [120], spatial learning, working memory [20], and saccharin or quinine consumption and preference [121]. Ethanol (EtOH) intake and preference were only increased in female FAAH$^{(-/-)}$ mice, which were less sensitive to the hypothermic and sedative effects of acute EtOH [121]. In an experiment of characterization of sleep/wake patterns, FAAH$^{(-/-)}$ mice were shown to possess higher values and more-intense episodes of slow-wave sleep than the wild-type mice, supporting the role of endocannabinoids in the modulation of sleep [122].

As mentioned above, FAAH$^{(-/-)}$ mice exhibit increased responsiveness to exogenous administration of AEA, but they recover from this treatment and finally show normal behavior indistinguishable from the wild-type mice several hours after AEA administration. This observation led *Mulder* and *Cravatt* to the hypothesis that FAAH$^{(-/-)}$ mice express alternative pathway(s) for AEA metabolism [123]. They

identified *O*-phosphorylcholine (PC)-AEA as a novel AEA-induced metabolite in the brains and spinal cords of AEA-treated FAAH$^{(-/-)}$ mice. In addition, PC-OEA and PC-PEA were identified as endogenous constituents of the central nervous system (CNS) of these mice, but are absent from the CNS of wild-type mice. Interestingly, PC-AEA was only detected from the CNS of AEA-treated FAAH$^{(-/-)}$ mice, but was absent (or under the limit of detection of the LC/MS/MS method used) from the CNS of untreated FAAH$^{(-/-)}$ mice. Moreover, PC-*N*-acylethanolamines were not detected in the CNS of wild-type mice after acute inhibition of FAAH by the potent FAAH inhibitor URB597, suggesting that significant levels of PC-*N*-acylethanolamines are only reached after long time of FAAH inactivation. The enzyme(s) responsible for the formation of PC-*N*-acylethanolamines from *N*-acylethanolamines is (are) still unidentified. Nevertheless, cholinephosphotransferase-1 and choline/ethanolaminephosphotransferase-1 enzymes are considered as good candidates to catalyze this transformation [123].

2.1.4. *Modulation of FAAH Expression in Pathological States.* Since the discovery of the endogenous ligands of the cannabinoid receptors, and the identification of the first enzyme responsible of their degradation, namely FAAH, there have been several reports dedicated to the changes in the endogenous contents of cannabinoid ligands (*i.e.*, the so-called 'endocannabinoid tone') and the concomitant modulations of FAAH activity and/or expression that occur in some diseases or disorders. A highly comprehensive review on this subject was published recently by *Pertwee* [91]. Therefore, in the present review, we will not cover again this area, which was comprehensively developed elsewhere. We will rather restrict ourselves to the latest studies published after the above review.

In 2005, the activity of FAAH was investigated in the peripheral platelets of human migraineurs, and revealed an increase in the FAAH activity restricted to the female migraineurs. Although conclusions must be drawn cautiously from this observation, it could give an explanation to the prevalence of migraine in women, caused by a decrease of the blood AEA concentration and, therefore, a reduced pain threshold [124]. In 2006, the FAAH expression was measured in visceral adipose tissue in human obese patients. The FAAH gene expression was shown to be negatively correlated with visceral fat mass, proving the dysregulation of the endocannabinoid system concomitant with human abdominal obesity [125]. However, a comparative study of FAAH activities in human epileptic and non-epileptic brain tissues failed to reveal any difference between the two groups, in spite of the increased brain endogenous level of AEA, which was observed in animal models of epilepsy [126].

2.1.5. *Another FAAH.* Very recently, a second membrane-associated FAAH enzyme containing the Ser-Ser-Lys catalytic triad was identified by *Cravatt* and coworkers [127]. 'FAAH-2' (originally designated as FLJ31204) was first detected in the membrane, but not in the cytosolic fraction of MCF-7 and OVCAR-3 cancer cells. FAAH-2 and the well-known FAAH, which had been identified first (and will be referred to now as 'FAAH-1'), share some 20% of amino acid residues. The two FAAH enzymes catalyze the hydrolysis of oleamide with similar rates, however FAAH-1 shows a greater activity with the *N*-acylethanolamine and *N*-acyltaurine substrates. FAAH-2 was found in several species, including human, primates, opossum, frog, chicken, pufferfish, and zebrafish. However, it is absent from murids. FAAH-2 is also

distinguished from FAAH-1 by its orientation on cell membranes. Whereas the C-terminal catalytic domain of FAAH-2 is predicted to be located in the luminal compartment of the cell, the FAAH-1 catalytic domain was shown to be located in the cytoplasmic area. Both FAAH enzymes have also distinct tissue distributions. FAAH-2 was detected in the heart and ovary, but not in the brain, small intestine, and testis, which are known to express FAAH-1. However, both FAAH-1 and FAAH-2 were detected in the prostate, lung, kidney, and liver (*Scheme 1*) [127].

