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Age-related changes in the metabolization of phosphatidic acid in rat cerebral cortex synaptosomes

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ABSTRACT

In this study, phosphatidic acid (PA) metabolization is found to generate diacylglycerol (DAG), monoacylglycerol (MAG) and glycerol by the sequential action of lipid phosphate phosphatase (LPP), diacylglycerol lipase (DAGL), and monoacylglycerol lipase (MAGL) in cerebral cortex (CC) synaptosomes. It is also demonstrated that PA is metabolized by phospholipases A (PLA)/lysophosphatidic acid phosphohydrolase (LPAPase) in synaptic endings. Age-related changes in the metabolization of PA have been observed in rat cerebral cortex synaptosomes in the presence of the alternative substrates for LPP, namely LPA, sphingosine 1-phosphate (S1P) and ceramide 1-phosphate (C1P). In addition, LPA and C1P up to concentrations of about 50 μM favor the metabolism in the direction of MAG and glycerol in aged and adult synaptosomes, respectively. At equimolecular concentrations with PA, LPA decreases DAG formation in adult and aged synaptosomes, whereas S1P decreases it and C1P increases it only in aged synaptosomes. Sphingosine (50 μM) or ceramide (100 μM) increase PA metabolism by the pathway that involves LPP/DAGL/MAGL action in aged membranes. Using RHC-80267, a DAGL inhibitor, we could observe that 50% and 33% of MAG are produced as a result of DAGL action in adult and aged synaptosomes, respectively. Taken together, our findings indicate that the ageing modifies the different enzymatic pathways involved in PA metabolization.

Introduction

Aging is accompanied by the impaired functioning of many systems, thus producing a gradual decline in the capacity of various cell types including neurons [1]. Lipids have broad information-carrying functions in the CNS. They form an integral part of membranes and provide messenger molecules that mediate communication among cells. Any modification in their metabolism and/or in the enzymatic activities that metabolize them may therefore affect cell function in physiological aging. Age-related changes in lipid content and in the enzymatic activities involved in lipid metabolism in different brain regions have been documented [2–10]. PA,[1] DAG and MAG are involved in signal transduction [11–13]. In eukaryote cells, these molecules have been associated with neurological disorders such as Alzheimer disease [14]. Previous research from our laboratory demonstrated that lipid phosphate phosphatase (LPP) hydrolyzes PA in synaptosomal cerebral cortex and that the generated DAG is metabolized to MAG by DAGL [6]. LPP also hydrolyzes LPA, S1P and C1P either on the cell surface or inside the cell so the degradation of lipid phosphates by LPP regulates cell signaling under physiological or pathological conditions. This cell signaling occurs via the attenuation of lipid phosphate signaling and the production of bioactive diacylglycerol, monoacylglycerol, sphingosine and ceramide [15]. The precise control of PA, DAG and MAG and the enzymes that metabolize them, LPP, DAGL and MAGL, are necessary for the correct functioning of these molecules in the signaling mechanism. The present study analyzes the formation of lipid mediators generated from [2-^{3}H]PA in synaptosomes prepared from the cerebral cortex (CC) of adult and aged rats. In all instances, [2-^{3}H]PA metabolization was analyzed in the presence of either LPA, S1P or C1P, which are alternative substrates for LPPs [16]. Our results demonstrate that aging modulates PA metabolization and indicate a different utilization of PA in the presence of LPA, S1P and C1P. Advances in our knowledge of lipid metabolism during aging will greatly contribute to a better understanding of the role of lipids in senescence. Imbalances in PA metabolism may be a key event in CNS injuries occurring during the aging process.

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[1] *Abbreviations used:* CC, cerebral cortex; C1P, ceramide 1-phosphate; DAG, diacylglycerol; DAGL, diacylglycerol lipase; DTT, dithiothreitol; 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]; LPA, lysophosphatidic acid, LPP, lipid phosphate phosphatase, MAG, monoacylglycerol; MAGL, monoacylglycerol lipase, NEM, *N*-ethylmaleimide; PA, phosphatidic acid; PLA, phospholipase A; RHC-80267, 1,6-bis(Cyclohexyloximinocarbonyl-amino) hexane; S1P, sphingosine 1-phosphate;TLC, thin-layer chromatography.

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Materials and methods

Male Wistar-strain rats were kept under constant environmental conditions and fed on a standard pellet diet. Animal handling was carried out in agreement with the standards stated in the *Guide to the Care and Use of Experimental Animals* published by the Canadian Council on Animal Care. [2-^{3}H]Glycerol (200 mCi/mmol) and Omnifluor were obtained from New England Nuclear-Dupont (Boston, MA, USA). The DAGL inhibitor, RHC-80267 (1,6-bis(Cyclohexyloximinocarbonyl-amino)) hexane was from Biomol, Horsholm, Denmark. Sphingosine 1-phosphate, ceramide 1-phosphate from bovine brain, oleoyl-L-α-lysophosphatidic acid, D-sphingosine, and non-hydroxy fatty acid ceramide from bovine brain were obtained from Sigma–Aldrich, St. Louis, MO, USA. All other chemicals used were of the highest purity available.

Preparation of synaptosomes

Four-month-old (adult) and 28-month-old (aged) rats were killed by decapitation and their CC was immediately dissected (2–4 min after decapitation).

