Research Communication

Aging Modifies the Enzymatic Activities Involved in 2-Arachidonoylglycerol Metabolism

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Abstract

One of the principal monoacylglycerol (MAG) species in animal tissues is 2-arachidonoylglycerol (2-AG), and the diacylglycerol lipase (DAGL) pathway is the most important 2-AG biosynthetic pathway proposed to date. Lysophosphatidate phosphatase (LPAase) activity is part of another 2-AG-forming pathway in which monoacylglycerol lipase (MAGL) is the major degrading enzyme. The purpose of this study was to analyze the manner in which DAGL, LPAase, and MAGL enzymes are modified in the central nervous system (CNS) during aging. To this end, diacylglycerols (DAGs) and MAGs of different composition were used as substrates of DAGL and MAGL, respectively. All enzymatic activities were evaluated in membrane and soluble fractions as well as in synaptic terminals from the cerebral cortex (CC) of adult and aged rats. Results related to 2-AG metabolism show that aging: (*a*) decreases DAGL-α expression in the membrane fraction whereas in synaptosomes it increases DAGL-β and decreases MAGL expression; (*b*) decreases LPAase activity in both membrane and soluble fractions; (*c*) decreases DAGL and stimulates LPAase activities in CC synaptic terminals; (*d*) stimulates membrane-associated MAGL-coupled DAGL activity; and (*e*) stimulates MAGL activity in CC synaptosomes. Our results also reveal that during aging the net balance between the enzymatic activities involved in 2-AG synthesis and breakdown is low availability of 2-AG in CC membrane fractions and synaptic terminals. Taken together, our results lead us to conclude that these enzymes play crucial roles in the regulation of 2-AG tissue levels during aging. © 2012 BioFactors, 39(2):209–220, 2013

Keywords: *2-arachidonoylglycerol; cerebral cortex; diacylglycerol; lysophosphatidic acid; monoacylglycerol; synaptosomes*

1. Introduction

The endocannabinoid system has been shown to be involved in a number of important physiological processes [1–3]. *N*-arachidonoylethanolamide (anandamide) was originally identified as an endocannabinoid [4] and 2-arachidonoylglycerol [5,6] was subsequently identified as the second endocannabinoid. These molecules are produced on demand in an activity-dependent manner and then released to the extracellular space, acting near the site of their synthesis. Several biochemical studies have demonstrated the stimulus-induced generation of 2-AG in various cell types, including neurons, and have revealed several pathways for 2-AG production, the most important of which involves the combination of phospholipase C (PLC) and DAGL. PLC firstly hydrolyzes arachidonic acid-containing membrane phospholipids such as phosphatidylinositol, and subsequently produces arachidonic acid-containing diacylglycerol [7]. In turn, 2-AG is produced from diacylglycerol by the action of DAGL. Other pathways for 2-AG production involve the conversion from 2-arachidonoyl lysophosphatidic acid into 2-AG by LPA phosphatase [8].

Abbreviations: *ABHD6, serine hydrolase alpha-beta-hydrolase domain 6; 2-AG, 2-arachidonoylglycerol; BSA, bovine serum albumin; CC, cerebral cortex; DAG, diacylglycerol; DAGL, diacylglycerol lipase; DTT, dithiotreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis (*β*-aminoethyl ether)-*N,N,N′,N′*-tetra acetic acid; HEPES,* N*-[2-hydroxyethyl]piperazine-*N′*-[2-ethanesulfonic acid]; LysoPtdOH, lysophosphatidic acid; LPAase, lysophosphatidate phosphohydrolase; MAG, monoacylglycerol; MAGL, monoacylglycerol lipase, NEM,* N*-ethylmaleimide; PtdCho, phosphatidylcholine; PLC, phospholipase C; TLC, thin-layer chromatography.*

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Endocannabinoids can be degraded through two different pathways, namely hydrolysis and oxidation [9]. Apart from the enzymes that catalyze the first pathway and which include mainly MAGL, additional 2-AG-hydrolyzing enzymes have been observed in the brain [10–12]. Furthermore, in the CNS, the molecules involved in endocannabinoid signaling are arranged around synapses and their signaling is mediated mostly by the CB1 receptor [13–15]. The principal endocannabinoid, 2-AG, in the developing and adult brain acts on cannabinoid receptors to regulate axonal growth and guidance, activity-dependent synaptic plasticity, and adult neurogenesis. It plays important roles in various aspects of neural functions including learning and memory, anxiety, depression, addiction, appetite and feeding behavior, pain, and neuroprotection. Exogenous and endogenous cannabinoids have been shown to exert neuroprotection in a variety of *in vitro* and *in vivo* models of neurodegeneration [16]. Although the role of 2-AG in neurodegenerative processes has been partially elucidated, its role in physiological aging remains unexplored. The main purpose of the present study was therefore to analyze the manner in which the enzymes involved in 2-AG metabolism are modified in the CNS during aging.

2. Experimental Procedure

2.1. Materials

[2-^{3}H]Glycerol (200 mCi/mmol or 2 Ci/mmol) and omnifluor were obtained from New England Nuclear-Dupont (Boston, MA); 2-arachidonoylglycerol [glycerol-1,2,3-3H] (40 Ci/mmol), unlabeled 2-arachidonoylglycerol, and lysophosphatidic acid, 1-oleoyl [oleoyl-9,10-^{3}H(N)]- (54 Ci/mmol) were obtained from American Radiolabeled Chemicals, Inc. (Saint Louis, MO). Oleoyl-L-α-lysophosphatidic acid, *N*-ethylmaleimide, and BSA were obtained from Sigma-Aldrich (St. Louis, MO). Polyclonal antibodies against MAGL protein and FAAH protein were obtained from Cayman (Ann Arbor, MI) and DAGL-α and DAGL-β antibodies were generously supplied by Dr. Patrick Doherty (King's College London, London, UK). The secondary antibody used for MAGL, FAAH, and DAGL-β enzyme detection was horse radish peroxidase (HRP)-conjugated anti-rabbit. HRP-conjugated anti-goat was used for DAGL-α enzyme detection. All other chemicals were of the highest purity available.

2.2. Animals

Male Wistar-strain rats were kept under constant environmental conditions and fed on a standard pellet diet. All procedures were carried out in accordance with the guidelines issued by the Animal Research Committee of the Universidad Nacional del Sur (Argentina) in accordance with the Guide of the Care and Use of Laboratory Animals of the Institute for Laboratory Animal Research (ILAR) of the National Academy of Science (Bethesda, MD). Adult rats (4 months old) and aged rats (28 months old) were killed by decapitation and the cerebral cortex (CC) was immediately dissected (2–4 min after decapitation).

2.3. Preparation of Rat Cerebral Cortex Membrane and Soluble Fractions

Cortices were homogenized (10%) in Tris HCl (20 mM, pH 8) containing 0.32 M sucrose and protease inhibitors (0.1 mM PMSF, 1 mg/mL aprotinin, and 2 mg/mL leupeptin) and centrifuged to eliminate nuclei and cellular debris (1,000*g*, 10 min, 4 °C). The pellet was discarded. Supernatants were ultracentrifuged (100,000*g*, 60 min, 4 °C) to obtain membrane and soluble fractions, both showing 2-AG-degrading activity [10].