2.2. *Monoacylglycerol Lipase* (MAGL). 2.2.1. *Molecular Structure, Distribution, and Substrates.* In spite of its earlier identification by *Tornqvist* and *Belfrage* in 1976 [128], and its cloning nearly simultaneous to that of FAAH [129], MAGL only aroused a belated interest in the cannabinoid field. Nowadays, MAGL is yet considered as the main enzyme catalyzing the hydrolysis of 2-AG *in vivo* (*Scheme 1*). It was confirmed by the comparison of the hydrolysis rates for 2-oleoylglycerol (2-OG) in $FAAH^{(-/-)}$ and $FAAH^{(+/+)}$ tissues, which were shown to be equivalent. Moreover, exogenous administration of 2-AG produced similar behavioral responses in both $FAAH^{(-/-)}$ and $FAAH^{(+/+)}$ mice, suggesting that an enzyme different from FAAH controls the metabolism of monoacylglycerides [130]. Mouse [129], rat [72], and human [131] MAGL were cloned, and all were reported to be 303 amino acids in length, sharing the catalytic triad Ser^{122}, Asp^{239}, and His^{269} [132]. The distribution of MAGL was studied in rat, and it was shown to be ubiquitous [129]. MAGL mRNA was reported to be present in adrenal gland, heart, adipose tissue, kidney, ovary, testis, spleen, lung, liver, skeletal muscle, and brain, especially in the areas that present high levels of CB_1 receptors: hippocampus, cortex, thalamus, and cerebellum [72]. The ultrastructural distribution of FAAH and MAGL in rat brain was studied by *Gulyas et al.* [133], who found that FAAH is preferably localized in the soma-dendritic compartment, whereas MAGL is present in axons. In the *amygdala*, the distribution of the two enzymes was shown to be complementary: MAGL is localized presynaptically, whereas FAAH is primarily a postsynaptic enzyme [133].

2.2.2. *Other MAGL-Like Enzymes.* In 2004, *Saario* and co-workers reported that the hydrolysis of monoacylglycerides such as 2-AG, 1-AG, or 2-OG does not only occur by the well-characterized cytosolic MAGL, but also by a membrane-bound MAGL-like enzyme (MAGLm) found in rat cerebellar membranes [134]. MAGLm is not yet characterized, but several studies have already shown that MAGLm and the well-known cytosolic MAGL differ in their sensitivities to inhibitors (*Scheme 1*) [135–138]. Furthermore, it must be mentioned that a third type of MAGL-like enzyme was recently identified by *Stella* in the microglial BV-2 cell line, which lacks MAGL mRNA [139].

2.3. N-*Acylethanolamine-Hydrolyzing Acid Amidase* (NAAA). 2.3.1. *Molecular Structure, Distribution, and Substrates.* In 1999, a novel hydrolase was identified by *Ueda* and co-workers in a human megakaryoblastic cell line (CMK). This enzyme differed from FAAH by its acidic optimal pH of 5, its preference to catalyze the hydrolysis of PEA, and its sensitivity to dithiothreitol (DTT), which increased its activity, but not those of FAAH [140]. Two years later, this hydrolase was purified by the same group from different rat tissues. Highest activities were reported in the lung, spleen, small intestine, thymus, caecum, and in peritoneal and alveolar macrophages. The enzyme of a molecular mass of 31 kDa was characterized for its ability to interact

with various *N*-acylethanolamines. PEA was confirmed to be the preferred substrate, followed by *N*-myristoylethanolamine (C14:0), *N*-stearoylethanolamine (C18:0), *N*-oleoylethanolamine (C18:1), *N*-linoleoylethanolamine (C18:2), and, finally, AEA, which was identified as a poor substrate (*Scheme 1*) [73].

Originally termed '*N*-palmitoylethanolamine-selective acid amidase' (NPAA), the enzyme was distinguished from FAAH by its activation by *Triton X-100*, its presence in the cytosol of RBL-1 cells that express FAAH in the particulate fraction [74], and its sensitivity to selected inhibitors [74]. *Ueda* and co-workers succeeded in cloning rat, mouse, and human NPAA in 2005, and finally renamed the enzyme as '*N*-acylethanolamine-hydrolyzing acid amidase' (NAAA) [75]. The amino acid sequences are 362 residues in length for rat and mouse, and 359 residues in length for human. They are highly conserved between rat and mouse, with 90.1% identity. Identity decreases to 76% between human and murids. No homology was observed between NAAA and FAAH, but similarity was revealed to acid ceramidase. The organ distribution of NAAA mRNA was in good agreement with those of activity (see above), and highest expression was, thus, detected in lung. RT-PCR allowed the confirmation of the expression of NAAA mRNA in the RAW264.7, P388-D1, and peritoneal macrophage-like cells of mouse and U937 and THP-1 macrophage-like cells of human, which also express FAAH. Surprisingly, the preference for PEA as substrate was not observed with NAAA isolated from RAW264.7 cells. NAAA mRNA Expression was also investigated in various mice tissues, and was revealed to be similar to the distribution in rats. Nevertheless, some differences were observed in mice brain and kidney, which showed high expression levels of NAAA, whereas those in rats were low [141].

3. Oxygenase Enzymes. – 3.1. *Cyclooxygenases* (COX). 3.1.1. *Molecular Structures and Distribution*. So far, three forms of cyclooxygenase (COX) enzymes have been characterized in mammalian tissues: COX-1, ubiquitously expressed; COX-2, inducible by various factors including growth factors, pyrogens, cytokines, and tumor promoters; and finally COX-3, which is rather considered as a splice variant of COX-1. COX-1 is constitutively expressed in most tissues and involved in several physiological processes, including homeostasis, gastric protection, and control of renal blood flow. COX-1 is, consequently, viewed as a 'housekeeping' enzyme [142], whereas COX-2 is involved in response-related activities and pathological processes [143]. Basal expression of COX-2 is not detectable in most tissues, except in the kidney and some area of the CNS, including the spinal cord, cortex, hypothalamus, and hippocampus. An increased activity of COX-2 was, however, recorded in several disorders, including ischemia [144], spinal-cord injury [145], amyotrophic lateral sclerosis [146], traumatic brain injury [147], *Creutzfeld–Jakob* disease [148], *Alzheimer* disease [149], *Parkinson* disease [150], cancer (colon, lung, breast, gastric, prostate, pancreatic adenocarcinomas [151]), ulcers [152], and gastritis [153].