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 1300*g* for 7 min and the supernatant was carefully poured into another tube. The nuclear pellet was re-suspended with the isolation medium and subsequently spun at 1300*g* for 7 min. The combined supernatant was then centrifuged at 17000*g* for 10 min to obtain the crude mitochondrial pellet (CM). The CM was re-suspended with the isolation medium and layered onto a two-step gradient of 7.5–13% Ficoll solution prepared in the isolation medium. The sample layered onto Ficoll discontinuous gradient was centrifuged at 99000*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].

Preparation of radioactive 1,2-diacyl-sn-glycerol-3-phosphate

Radioactive PA was obtained from [2-^{3}H]glycerol-PC, synthesized from bovine retinas incubated with [2-^{3}H]glycerol (200 mCi/mmol) as previously described [18]. Lipids were extracted from the tissue as described elsewhere [19]. [2-^{3}H]glycerol-PC was isolated by mono-dimensional thin-layer chromatography (TLC), eluted [20] and hydrolyzed with phospholipase D [21]. The hydrolysis product [2-^{3}H]glycerol PA was then purified by one-dimensional TLC on silica gel H developed with chloroform/methanol/acetic acid/acetone/water (9:3:3:12:1.5, by vol). The substrate was eluted from silica gel with neutral solvents to avoid the formation of lysophosphatidic acid and was subsequently converted into free acid by washing firstly with an upper phase containing 0.1 M sulfuric acid and then with an upper phase containing water. Radioactivity and phosphorus content [22] were measured to determine specific radioactivity. [2-^{3}H]PA with a specific radioactivity of 0.1–0.2 μCi/μmol was obtained.

Enzymatic assays

For the determination of LPP activity, each assay contained 50 mM Tris–maleate buffer, pH 6.5, 1 mM EDTA plus EGTA, 4.2 mM NEM, and 100 μg synaptosomal protein in a volume of 200 μl. The reaction was started by the addition of 100 μM of [2-^{3}H]-phosphatidate/Triton X-100 mixed micelles in a constant 1:50 M ratio of lipid to Triton X-100. The effect of the alternative substrates on PA metabolization by LPP activity was evaluated using 100 μM [2-^{3}H]PA/Triton X-100 mixed micelles in the presence of unlabeled LPA, S1P or C1P (previously re-suspended in the buffer assay containing Triton X-100) [23]. Radiolabel PA was mixed with unlabeled substrates before drying and re-suspension. The aqueous microdispersions were sonicated by a sonication tip until clarity. Sphingosine and ceramide were solubilized in 0.1% dimethyl sulfoxide (DMSO) as vehicle; the respective controls were made with 0.1% DMSO alone. DAGL activity was determined by monitoring the formation rate of monoacyl [2-^{3}H]glycerol, using diacyl[2-^{3}H]glycerol generated from [2-^{3}H]glycerol-PA by LPP action as substrate [24]. [2-^{3}H]glycerol was determined from [2-^{3}H]glycerol-PA. Standard assays, pH conditions, protein concentration, time and the final volume of incubation were the same as those described for LPP. All the assays were conducted at 37 °C during 30 min. The enzymatic reactions were stopped by adding chloroform/methanol (2:1, by vol). Blanks were prepared identically except that the membrane fraction was boiled for 5 min before use.

Lipids were extracted with chloroform/methanol (2:1, by vol) and washed with a 0.2 volume of $CaCl_2$ (0.05%) [19]. Neutral lipids were separated by TLC on silica gel G [25] and developed with hexane/diethyl ether/acetic acid (35:65:1, by vol). To separate MAG from phospholipids, the chromatogram was rechromatographed up to the middle of the plate by using hexane/diethyl ether/acetic acid (20:80:2.3, by vol) as developing solvent. Once the chromatogram was developed, [2-^{3}H]glycerol-PA and phospholipids were retained at the spotting site. To separate [2-^{3}H]glycerol, the aqueous phase from a Folch extraction was chromatographed by TLC on silica gel G using chloroform/acetone/5 NH_3 (10:80:10) as developing solvent [26] and the chromatograms visualized as described [27]. The chromatograms were visualized by exposure to iodine vapors and scraped off for counting by liquid scintillation after the addition of 0.4 ml water and 10 ml 5% Omnifluor in toluene/Triton X-100 (4:1, by vol) using a WALLAC 1214 RACKBETA liquid scintillation counter.

Other methods

Protein and lipid phosphorus were determined according to Bradford [28] and Rouser [22], respectively.

Statistical analysis

All data are given as means ± SD and were evaluated using the Student́s *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. The statistical analysis was performed using GraphPad software, San Diego, CA, USA, www.graphpad.com. Statistical significance was set at $p < 0.05$.

Results

Metabolization of [^{3}H]-phosphatidic acid in the presence of LPA in CC synaptosomes from adult and aged rats

LPP hydrolyzes [^{3}H]-phosphatidic acid generating DAG which is, in turn, partially hydrolyzed by DAGL, thus yielding MAG. DAG generation and its partial degradation by DAGL occur immediately and have been extensively studied in our laboratory [18,24]. In our research, it was observed that MAG is hydrolyzed by MAGL to glycerol. DAGL and MAGL are coupled to LPP and these enzymes appear to work as an enzymatic complex. The assay conditions for the determination of LPPs (time, substrate and protein concentration) were usually used for this enzymatic assay [8,9,15,29,30]. The LPPs