2.4. Preparation of Synaptosomes

CC homogenates were prepared in the following way: 20% (w/v) in 0.32 M sucrose, 1 mM EDTA, 5 mM buffer HEPES-Na (pH 7.4). The CC homogenate was centrifuged at 1,300*g* for 7 min and the supernatant was carefully transferred to another tube. The nuclear pellet was resuspended with the isolation medium and subsequently centrifuged at 1,300*g* for 7 min. The combined supernatant was then centrifuged at 17,000*g* for 10 min to obtain the crude mitochondrial pellet (CM). The CM was resuspended with the isolation medium and layered onto a two-step gradient of 7.5 to 13% Ficoll solution prepared in the isolation medium. The sample layered onto Ficoll discontinuous gradient was centrifuged at 99,000*g* for 30 min using an ultracentrifuge Beckman, model LS-50, with a swinging bucket rotor (SW28). The myelin fraction band is at the interphase between the isolation medium and 7.5% Ficoll medium, the synaptosomal fraction bands are at the interphase between 7.5% and 13% Ficoll medium, and the free mitochondrial fraction is the pellet below 13% Ficoll medium [17].

2.5. Preparation of Radioactive 1,2-diacyl-sn-glycerol

DAGs from different origins were used as substrate:

1. In one case, DAG was obtained from [^{3}H]PtdCho (0.15 μCi/μmol) after hydrolysis by phospholipase C from *Clostridium welchii* (Grade B, Calbiochem, Los Angeles, CA). [2-^{3}H]Glycerol-PtdCho ([^{3}H]PtdCho) was synthesized from bovine retinas incubated with [2-^{3}H]glycerol (200 mCi/mmol) as previously described [18]. [^{3}H]PtdCho was dried under a stream of nitrogen and dissolved in 2% ethanol in diethyl ether (v/v) [19]. The enzyme was dissolved in 50 mM Tris-HCl buffer, pH 7.3, containing 3 mM $CaCl_2$ and added to the solution of lipid; the mixture was then incubated at room temperature for 4 h. DAG was extracted from the hydrolysis mixture with diethyl ether containing 1% water and isolated by one-dimensional TLC on silica gel G in the solvent hexane:diethyl ether:acetic acid (45:55:1.5, v/v) [20]. DAG was extracted from silica gel with *n*-hexane: 2-propanol (3:2 v/v) to avoid isomerization and stored at −20 °C. [^{3}H]DAG specific activity was 0.15 mCi/mmol.

2. In another case, DAG was synthesized from bovine retinas incubated with [2-^{3}H]glycerol (2 Ci/mmol) as previously described [18]. DAG was extracted from the tissue as described elsewhere [21] and isolated and extracted from silica gel as specified in (1). [^{3}H]DAG specific activity was 2.35 mCi/mmol.

2.6. Preparation of Radioactive Monoacyl-*sn*-Glycerol

MAG was obtained from [2-^{3}H]triacylglycerol (TAG) by incubation with pancreatic lipase in Tris-HCl 50 mM (pH 7), NaCl 3 mM, $CaCl_2$ 10 mM, and Triton X-100 15 mM, at 37 °C for 30 min [22]. [2-^{3}H]TAG was synthesized from bovine retinas incubated with [2-^{3}H]glycerol (200 mCi/mmol). MAG was extracted from the hydrolysis mixture following Folch et al. [21] and isolated by one-dimensional TLC on silica gel G in the solvent hexane:diethyl ether:acetic acid (45:55:1.5, v/v) [20]. MAG was extracted from silica gel with *n*-hexane: 2-propanol (3:2 v/v) to avoid isomerization and stored at −20 °C. [^{3}H]MAG specific activity was 0.5 mCi/mmol.

2.7. Fatty Acid Analysis

Fatty acid composition of DAG and MAG substrates was determined by gas chromatography (GC) of their fatty acid methyl ester derivatives. The fatty acid composition of lipids was determined by GC of their fatty acid methyl ester derivatives, which were prepared by placing lipid samples with 14% BF3 in methanol overnight at 45 °C under N_2 in Teflon® sealed tubes [23]. Before GC, methyl esters were purified by TLC using hexane/ether (95:5, v/v) on silica gel G plates prewashed with methanol/ether (75:25, v/v). They were subsequently recovered from the silica gel plates after thorough mixing and partitioning to obtain water/methanol/hexane (1:1:1, by vol; three hexane extractions). PUFA fractions were spotted and resolved into fractions by means of chloroform/methanol (90:10, v/v). HPLC grade solvents were used throughout. A Varian 3700 gas chromatograph equipped with two (2 m × 2 mm) glass columns packed with 10% SP 2330 on Chromosorb WAW 100/120 (Supelco, Inc., Bellefonte, PA) was used. The column oven temperature was programmed from 155 to 230 °C at a rate of 5 °C/min and then kept at the highest temperature for approximately 30 min to allow VLCPUFA to elute from the column. Injector and detector temperatures were set at 220 and 230 °C, respectively, and N_2 (30 ml/min) was the carrier gas. FA peaks were detected using FID operated in the dual-differential mode and quantified by electronic integration (Varian Workstation). Methyl heneicosanoate was added as an internal standard for quantitative analysis.

2.8. DAGL Activity Assay

DAGL activity was determined using exogenously added [^{3}H]DAG as substrate. Assays using [^{3}H]DAG were performed in 50 mM MOPS buffer (pH 7.4) containing 0.25% fatty acid-free bovine serum albumin (BSA) in a final volume of 100 μL. The DAGL assay (50 μg protein per assay) was initiated with the addition of [^{3}H]DAG suspensions (300 μM, 10,000 DPM) to either membrane or soluble fractions. [^{3}H]DAG suspensions were prepared by separately sonicating with equimolecular concentrations of lysoPtdCho in 50 mM MOPS buffer (pH 7.4).

2.9. LPAase Activity Assay

Incubations contained unlabeled 1-oleoyl LysoPtdOH and LysoPtdOH,1-oleoyl [oleoyl-9,10-^{3}H(N)]- (20 μM, 6×10^4 DPM), 100 mM Tris-HCl (pH 7.4), 1.2 mM DTT, 2 mM EDTA, and membrane or soluble fractions (50 μg protein) preincubated in the presence of 4.4 mM NEM in a final volume of 100 μL [24]. The substrate was added in phosphate buffer saline solution (pH 7.4) containing BSA 0.1%.

2.10. MAGL Activity Assay

MAGL activity was assessed by incubating sample fractions (50 μg protein) in a buffer solution of Tris-HCl (50 mM, pH 7.5) containing 1 mM EDTA in a final volume of 200 μL. When [^{3}H]-MAG (20 μM, 5,000 DPM) was used as substrate, it was vehiculized in fatty acid-free BSA (1.25 mg/mL). When 2-AG was used as substrate, it was prepared using [^{3}H]2-AG ([1,2,3-^{3}H]glycerol) plus unlabeled 2-AG (10 μM, 5,000 DPM) in acetonitrile.