COX-1 and COX-2 are heme-containing enzymes with two catalytic sites. The two COX isozymes have been cloned in different species, including human, sheep, chicken, fish, and murids [154–156]. The amino acid sequences are *ca*. 600 residues in length in all species, and exhibit *ca*. 60% identity. Crystallographic structures of COX-1 [157] and COX-2 [158–160] have been obtained from different research groups, and exhibit marked similarities, showing that both isozymes share the same four functional

domains: *i*) an amino-terminal signal peptide, *ii*) a dimerization domain, *iii*) a membrane-binding domain, and *iv*) a catalytic domain. The role of the amino-terminal signal peptide is to direct the nascent COX-1 and COX-2 polypeptides into the lumen of the endoplasmic reticulum. The signal peptides have lengths of 22–26 amino acids for COX-1, and of 17 amino acids for COX-2. The dimerization domain, which is *ca*. 50 amino acids long, holds together the COX-1 and COX-2 monomers in a dimeric structure. The membrane-binding domain is constituted by a group of four amphipathic helixes encoded by *ca*. 50 amino acids. They penetrate into the intraluminal surface of microsomal membranes and allow the attachment of the COX isozymes. They also frame the entrance of the COX active site. The catalytic domain is *ca*. 480 amino acids long and includes two active sites: the peroxidase and the COX active sites.

Both COX-1 and COX-2 recognize arachidonic acid (AA; **7**) as preferred substrate. The COX active site adds two O-atoms at the level of the C(15) atom of the arachidonic chain, to form the endoperoxide-containing prostaglandin PGG_2 (**8**). The hydroperoxyl group of PGG_2 is then reduced to an OH group by the peroxidase active site to yield the endoperoxide-containing prostaglandin PGH_2 (**9**). The process is initiated by oxidation of the heme group, which is bound at the level of the catalytic domain. PGH_2 acts as a precursor to several prostaglandins (PGA_2 (**10**), PGB_2 (**11**), PGD_2 (**12**), $PGF_{2\alpha}$ (**13**), and PGE_2 (**14**)), prostacyclins (PGI_2 (**15**)), and thromboxanes (TXA_2 (**16**), TXB_2 (**17**)), as shown in *Scheme 1*. They interact with their respective receptors to modulate cell functions (for a comprehensive review on COX isozymes, see the work of *Simmons et al.* [161]).

The glycoprotein COX-3 does not seem to be involved in the generation of prostaglandins. COS-7 Cells transfected with rat COX-3 exhibit very low endogenous COX activity [162]. Nevertheless, more experiments are needed to elucidate the exact function of COX-3. Thus, it was reported that canine COX-3 exhibits COX activity, suggesting that COX genes may produce different splice variants, with COX activity or not [163]. Nevertheless, the current knowledge of COX-3 does not allow to include the endocannabinoids in the list of substrates recognized by this enzyme.

3.1.2. *Endocannabinoids as COX Substrates.* As mentioned above, COX-1 and COX-2 preferably recognize AA as substrate, but they are also capable to catalyze the oxygenation of other fatty acids such as eicosapentaenoic acid, docosahexaenoic acid, and γ-linolenic acid [164]. However, in early works performed at the end of the 1960s, it was reported that COX enzymes do not only act on free fatty acids [165][166]. When incubated with AEA or 2-AG, purified human COX-2 (but not human COX-1) produces prostaglandins retaining the ethanolamide or glycerol ester heads of their precursors.

In 1997, *Yu et al.* [167] confirmed by HPLC/MS analysis that incubation of purified human COX-2 with [1-^{14}C]AEA (40 μM) yields prostaglandin E_2 ethanolamide (PGE_2-EA; **18**). Similar results were obtained when COX-2-expressing human-foreskin-fibroblast (HFF) cells, in which COX-2 was induced by treatment with phorbol 12-myristate 13-acetate and interleukin-1α, were used instead of purified human COX-2. Nevertheless, hCOX-2 was shown to exhibit lower affinity for AEA than AA, with K_m values of 5.8 and 23.7 μM towards AA and AEA as substrates, respectively [167].