All enzymatic reactions were conducted at 37 °C for 20 min. The enzymatic reactions were stopped by adding chloroform:methanol (2:1, v/v) or chloroform:methanol (1:1, v/v) when MAGL was assayed. Blanks were prepared identically to each enzymatic assay except that the membrane or soluble fraction was either boiled for 10 min or inactivated by the addition of chloroform:methanol (2:1, v/v) before use. Blanks without protein were also prepared and no differences were observed among the different blanks. Blank values were subtracted from each enzyme activity. Lipid products derived from DAGL and LPAase activities were extracted with chloroform:methanol (2:1, v/v) and washed with 0.2 volume of $CaCl_2$ (0.05%) [21].

2.11. Separation of Enzymatic Reaction Products

The DAGL product, MAG, was separated by gradient-thickness thin layer chromatography on a silica gel G plate and developed with hexane:diethyl ether:acetic acid (45:55:1.5, v/v) [20]. In this solvent system, DAG migrates with an R_f of 0.65 and MAG remains near the origin. LPAase products, MAG and free fatty acids were chromatographed by TLC on a silica gel H plate and developed with chloroform:acetone:methanol:acetic acid:water (30:40:10:10:4, v/v) up to the middle of the plate. The chromatogram was subsequently rechromatographed up to the top of the plate using hexane:diethyl ether:acetic acid (45:55:1.5, v/v) as developing solvent. Chromatograms were visualized by exposure to iodine vapors and scraped off for counting by liquid scintillation.

Glycerol, the MAGL product, was obtained in the upper phase after interrupting the enzymatic reaction. The aqueous phase containing radiolabel glycerol was concentrated to dryness and counted by liquid scintillation. Radiolabel samples were counted after the addition of 0.4 mL water and 10 mL 5% Omnifluor in toluene/Triton X-100 (4/1, v/v).

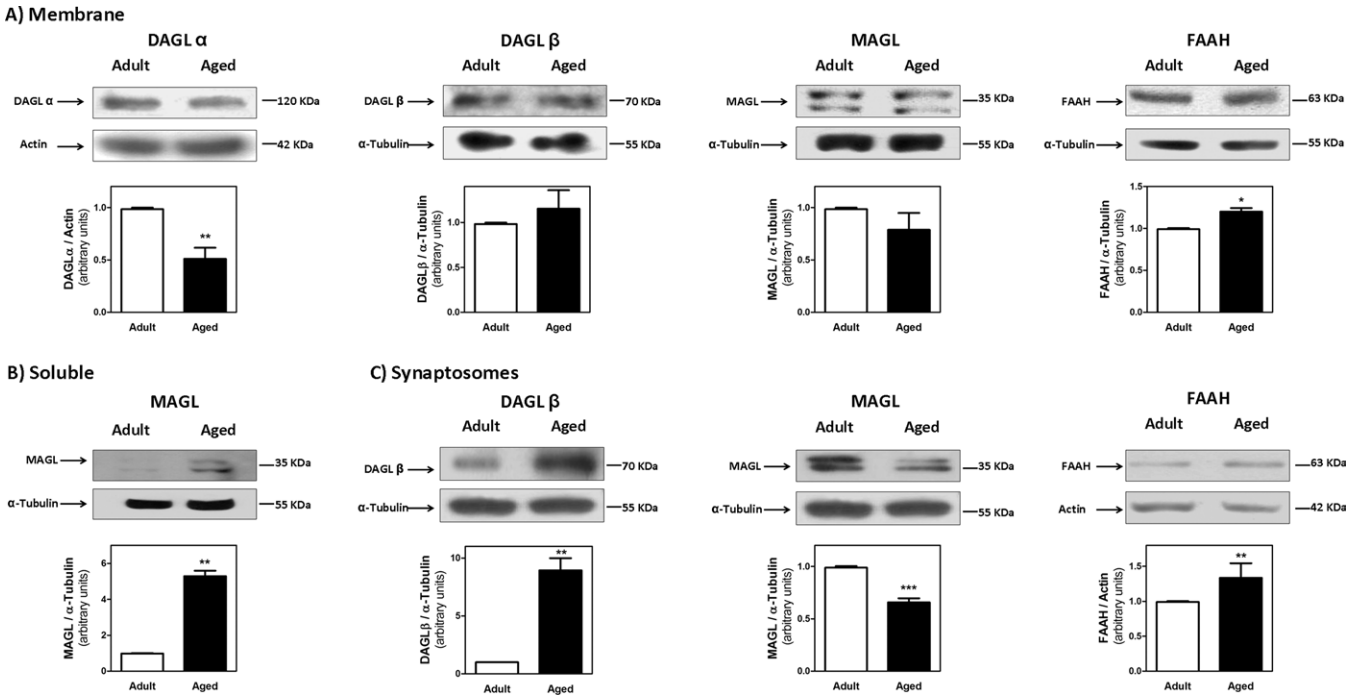

FIG 1

*Immunoblot analysis in CC from adult and aged rats. Proteins (40 μg) were boiled in Laemmli buffer, resolved in a 10% SDS-PAGE and transferred to a PDVF membrane for further WB assays. Membranes were blocked and incubated with primary and secondary antibodies as detailed in Materials and Methods. Immunoreactive bands were detected by enhanced chemiluminescence. Numbers on the right indicate molecular weights and the data shown represent the results of three independent experiments. The bar graph shows relative density corresponding to protein expression expressed as a ratio of loading control (actin or α-tubulin). Asterisks indicate significant differences compared to adult condition. ***P < 0.0001; **P < 0.001; and *P < 0.01.*

2.12. SDS-PAGE and Immunoblot

SDS-PAGE was carried out using 10% gels according to Laemmli [25]. Resolved proteins were transferred to immobilon P membranes using a Mini Trans-Blot cell electro blotter (BIO-RAD Life Science Group, CA) for 75 min. Membranes were blocked for 2 h with Tris-buffered saline (20 mM Tris–HCl, 150 mM NaCl) pH 7.5, containing 0.1% Tween 20 and 5% crystalline grade bovine serum albumin (BSA) (TTBS). Incubations with primary antibodies (anti-MAGL (1:2,000), anti-FAAH (1:2,000), anti-DAGL-α (1:2,000), anti-DAGL-β (1:2,000), and anti-Tubulin (1:5,000)) were carried out at room temperature for 2 h and with anti-Actin (1:1,000) at 4 °C overnight. Membranes were washed with TTBS and subsequently exposed to the appropriate HRP-conjugated secondary antibody (anti-rabbit or anti-goat) for 1 or 2 h. The membranes rewashed with TTBS and immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham Biosciences) using standard X-ray film (Kodak X-Omat AR).

2.13. Other Methods

Protein and lipid phosphorus content was determined following Bradford [26] and Rouser et al. [27], respectively.

2.14. Statistical Analysis

All data are given as means ± SD. Statistical analyses were carried out using GraphPad software (San Diego, CA; available at: www.graphpad.com) and were analyzed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Immunoblot Analysis in CC Membrane and Cytosolic Fractions and in Synaptosomes from Adult and Aged Rats

Immunoblot analysis with the respective antibodies against DAGL-α and −β, MAGL and FAAH is shown in Fig. 1. DAGL-α expression, which was found to decrease (48%) with aging, could be observed only in the membrane fraction (Fig. 1A). DAGL-β, on the other hand, was observed both in membranes (Fig. 1A) and in synaptosomes (Fig. 1C). No changes were observed in its expression in membranes during aging (Fig. 1A) but in synaptosomes, aging led to an eightfold increase (Fig. 1C). Western blot (WB) analysis revealed the presence of MAGL in the membrane (Fig. 1A), soluble (Fig. 1B) and synaptosomal fraction (Fig. 1C) from CC. A high expression (fourfold) of MAGL was also observed in the soluble fraction (Fig. 1B), whereas no changes were detected in the membrane fraction (Fig. 1A) during aging. In contrast, MAGL expression in aged synaptosomes was low (33%) (Fig. 1C). A 20% and 34% increase in FAAH expression was observed in membrane

TABLE 1 *[^{3}H]-DAG fatty acid composition used to determine DAGL activity*

Fatty acid	*% (a)*	*% (b)*
16:0	35	32
16:1	2	4
18:0	20	20
18:1	19	16
18:2	1	1
20:4	5	13
22:5	1	2
22:6	17	12

(a), DAG was prepared by hydrolysis of [^{3}H]-PtdCho; (b) [^{3}H]-DAG was obtained incubating retinas with [^{3}H]-glycerol as was specified in the Materials and Methods. The data therein represent the percentage distribution of each fatty acid.

(Fig. 1A) and synaptosomal (Fig. 1C) fractions, respectively, as a result of aging.

3.2. DAGL and LPAase Activity in CC Membrane and Cytosolic Fractions from Adult and Aged Rats

The DAGL and LPAase enzymes involved in the formation of 2-AG were assayed in terms of the effect of aging on their activities. The following substrates were used for the determination of DAGL activities: (i) DAG, composed mainly of fatty acids 16:0, 18:0, 18:1 and 22:6 (Table 1, see (a)); and (ii) DAG with a saturated and monoenoic fatty acid composition, that is, similar to substrate (i) but with a higher percentage of 20:4 (Table 1, see (b)). No DAGL activity was observed in the membrane and soluble fractions when using substrate (i) (data not shown) but activity was observed in membrane fractions when using DAG with a high percentage of 20:4 (Fig. 2A). Soluble LPAase activity represented 11% of the total activity in the preparations from adult animals. Aging decreased membrane and soluble activity by 32% and 29%, respectively (Fig. 2B).

3.3. DAGL and LPAase Activity in CC Synaptosomes from Adult and Aged Rats

DAGs of different composition gave rise to different DAGL activities. When the DAG specified in Table 1, (a) was used as substrate, MAG production was stimulated fivefold during aging (Fig. 3A) whereas in the case of the DAG specified in Table 1, (b), MAG production was diminished by 38% during aging (Fig. 3B). Furthermore, the contribution of LPAase to MAG production in adult animals was found to be higher than that derived from DAGL, and LPAase activity was observed to increase by 12% during aging (Fig. 3C).

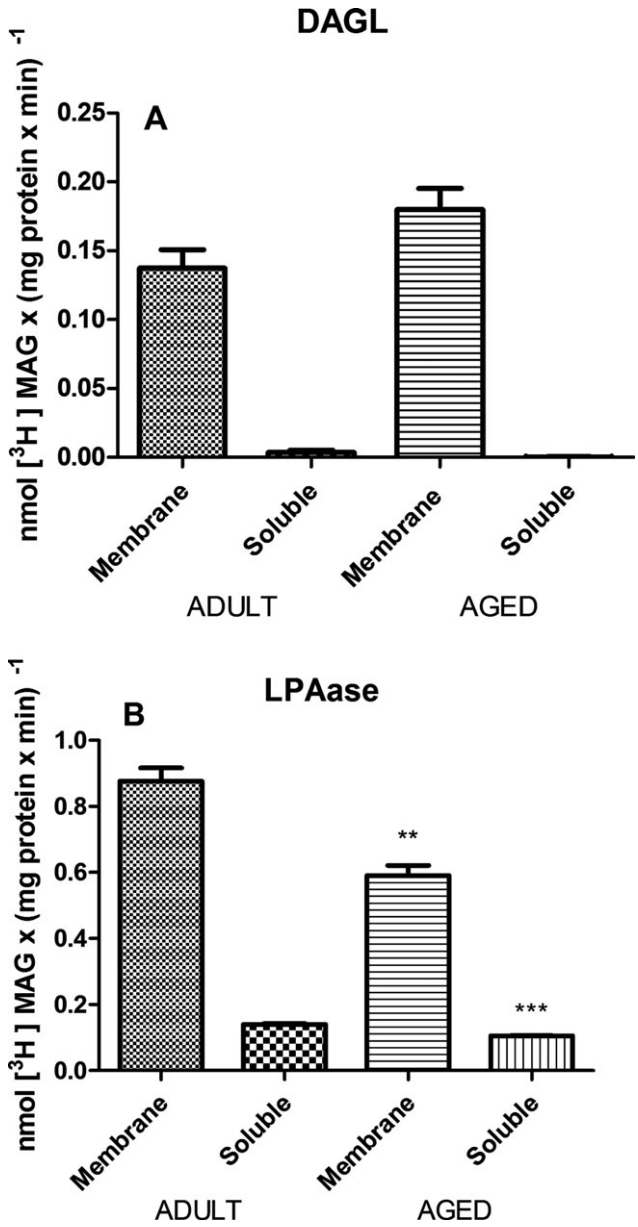

FIG 2 *DAGL and LPAase activity in CC membrane and cytosolic fraction from adult and aged rats. (A) DAGL activity was assayed using exogenously added [^{3}H]DAG (substrate (a) in Table 1) in the presence of membrane or soluble fraction in a final volume of 100 μL. (B) LPAase activity was assayed using unlabeled LysoPtdOH and LysoPtdOH,1-oleoyl [oleoyl-9,10-^{3}H(N)]- (20 μM, 6 $\times$ 10^4 DPM) in the presence of the membrane or cytosolic fraction, preincubated with 4.4 mM NEM in a final volume of 100 μL. The enzymatic activities were measured almost under saturated conditions and were stopped by adding chloroform:methanol (2:1, v/v). The subsequent procedure is specified in the Experimental Procedure section. Results represent the mean $\pm$ SD of three individual samples. ***P $<$ 0.0001 and **P $<$ 0.001 with respect to adult soluble and adult membrane, respectively.*