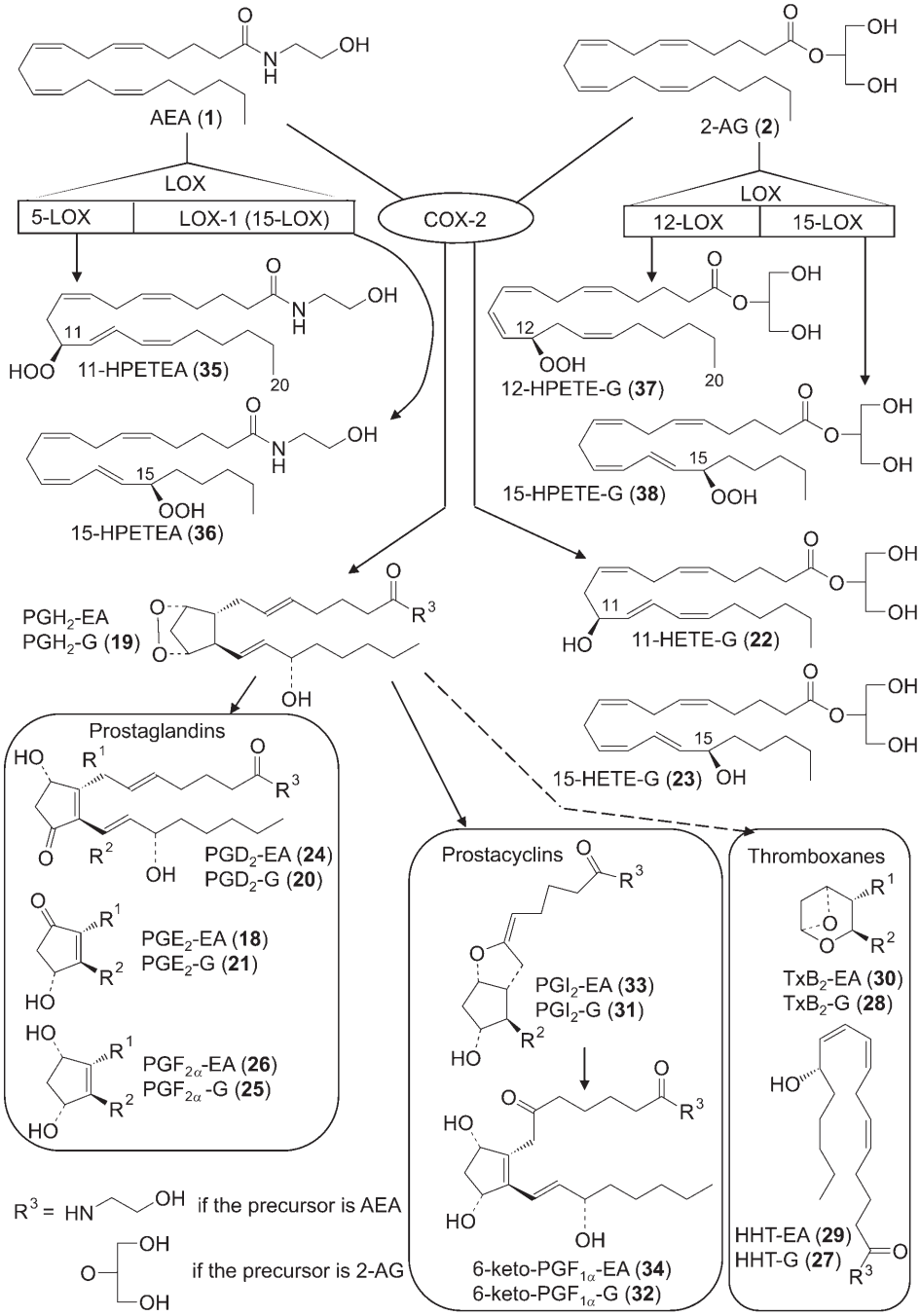
In 2000, *Kozak* and co-workers investigated the oxidative metabolism of 2-AG (15 μg) by purified human COX-2 (15–30 μg), and the products of 2-AG oxygenation were indentified by MS analysis as prostaglandin H_2 glycerol ester (PGH_2-G; **19**),

prostaglandin D_2 glycerol ester (PGD_2-G; **20**), prostaglandin E_2 glycerol ester (PGE_2-G; **21**), 11-hydroxyeicosatetraenoic acid glycerol ester (11-HETE-G; **22**)), and 15-hydroxyeicosatetraenoic acid glycerol ester (15-HETE-G; **23**), as shown in *Scheme 2*. Unlike AEA, 2-AG was reported to be a substrate as effective as AA for COX-2, exhibiting similar K_m values (6.1 and 4.4 μM, resp., when AA and 2-AG were used as substrates in human COX-2). The ability of 2-AG to serve as a substrate for COX-2 was investigated in the murine macrophage cell line RAW264.7, in which COX-2 was induced by interferon-γ (IFN-γ) and lipopolysaccharide (LPS) treatment. Under these conditions, fewer oxygenation metabolites of 2-AG were detected. Incubation of 2-AG (20 μM) with activated RAW264.7 macrophages released PGD_2-G as major metabolite, which, however, suggested the former release of its precursor PGH_2-G [168], which was not directly detected.

In 2002, a systematic study on COX-2 oxygenation of endocannabinoids in cellular and subcellular systems was reported by *Kozak* and co-workers [169], who clarified the diversity of endocannabinoid-derived prostaglandins. Incubation of AEA (20 μM) with RAW264.7 activated by IFN-γ and LPS released prostaglandin D_2 ethanolamide (PGD_2-EA; **24**) as major metabolite. In contrast, both PGE_2-EA (**18**) and PGD_2-EA (**24**) were detected when AEA was incubated with purified human COX-2. To investigate the formation of endocannabinoid-derived prostaglandins of the E- and F-series, the authors chose the human colon adenocarcinoma cell line HCA-7, which expresses COX-2 constitutively, producing primarily PGE_2 (**14**) and $PGF_{2\alpha}$ (**13**), when treated with AA [170]. Treatment of HCA-7 cells with 2-AG and AEA, respectively, yielded the corresponding PGE_2 and $PGF_{2\alpha}$ glycerol esters (PGE_2-G (**21**) and $PGF_{2\alpha}$-G (**25**)) and ethanolamides (PGE_2-EA (**18**) and $PGF_{2\alpha}$-EA (**26**)). The formation of these products required exogenous 2-AG and AEA, and was inhibited by selective COX-2 inhibitors. To determine if endocannabinoid-derived thromboxanes might be produced by isomerization of endocannabinoid-derived PGH_2-like lipids, the authors incubated purified human COX-2 in the presence of recombinant thromboxane synthase (TXAS), with 2-AG or AEA, respectively. Under these conditions, new metabolites were observed, corresponding to 12-hydroxyheptadecatrienoic acid (HHT) and thromboxane B_2 (TxB_2) glycerol esters (HHT-G (**27**) and TxB_2-G (**28**)) as well as ethanolamides (HHT-EA (**29**) and TxB_2-EA (**30**)). However, comparison of the rates of isomerization of PGH_2 (**9**) (produced from the natural substrate AA) and PGH_2-G (produced from 2-AG) into TxB_2 (**17**) and TxB_2-G (**28**), respectively, by human TXAS revealed that TxB_2 production was strongly reduced (*ca.* 20-fold) when 2-AG was employed as substrate. This observation suggests that the formation of endocannabinoid-derived TxB is not physiologically relevant.