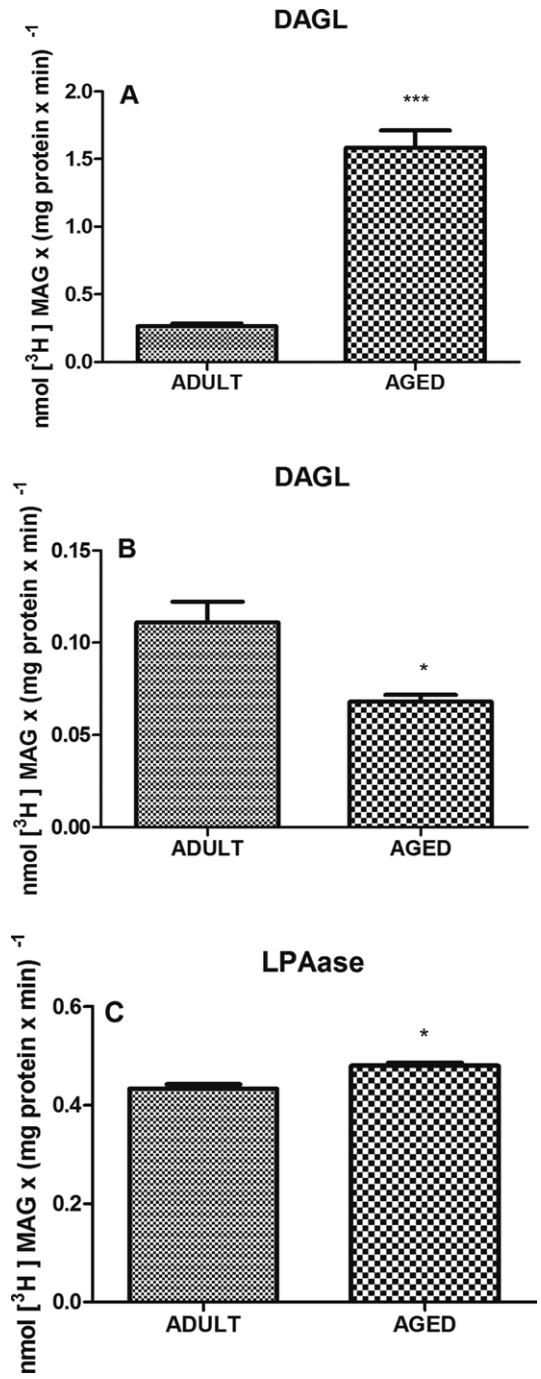

FIG 3

*DAGL and LPAase activity in CC synaptosomes from adult and aged rats. DAGL activity was assayed using as substrate [^{3}H]DAG. (A): substrate (a) in Table 1 and (B): substrate (b) in Table 1. The assay conditions for DAGL and LPAase (C) are specified in Fig. 2. Results represent the mean ± S.D. of 3 individual samples. ***P < 0.0001; *P < 0.01 with respect to adult condition.*

3.4. MAGL Activity in CC Membrane and Cytosolic Fractions from Adult and Aged Rats

Monoacylglycerol hydrolysis was analyzed using the following substrates: (i) 2-AG (Fig. 4A), (ii) saturated monounsaturated MAGs (Table 2, Fig. 4B), and (iii) MAGs generated from DAG containing a higher percentage of 20:4, by DAGL activity (Fig. 4C). The hydrolysis of 2-AG was observed to be equally distributed between membrane and soluble fractions. Neither total activity (membrane plus soluble) nor the distribution between fractions was modified as a result of aging (Fig. 4A).

Figure 4B shows MAG hydrolysis using substrate (ii). The principal degradative activity in MAG was associated with the membrane fraction (75%) in adult CC. MAG hydrolysis decreased by 38% in the membrane fraction whereas it underwent no changes in the soluble fraction during aging (Fig. 4B). It was also observed that MAG generated by DAGL acting on DAG substrate (iii) was further metabolized to glycerol in membrane fraction. The hydrolysis of the resulting MAG was 40% higher as a result of aging (Fig. 4C).

3.5. MAGL Activity in CC Synaptosomes from Adult and Aged Rats

MAG hydrolysis was analyzed using the following substrates: (i) 2-AG (Fig. 5A), (ii) saturated-monounsaturated MAGs (Fig. 5B), (iii) MAG generated from the enzymatic hydrolysis of DAG, containing a higher percentage of 20:4, by DAGL (Fig. 5C), and (iv) MAG generated from the enzymatic hydrolysis of DAG containing a higher percentage of 22:6, by DAGL (Fig. 5D). In adult CC synaptosomes, the highest hydrolytic activity was obtained from MAG with a high degree of saturation (Fig. 5B). MAG generated from DAG containing a high proportion of 20:4 showed low hydrolysis efficiency (Fig. 5C). When substrates (i), (ii) and (iv) were used, aging was observed to stimulate MAG hydrolysis by 43%, 31% and 63%, respectively (Figs. 5A, 5B, and 5D). However, MAG hydrolysis was found to decrease by 41% with substrate (iii) (Fig. 5C).

3.6. Degradation of 2-AG in the Presence of Inhibitors in CC Membrane and Synaptosomes from Adult and Aged Rats

Multiple enzymes, including MAGL, FAAH, and ABHD6, contribute to 2-AG degradation in the adult brain [10]. The rate of [^{3}H]2-AG degradation was therefore measured in the presence of MAGL inhibitors such as URB602 [28] and KML29 [29] and FAAH inhibitor URB597 [30] in the CC membrane fraction (Figs. 6A and 6B) and in CC synaptosomes (Figs. 6C and 6D). URB602 was observed to inhibit adult membrane-associated MAGL activity by 16% (Fig. 6A). However, when KML29 was used, inhibition reached 78% (Fig. 6A). The hydrolysis of 2-AG was found to be inhibited by 40%, 76%, and 25% in the presence of URB602, KML29, and URB597, respectively, in CC adult synaptosomes (Fig. 6C). It was also observed that 2-AG

hydrolysis was totally inhibited during aging in the presence of URB602 inhibitor, and by 75% and 18% in the presence of KML29 and URB597, respectively (Fig. 6D).

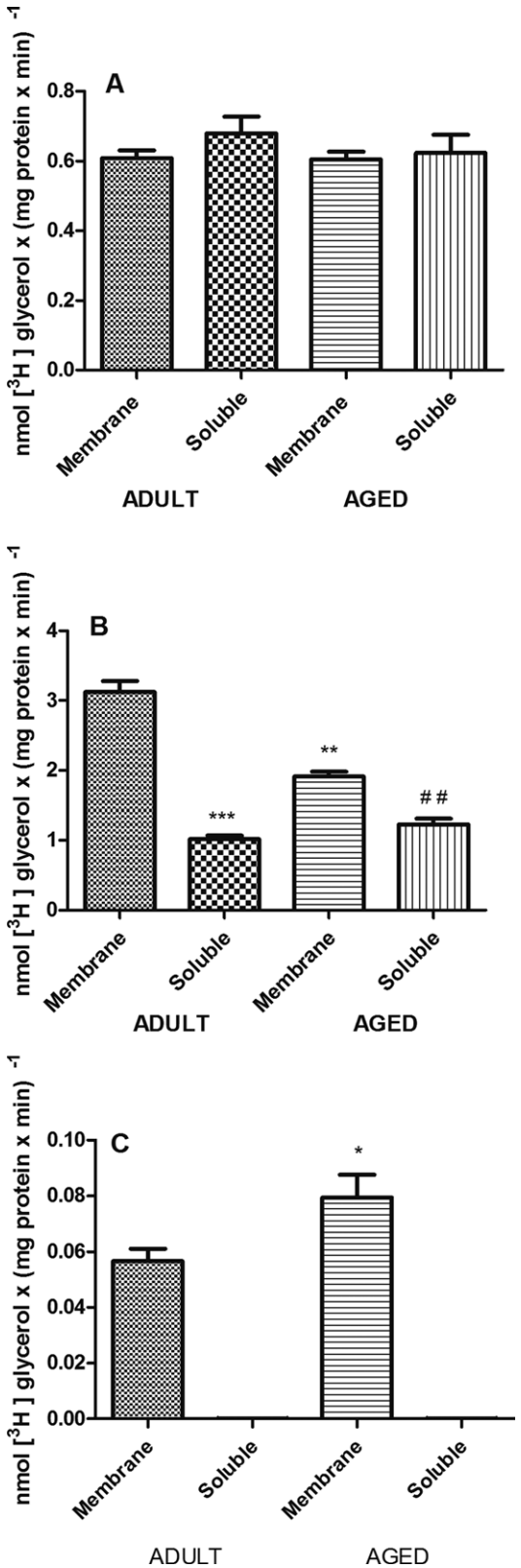

TABLE 2 *[^{3}H]-MAG fatty acid composition used to determine MAGL activity*

Fatty acid	*%*
14:0	8
16:0	25
16:1	10
18:0	44
18:1	13

The preparation of substrate was specified in the Materials and Methods. The data therein represent the percentage distribution of each fatty acid.