Finally, the isomerization of endocannabinoid-derived PGH_2-like lipids into prostacyclins (PGI) was investigated by incubation of the endocannabinoids with PGI synthase (PGIS) prepared from mature bovine aorta. New metabolites with the structures of 6-keto-prostaglandin $F_{1\alpha}$ glycerol ester (6-keto-$PGF_{1\alpha}$-G; **32**) and 6-keto-prostaglandin $F_{1\alpha}$ ethanolamide (6-keto-$PGF_{1\alpha}$-EA; **34**) were identified as the products of spontaneous nonenzymatic hydrolyses of the respective prostacyclins PGI_2-G (**31**) and PGI_2-EA (**33**) (*Scheme 2*). Unlike TXAS, PGIS was shown to catalyze the hydrolysis of PGH_2 (produced from AA) and PGH_2-G (produced from 2-AG) into the respective 6-keto-$PGF_{1\alpha}$ metabolites with similar efficiency (*Scheme 2*) [169].

Scheme 2. *Graphical Representation of the Oxygenase Pathway of Endocannabinoid Metabolism by Cyclooxygenase* (COX) *and Lipoxygenase* (LOX) *Enzymes*



In vivo, the oxidative metabolism of AEA was investigated in $FAAH^{(-/-)}$ mice, in which a single high dose of AEA (50 mg/kg i.v.) was administered, 30 min after injection of AEA. Here, the metabolites $PGF_{2\alpha}$-EA (**26**), PGE_2-EA (**18**), and PGD_2-EA (**24**) were detected in the liver, kidney, lung, and small intestine of the mice. In contrast, only small amounts of PGE_2-EA (**18**) and PGD_2-EA (**24**) were detected in the kidney and lung, and even none in the liver and small intestine of wild-type $FAAH^{(+/+)}$ mice. $PGF_{2\alpha}$-EA (**26**) was not detected (at the HPLC/MS detection limit) in wild-type mice [171].

Site-directed mutagenesis studies were conducted to identify the amino acid determinants in COX-2 oxygenation of 2-AG and AEA. They demonstrated that Tyr^{385} is required to initiate the oxygenation of the three substrates AA, AEA, and 2-AG. Furthermore, the COX-2 side pocket and the Arg^{513} residue are determinant to generate the metabolites PGH_2-EA and PGH_2-G, and might rationalize the incapability of COX-1 to generate endocannabinoid-derived prostaglandins, since the Arg residue is absent from the position 513 of this isoform. Mutation of Leu^{531} to Ile, Val, or Ala afforded mutants with decreased ability to oxygenate AA, AEA, and 2-AG. Finally, the two polar residues Arg^{120} and Glu^{524} present within the COX-2 active site were shown to play critical roles in 2-AG oxygenation. The presence of a Gly^{533} residue, located near the end of the hydrophobic alcove in which the ω-terminus of arachidonic acid binds, was also found to be critical for AA, AEA, and 2-AG oxygenation. Replacement of Gly^{533} with bulkier amino acids prevented substrate binding and oxygenation [172][173].

3.1.3. *Biological Actions of Endocannabinoid-Derived COX Metabolites.* The COX-2-induced metabolites of AEA and 2-AG exhibit their own biological activity, and their investigation was facilitated since the commercial availability of some of them (PGD_2-1-G, PGE_2-EA, PGD_2-EA). $PGF_{2\alpha}$-EA (**26**) was reported to potently contract the feline iris sphincter, with an EC_{50} value of 57 nM. PGE_2-EA (**18**) and PGD_2-EA (**24**) were found to be much less potent, with EC_{50} values of 564 and 499 nM, respectively, while the corresponding free acids $PGF_{2\alpha}$, PGE_2, and PGD_2 (produced from AA) exhibited EC_{50} values of 11, 260, and 150 nM, respectively. Surprisingly, the prostaglandin ethanolamides $PGF_{2\alpha}$-EA (**26**), PGE_2-EA (**18**), and PGD_2-EA (**24**) are devoid of affinity for the human prostaglandin receptors TP, DP, EP_1, EP_2, EP_3, FP, and IP. $PGF_{2\alpha}$-EA (**26**) was shown to activate the TRPV-1 receptor, with an EC_{50} value of 15 μM and low efficacy. These results suggest that the contracting action of the three PG-EA on the feline iris sphincter is mediated by a novel receptor, which is functional in the cat iris. Additionally, the three PG-EA were reported to lack any inhibitory effect upon FAAH, suggesting that these prostaglandins are unlikely capable of enhancing the AEA endogenous levels by inhibition of its major hydrolytic metabolic pathway [174]. Similarly, the prostaglandin $F_{2\alpha}$-, E_2-, and D_2-1-glycerol esters were shown to loose the ability of their parent compound 1-AG to inhibit the hydrolysis of AEA and 2-oleoylglycerol by membrane and cytosolic fractions of rat cerebella, respectively, sources of FAAH and MAGL [76]. Consistent with the lack of affinity of prostaglandin ethanolamides for the prostaglandin receptors, the 2-AG-derived metabolite PGE_2-G (**21**) was shown to exhibit no binding to TP, IP, DP, and FP receptors, but weakly binds to the EP_1 and EP_3 receptors. PGE_2-G (**21**) induced a concentration-dependent release of Ca^{2+} in RAW264.7 cells, with an EC_{50} value of 1 pM and an increase in extracellular-

signal-regulated kinase (ERK) phosphorylation in a PKC-dependent way. These results were obtained at doses of PGE_2-G (**21**) (< 50 nM) much lower than those required for the binding to EP receptors, suggesting that the contribution of these receptors is unlikely to rationalize the PGE_2-G-mediated signaling. These observations raised the possibility that PGE_2-G effects are mediated by a novel receptor [175]. Very recently, PGE_2-G (**21**) was reported to induce a concentration-dependent increase in the frequency of miniature inhibitory postsynaptic currents (mIPSCs) in primary cultured hippocampal neurons, with an EC_{50} value of 1.71 μM. The other metabolites, PGD_2-G (**20**), $PGF_{2\alpha}$-G (**25**), and PGD_2-EA (**24**), also induced an increase in the frequency of mIPSCs, while PGE_2-EA (**18**) and $PGF_{2\alpha}$-EA (**26**) were devoid of any effect. Interestingly, the effect observed with the parent compounds 2-AG and AEA was opposite: namely a decrease in the frequency of mIPSCs. A similar decrease was observed when the AA-derived prostaglandins PGE_2 (**14**) and PGD_2 (**12**) were applied. Finally, the effect of PGE_2-G (**21**) was significantly attenuated by application of an IP_3 inhibitor or a PKA inhibitor, suggesting the implication of these pathways in the PGE_2-G-induced enhancement of synaptic transmission [176].

In addition to these studies in which the involvement of an oxidative metabolite of endocannabinoids was clearly identified, some reports suggest the implication of a COX metabolite in some AEA-induced effects. Thus, the inhibition of interleukin-2 secretion by AEA in primary splenocytes is partially reversed by a COX-2-specific inhibitor, and by a PPARγ-specific antagonist, but not by CB_1 or CB_2 antagonists [177]. A possible role for a COX metabolite was also suggested in the AEA-induced cell death of colorectal carcinoma cells (CRC). AEA inhibits the growth of the CRC cell lines HT29 and HCA7/C29, which exhibit moderate and high COX-2 expression, respectively, but has only a weak effect upon the growth of the SW480 CRC cell line, which has low expression of COX-2. Moreover, the application of an FAAH inhibitor only potentiated the non-apoptotic cell death, suggesting the implication of the COX-2 pathway in the antitumoral activity of AEA in CRC [12]. Finally, the AEA- and 2-AG-derived prostaglandins were reported to be significantly more stable than the AA-derived prostaglandins, suggesting that these oxygenated lipids have half-lives long enough to act as signal mediators or pro-drugs [77].

3.1.4. *Generation of COX-1$^{(-/-)}$ and COX-2$^{(-/-)}$ Mice.* The generation of COX-2$^{(-/-)}$ mice [178][179] was succeeded simultaneously to the generation of COX-1$^{(-/-)}$ mice [180] in 1995. Compared to COX-2$^{(+/+)}$ wild-type mice, the COX-2$^{(-/-)}$ mice have reduced life expectancy due to severe nephropathy and progressive renal deterioration. COX-1$^{(-/-)}$ and COX-2$^{(-/-)}$ mice show higher susceptibility to ischemic brain injury [181] and pulmonary fibrogenesis [182], respectively. Only COX-1$^{(-/-)}$ mice exhibit increased crypt epithelial cell apoptosis and decreased clonogenic stem-cell survival after γ-irradiation, while the COX-2$^{(-/-)}$ mice are undistinguishable from wild-type mice after irradiation [183]. Conversely, disruption of the COX-2, but not the COX-1 gene, leads to impaired wound healing of gastric ulcers induced by cryoprobe [184]. However, both COX-1$^{(-/-)}$ and COX-2$^{(-/-)}$ mice demonstrated decreased recovery of left-ventricular-developed pressure after cardiac ischemia [185]. The investigation of the COX-1 or COX-2 gene disruption on inflammation and inflammatory pain highlighted sex differences. Thus, only COX-2$^{(-/-)}$ females exhibited reduction in edema and joint destruction, but both males and females lacked to develop thermal