4. Discussion

The 2-AG is one of the principal MAGs in animal tissues. Before its identification as an endocannabinoid, this molecule was regarded as a mere intermediate in the breakdown of DAG [31]. In the present study, the expression and enzyme activities involved in endocannabinoid synthesis (DAGL and LPAase) and breakdown (MAGL) were analyzed in the membrane fraction (free from the nuclear fraction) and in the CC soluble fraction from adult and aged rats. Taking into account the role that these molecules play in neuronal synapses and since their synthesis and hydrolysis were observed to occur in the postsynapse and presynapse, respectively, we considered it relevant to also evaluate the effect of aging on the metabolism of MAGs at synaptic terminals. The biosynthetic pathways for 2-AG not only differ in different tissues and cells but also depend on the conditions of stimulation [32]. DAGL was analyzed in the present study, using DAGs with different acyl

FIG 4 *MAGL activity in CC membrane and cytosolic fractions from adult and aged rats. MAGL activity was assayed using (A) [^{3}H]2-AG ([1,2,3-^{3}H]glycerol) plus unlabeled 2-AG (10 μM, 5,000 DPM); (B) [^{3}H]MAG (20 μM, 5,000 DPM) obtained from the hydrolysis of bovine retina [^{3}H]TAGs; or (C) [^{3}H]MAG endogenously generated from the enzymatic hydrolysis of DAGL on [^{3}H]DAG containing a high proportion of 20:4. All assays were carried out in the presence of membrane or cytosolic protein in a final volume of 200 μL. The enzymatic reaction was conducted at 37 °C and was stopped by adding chloroform:methanol (1:1, v/v). MAGL product, [^{3}H]glycerol, which was obtained in the upper phase, concentrated to dryness and counted by liquid scintillation. Results represent the mean ± SD of three individual samples in (A) and (B) and of five individual samples in (C). ***P < 0.0001; **P < 0.001; and *P < 0.01 with respect to adult membrane; ##P < 0.001 with respect to aged membrane.*

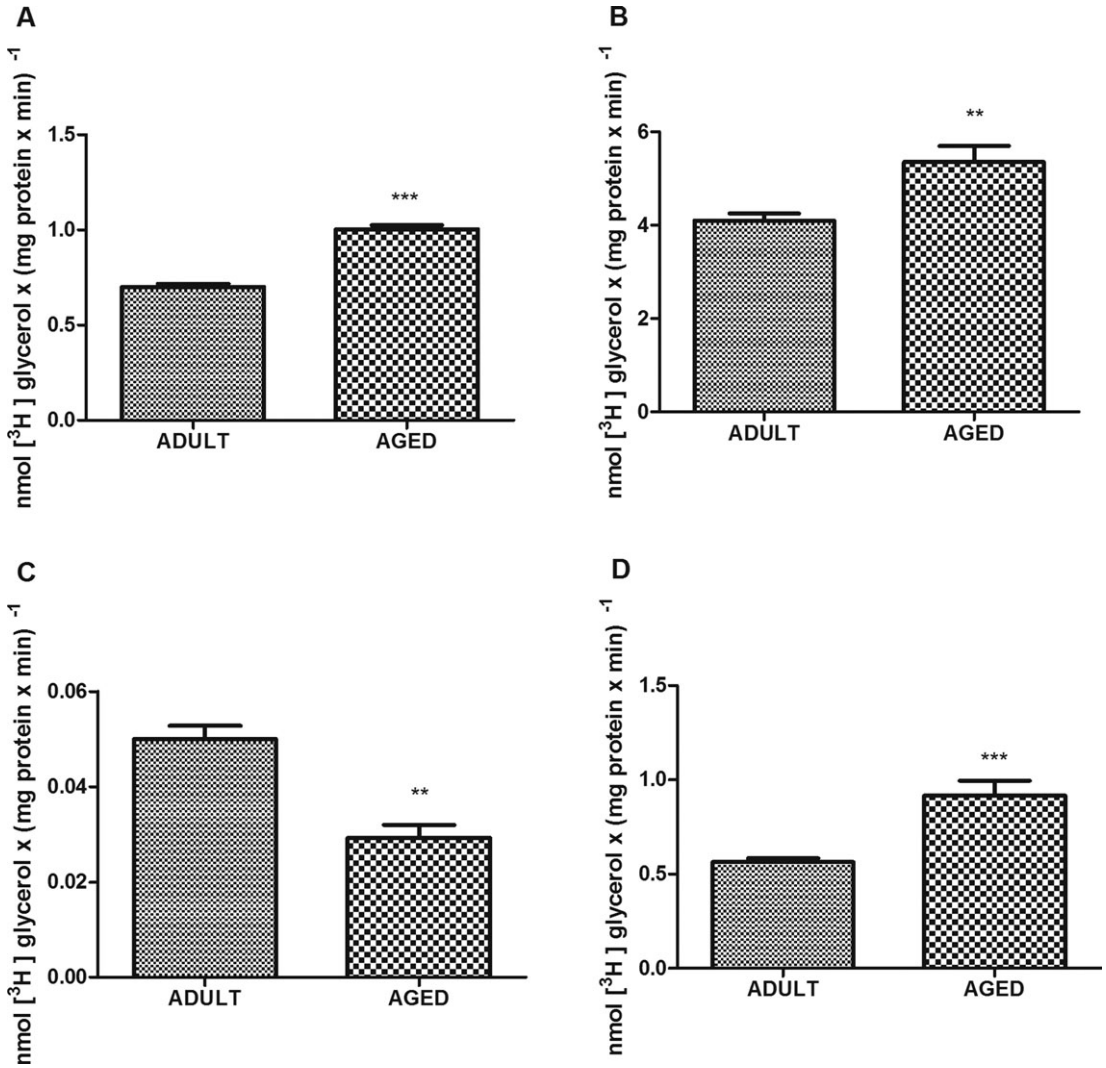

FIG 5

MAGL activity in CC synaptosomes from adult and aged rats. The assay conditions for MAGL and the substrates used in A, B, and C are specified in Fig. 4 (D) [^{3}H]MAG endogenously generated from [^{3}H]DAG containing a high proportion of 22:6 was used as substrate. Results represent the mean ± SD. (A) n = *12; (B)* n = *5; (C)* n = *4; and (D)* n = *8.* ***P < *0.0001 and* **P < *0.001 with respect to adult synaptosomes.*

composition. The key difference between the two substrates lies in their polyunsaturated fatty acid content: one has a high content of 20:4 whereas the other has almost exclusively 22:6. Previous research on DAGL-α and $-\beta$ substrate selectivity in the brain led to the conclusion that isoform β appears to prefer DAGs with 18:2 > 18:1 > 20:4 > 18:0 in position 2 whereas the α form appears to work equally well with all fatty acids [33]. Our results with DAG substrate selectivity suggest that the two isoforms coexist and contribute to 2-AG production in membranes.