hyperalgesia and mechanical allodynia in the model of chronic *Freund*'s adjuvant-induced arthritis. Moreover, the COX-1$^{(-/-)}$ females, but not the males, exhibited reduced inflammatory edema and joint destruction. Taken together, these results suggest that both COX genes contribute to pain and inflammation in a model of inflammatory arthritis, and that the therapeutic effects of COX inhibitors in this disorder might be sex-dependent [186]. Furthermore, the disruption of the COX-2 gene was shown to be beneficial and to prevent the development of some disorders. Among others, COX-2$^{(-/-)}$ mice were reported to be more resistant to influenza-A viral infection [187], to exhibit reduced susceptibility to ischemic brain injury and *N*-methyl-D-aspartate-mediated neurotoxicity [188], reduced sulfur-mustard-induced skin toxicity [189] and susceptibility to autoimmune arthritis [190]. More details on the characteristics of COX-1$^{(-/-)}$ and COX-2$^{(-/-)}$ mice can be found in the reviews of *Langenbach et al.* [191], and *Loftin et al.* [192]. Unfortunately, the effect of endocannabinoids in COX-2$^{(-/-)}$ mice is not known. To our knowledge, no study assessing the effect of endocannabinoids in these knock-out mice has been reported.

3.2. *Lipoxygenase* (LOX). 3.2.1. *Molecular Structures, Distribution, and Catalytic Mechanism.* Lipoxygenases (LOXs) are a family of monomeric non-heme ferroproteins that catalyze the regio- and stereospecific conversion of polyunsaturated acids into hydroperoxides. LOXs are widely expressed in mammals and plants, in which their products were shown to be involved in germination, wound healing, and disease resistance [193]. In humans, six LOXs have been characterized: 5-LOX, e-LOX-3, platelet-type 12-LOX, 12(*R*)-LOX, 15-LOX-1, and 15-LOX-2. The prefixes '5', '12', and '15' in these names refer to the ability of the enzyme to add a hydroperoxy (OOH) group at C(5), C(12), and C(15), respectively, of AA. However, the leukocyte-type 12-LOX, which is highly related to 15-LOX, is capable to form 15-hydroperoxyeicosatetraenoic acid (15-HPETE; **39**) in addition to 12-HPETE (**40**) from AA as substrate (*Scheme 1*). The leukocyte-type 12-LOX is found in porcine leukocytes, mouse macrophages, and bovine tracheal epithelium, while 15-LOX is present in human airway epithelium, eosinophils, and reticulocytes [194][195]. Soybean LOX-1 is a 15-LOX widely used as model for the study of all lipoxygenases. The three-dimensional structure of LOX-1 was elucidated in 1993 by *Boyington et al.* [196], revealing two domains. Domain I comprises 146 residues at the N-terminus; and domain II, which consists of 693 residues at the C-terminus, contains the active site in its center. Two faces of the Fe center face large cavities. The first cavity is shaped like a funnel and allows the access of molecular oxygen (O_2) to the Fe centers. The second cavity is the center of the catalytic events, and its entrance is blocked by Leu^{480} and Met^{341}. Small movements of the side chains of these residues allow the entrance of the substrates [196].

In the catalytic mechanism of LOXs, one electron of the 1,4-diene system of the substrate is abstracted by the Fe^{3+} form of the enzyme, to yield the free-radical form of the 1,4-diene and the Fe^{2+} form of the enzyme. The free radical reacts with O_2 to form a peroxyl radical ($HOO^{\bullet}$), which then abstracts an electron from the metal. A peroxidate radical anion is produced, and Fe^{3+} is regenerated. Finally, H^+ is transferred from the base to the radical anion, producing the hydroperoxide as final product [197]. More than 50 LOXs have been sequenced and expressed in bacterial systems. The amino acid sequences are 661 to 923 residues long, and the shortest sequences are found in

mammals lacking the N-terminal domain I present in LOX-1 [198]. Unlike their beneficial actions in plants, LOXs have been associated to the development of several disorders in mammals, including asthma, atherosclerosis, cancer, HIV infection, brain aging, psoriasis, and arthritis [199–202]. In mammals, most studies have been dedicated to 5-LOX, which is expressed in macrophages, mast cells, and polymorphonuclear leukocytes [203].

3.2.2. *Endocannabinoids as LOX Substrates.* LOXs do not only catalyze the hydroperoxidation of AA, but also of other fatty acids containing one or more (1*Z*,4*Z*)-penta-1,4-diene systems such as linoleic (C18 : 2) and linolenic (C18 : 3) acids. The two endocannabinoids AEA and 2-AG were reported to be substrates of several kinds of LOXs. Unfortunately, they were not tested in the same LOX models. The knowledge on the LOX pathways, which might involve endocannabinoids as substrates, is, thus, probably incomplete. AEA was mainly assessed in plant-derived LOX systems. Thus, incubation of AEA with purified 5-LOX from barley and tomato yielded (11*S*)-11-hydroxyperoxyeicosa-5,8,12,14-tetraenoylethanolamide (11*S*-HPETEA; **35**) as major product, while the (5*S*)-configured product had been expected. However, incubation of soybean 15-LOX with AEA formed 15*S*-HPETEA (**36**) as major product. Taken together, these results suggest that the regiospecificity of plant 5-LOX is strongly altered when AEA is used as substrate instead of AA, while that of plant 15-LOX is only slightly affected [204]. Conversely, the metabolism of 2-AG by LOX enzymes was investigated with mammal 12-LOX and 15-LOX. Incubation of 2-AG with porcine leukocyte-type 12-LOX, but not with platelet-type 12-LOX, yielded (12*S*)-12-hydroxyperoxyeicosa-5,8,10,14-tetraenoic acid glycerol ester (12*S*-HPETE-G; **37**). A similar result was obtained when 2-AG was incubated with COS-7 cells transfected with leukocyte-type 12-LOX. When compared to AA, 2-AG is, however, considered as a weak substrate of 12-LOX: the k_{cat}/K_m ratio for oxygenation of 2-AG is only 40% of that for AA [205]. In contrast, kinetics analysis demonstrated that soybean 15-LOX, human 15-LOX-1, and human 15-LOX-2 oxygenate 2-AG comparably to AA. Incubation of 2-AG with 15-LOX released 15-HPETE-G (**38**) as single predominant product, which acts as a PPARα agonist. The metabolism of 2-AG was also investigated with potato and human 5-LOX. Surprisingly, unlike AEA, 2-AG was shown to be unaffected by these enzymes (*Scheme 2*) [79].

3.2.3. *Generation of 5-LOX$^{(-/-)}$ and 12/15-LOX$^{(-/-)}$ Mice.* The generation of 5-LOX$^{(-/-)}$ mice was achieved simultaneously by *Goulet et al.* [206], and *Chen et al.* [207]. The 5-LOX$^{(-/-)}$ mice are indistinguishable in growth, size, life expectancy, and cage behavior from their 5-LOX$^{(+/+)}$ wild-type littermates. With the exception of the models of acute vesicular stomatitis virus encephalitis [208], *Klebsiella pneumoniae* airway infection [209], and kidney transplantation [210], the 5-LOX$^{(-/-)}$ mice generally fare much better than the wild-type mice. Thus, the 5-LOX$^{(-/-)}$ mice exhibit more resistance to the lethal effects of shock induced by platelet-activating factor [209], higher resistance to pleurisy and lung injury caused by carrageenan [211], and exhibit reduced degree of renal dysfunction caused by renal ischemia/reperfusion injury [212].

Leukocyte-type 12/15-LOX$^{(-/-)}$ mice (L-12/15-LOX$^{(-/-)}$ mice) were generated in 1996 by *Sun* and *Funk* [213]. They appear morphologically normal, grow normally, and are indistinguishable from their wild-type littermates. However, they have higher serum-insulin levels compared to wild-type mice [214], which confers a resistance to

streptozotocin-induced diabetes [215]. Disruption of the 12/15-LOX gene also leads to atheroprotection [216], but also to an impaired ischemic preconditioning-induced cardioprotection [217]. Remarkably, L-12/15-LOX$^{(-/-)}$ mice were shown to exhibit enhanced responses following acute, but not chronic, administration of morphine or cocaine [218]. Unfortunately, the effect of endocannabinoids was not assessed in the 5-LOX$^{(-/-)}$ or L-12/15-LOX$^{(-/-)}$ knock-out mice.

4. Conclusions. – The endocannabinoids are metabolized *via* two main pathways. The 'hydrolase' pathway was first identified and, consequently, is the most-studied and best-characterized one. FAAH and MAGL belong to the hydrolase pathway of the endocannabinoid metabolism and catalyze the hydrolysis of the amide and ester bonds of AEA and 2-AG, respectively. They were recently joined by NAAA, which preferably recognizes PEA as substrate. FAAH, MAGL, and NAAA are now very well-characterized. They are the targets of researchers who want to promote the therapeutic potential of endocannabinoids by enhancement of their endogenous half-life, following chemical inactivation of their degrading enzymes by selective inhibitors. Such a strategy was already crowned with success and led to the discovery of new analgesic, anxiolytic, and anti-inflammatory drugs [6][219]. Nevertheless, the endocannabinoids are also subject to oxidative metabolism by the enzymes of the 'oxygenase' pathway, including enzymes of the COX and LOX family. However, very little is known on the physiological relevance of the 'oxygenase' pathway in the endocannabinoid metabolism. The formation of endocannabinoid-derived oxidative metabolites was observed after incubation of the endocannabinoid precursors with purified oxygenase enzymes, or in activated macrophages, followed by direct MS analysis. *In vivo*, significant levels of oxidative metabolites of endocannabinoids were only reached in knock-out mice in which the competing FAAH pathway is lacking. Even under these conditions, the endogenous levels of AEA-derived prostaglandins $F_{2\alpha}$, E_2, and D_2 are more than 20-fold lower than those of AEA in the same tissues of FAAH$^{(-/-)}$ mice [171]. In wild-type mice, the non-selective COX inhibitor ibuprofen failed to block the effects of AEA in the tetrad test, but efficiently blocked the effects of AA [220]. So far, no significant level of endocannabinoid-derived oxidative metabolite was detected in a biological system expressing a competitive hydrolase pathway, which questions the physiological relevance of the oxygenase-metabolism pathway for endocannabinoids. Moreover, all the observations of oxygenated metabolites of endocannabinoids required exogenous administration of the precursor. New experiments are, thus, needed to clarify the physiological relevance of the oxygenase metabolism of endocannabinoids. However, some connections likely exist between the FAAH/MAGL hydrolase pathways and the COX/LOX oxygenase pathways. It must be kept in mind that when the hydrolase pathway was mainly presented in this review as a termination of endocannabinoid signaling, it might also be viewed as a starting point of the COX and LOX pathways. Indeed, hydrolyses of AEA and 2-AG by FAAH and MAGL release AA, which is the natural substrate of COX and LOX enzymes. This interconnection between the hydrolase and oxygenase pathways should not be forgotten in the quest of 'selective' drugs acting on specific enzymes responsible of endocannabinoid metabolism. *In vivo*, these compounds will probably have more effects than the specific role for which they were designed.

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