Interestingly, whereas the expression of both DAGL isoforms was observed in the membrane fractions, DAGL-β was the only isoform found in synaptosomes. This would appear to be in disagreement with previous research according to which the bulk of retrograde endocannabinoid signaling throughout the brain is a consequence of 2-AG derived from DAGL-α [34]. However, our results indicate that DAGL-α expression in synaptosomes is minimal and may gain relevance in response to a stimulus. An alternative explanation is that DAGL-α remains as a reservoir in some neuron organelles and migrates to the synaptic terminal when 2-AG synthesis is required. Previous research with DAGL-α–/– mice and DAGL-β–/– showed that both isoforms play a role in 2-AG synthesis in the CNS [35], which is also consistent with our findings. The absence of DAGL activity in the soluble fraction coincides with the absence of expression of soluble DAGLα/β proteins revealed by WB.

Although DAGL expression and activity in synaptosomes underwent significant changes as a result of aging, membrane-associated DAGL activity and β isoform enzyme expression exhibited no aging effects. This, together with the significant decrease observed in α isoform enzyme expression, would

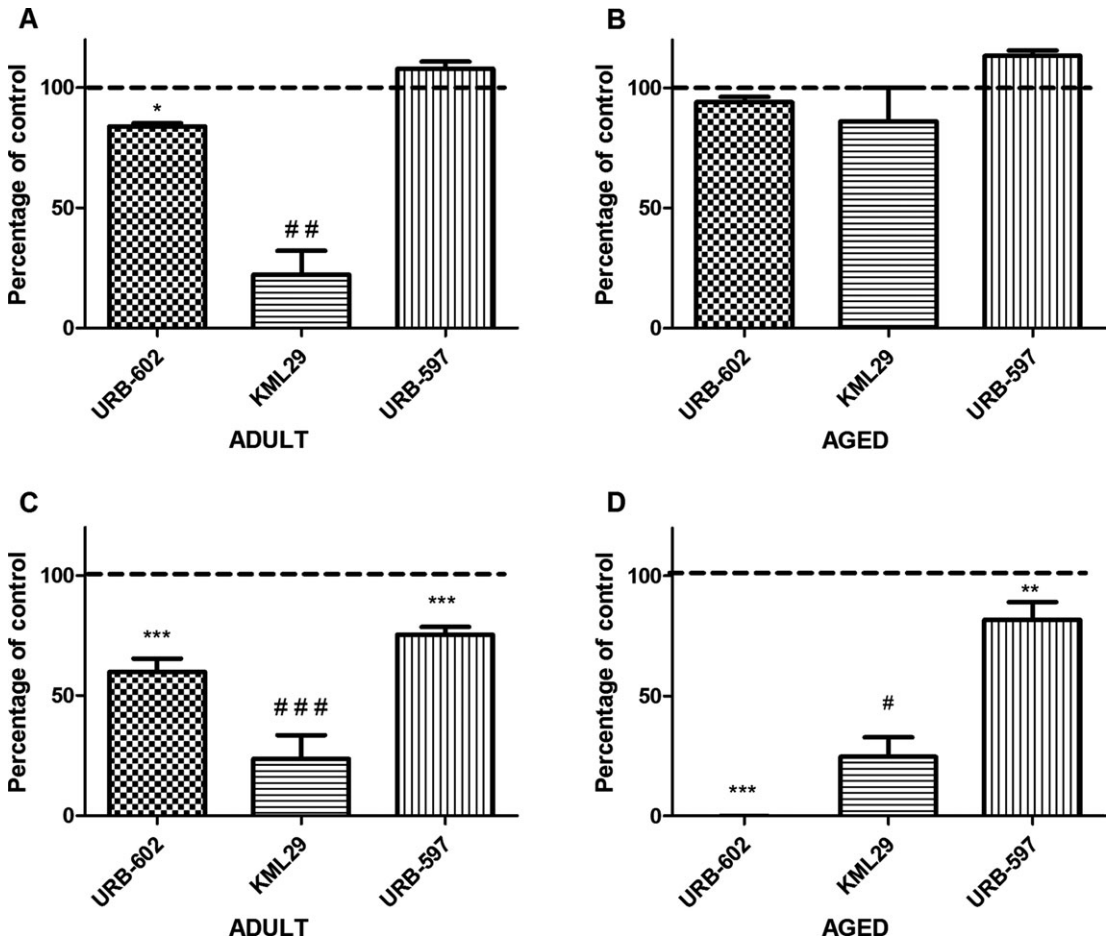

FIG 6

Degradation of 2-AG in the presence of inhibitors in CC membrane and synaptosomes from adult and aged rats. Membrane (A and B) or synaptosomes (C and D) were preincubated either with MAGL inhibitors, URB602 (1 mM), KML29 (1 μM), or with FAAH inhibitor, URB597 (0.1 μM) for 10 min (URB602 and URB597) and 30 min (KML29); 2-AG was subsequently added and MAGL was assayed. Results are expressed as a percentage of the corresponding control values (controls represent 100%) and represent the mean ± SD of six individual samples. $^{***}P < 0.0001$, $^{**}P < 0.001$, and $^{*}P < 0.01$ with respect to vehicle control for URB602 and URB597; $^{\#\#\#}P < 0.0001$, $^{\#\#}P < 0.001$, and $^{\#}P < 0.01$ with respect to vehicle control for KML29.

appear to indicate either that 2-AG synthesis is carried out by the β isoform or that the absence of the α isoform is compensated by higher DAGL-β expression and activity. Aging also produced a differential effect when DAGL activity in synaptosomes was assayed with DAGs containing either a high proportion of 22:6 or 20:4. In the former, enzyme activity was stimulated concomitantly with increased expression of DAGLβ, whereas in the latter it was diminished. Based on our findings relating to DAGL expression and activity we conclude that DAGL-β could be primarily responsible for 2-AG synthesis during aging. In addition, an alternative pathway which could lead to 2-AG synthesis and whose contribution was observed to be higher than that of DAGL in membrane fractions and synaptosomes, was found to be mediated by LPAase. The contribution of this latter to MAG production was also found to be differentially modulated by aging, being highly diminished in membrane fractions and slightly increased in synaptosomes. Our results therefore demonstrate a differential modulation mechanism exerted by aging in membrane fractions and in synaptosomes.

Furthermore, as is typical of neuromodulators, 2-AG is efficiently metabolized to ensure rapid signal inactivation. The action of 2-AG is generally terminated by uptake into responsive cells and by hydrolysis. MAGL, ABHD6, and ABHD12 are three serine hydrolases that together account for 99% of brain 2-AG hydrolase activity. MAGL is mainly responsible for 2-AG hydrolysis and ABHD6 and ABHD12 account for 4% and 9% of brain 2-AG hydrolase activity, respectively [36,37]. Fatty acid amide hydrolase (FAAH) also appears to contribute to 2-AG inactivation [10].

Previous research has demonstrated that MAGL has many substrates [38]. In our study, MAG hydrolysis was assessed on the basis of substrates with different acyl compositions. The main hydrolytic activity was observed using MAGs with a high degree of saturation in CC membrane, soluble and synaptosomal fractions. Hydrolysis of saturated MAGs was diminished in

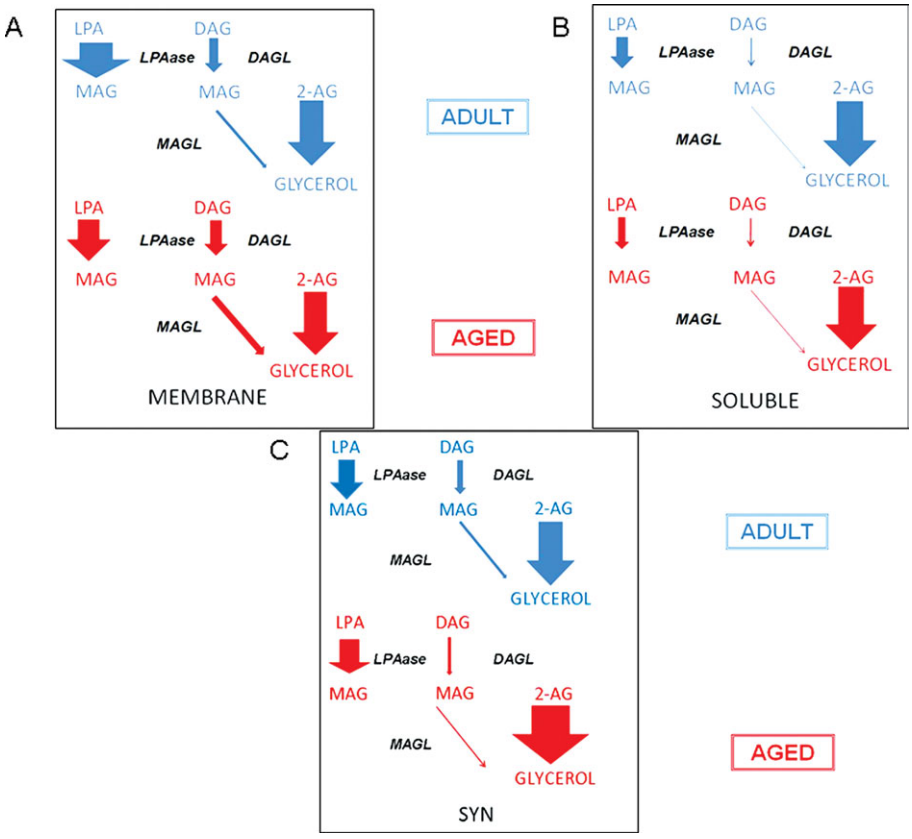

FIG 7

Age-related changes in the enzymes involved in 2-arachidonoylglycerol metabolism in CNS. The relative size of arrows indicates the predominance of MAGs in their synthesis and/or breakdown. LPA: 1-oleoyl lysophosphatidic acid; DAG: diacylglycerol containing mainly arachydonoyl at position 2; 2-AG: 2-arachydonoyl glycerol. The enzyme name is in italic script. LPAase: lysophosphatidic acid phosphohydrolase; DAGL: diacylglycerol lipase; MAGL, monoacylglycerol lipase.

the membrane fraction during aging. The changes observed in the hydrolysis of MAG generated from DAG with a high content of 20:4 in membranes and synaptosomes were a consequence of the aging effects on DAGL, for which the substrate availability is different.

The hydrolysis of 2-AG was not modified in the membrane or in the soluble fractions during aging. In the membrane fraction this coincided with the absence of changes in MAGL expression during aging whereas in the soluble fraction a high expression of MAGL was detected. This suggests that the soluble fraction behaves as a reservoir for MAGL which could be activated by translocation to the membrane fraction. Furthermore, the fact that MAGL remains in the soluble fraction appears to ensure higher 2-AG availability during aging. The hydrolysis of 2-AG was observed to be higher in synaptic endings, whereas MAGL expression was found to decrease during aging. This would appear to indicate that a signaling factor related to this mechanism stimulates enzyme activity, which, in turn, compensates MAGL deficiency during aging. Our results therefore demonstrate that in membrane and cytosolic fractions as well as in synaptosomes, the hydrolytic activities involved in the metabolism of endocannabinoid 2-AG and other monoacylglycerols are differently modulated by aging, thus suggesting that they may have preferred access to different MAG pools.

To assess the specific role of MAGL, FAAH, and ABHD in altered 2-AG hydrolysis during aging, the rate of 2-AG degradation was measured in the presence of MAGL inhibitors URB602 [28] and KML29 [29] and FAAH inhibitor URB597 [39]. It has been demonstrated that KML29 is a potent and selective MAGL inhibitor [29] whereas URB602 shows a lower capacity to disrupt MAGL hydrolysing activity, depending on the source of the enzyme [40]. Our results suggest that (i) 2-AG hydrolysis is produced mainly by MAGL (78%) and partly by ABHD6 in CC adult membranes and (ii) ABHD is the only enzyme responsible for 2-AG hydrolysis in CC aged membranes. Previous research has, in fact, reported that ABHD6 protein is present in the aged human hippocampus [41]. Although increased expression of FAAH has been observed in aged membranes, it seems to remain inactive in the presence of its inhibitor. A different profile in the presence of inhibitors was observed in CC synaptosomes. These data suggest that (i) in adult synaptosomes, 75% of 2-AG hydrolysis corresponds to MAGL and the remaining 25% to FAAH and (ii) although

MAGL and FAAH are mainly responsible for 2-AG hydrolysis in aged synaptosomes, we cannot discard the participation of other URB602-sensitive serine hydrolases, such as ABHD. Furthermore, whereas FAAH expression in aged synaptosomes increases, its participation in 2-AG hydrolysis is similar to that observed in adults, as indicated by results using URB597. This suggests that a high level of the enzymatic protein is necessary in order to generate the same rate of 2-AG hydrolysis as that observed in adult synaptosomes.

Results from the present study (summarized in Fig. 7) provide the first evidence of the regulation of enzymes involved in 2-AG metabolization during aging.

In summary, the net balance between the enzymatic activities involved in MAG synthesis and breakdown reveals low availability of 2-AG in CC membrane fractions and synaptic terminals during aging. Taking in account the neuromodulatory action of endocannabinoid signaling in inhibiting the release of neurotransmitters [42–44] and that endocannabinoids are neuroprotective in a number of experimental situations and/or neurodegenerative diseases [45–47], we conclude that the low 2-AG availability in neuronal membranes could be partly responsible for the dysfunction in synaptic processes during physiological aging. In view of the above, the enzymes involved in 2-AG synthesis and degradation constitute promising therapeutic targets in the aging process.

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