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TRANSLATIONAL NEUROSCIENCES - ORIGINAL ARTICLE

Conjugated linoleic acid-enriched butter improved memory and up-regulated phospholipase A_2 encoding-genes in rat brain tissue

Marco A. S. Gama[1] · Nádia R. B. Raposo[3] · Fábio B. Mury[2] · Fernando C. F. Lopes[1] · Emmanuel Dias-Neto[2] · Leda L. Talib[2] · Wagner F. Gattaz[2]

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Abstract Reduced phospholipase A_2 (PLA_2) activity has been reported in blood cells and in postmortem brains of patients with Alzheimer disease (AD), and there is evidence that conjugated linoleic acid (CLA) modulates the activity of PLA_2 groups in non-brain tissues. As CLA isomers were shown to be actively incorporated and metabolized in the brains of rats, we hypothesized that feeding a diet naturally enriched in CLA would affect the activity and expression of *Pla*$_2$-encoding genes in rat brain tissue, with possible implications for memory. To test this hypothesis, Wistar rats were trained for the inhibitory avoidance task and fed a commercial diet (control) or experimental diets containing either low CLA- or CLA-enriched butter for 4 weeks. After this period, the rats were tested for memory retrieval and killed for tissue collection. Hippocampal expression of 19 *Pla*$_2$ genes was evaluated by qPCR, and activities of PLA_2 groups (cPLA$_2$, iPLA$_2$, and sPLA$_2$) were determined by radioenzymatic assay. Rats fed the high CLA diet had increased hippocampal mRNA levels for specific PLA_2 isoforms (*iPla*$_2$*g6γ*; *cPla*$_2$*g4a*, *sPla*$_2$*g3*, *sPla*$_2$*g1b,* and *sPla*$_2$*g12a*) and higher enzymatic activity of all PLA_2 groups as compared to those fed the control and the low CLA diet. The increment in PLA_2 activities correlated significantly with memory enhancement, as assessed by increased latency in the step-down inhibitory avoidance task after 4 weeks of treatment ($r_s = 0.69$ for iPLA$_2$, $P < 0.001$; $r_s = 0.81$ for cPLA$_2$, $P < 0.001$; and $r_s = 0.69$ for sPLA$_2$, $P < 0.001$). In face of the previous reports showing reduced PLA_2 activity in AD brains, the present findings suggest that dairy products enriched in *cis*-9, *trans*-11 CLA may be useful in the treatment of this disease.

Keywords Conjugated linoleic acid · Dairy fat · Immunomodulation · Phospholipase A_2 · Brain tissue · Alzheimer disease

Abbreviations

CLA	Conjugated linoleic acid
PLA_2 or *Pla*$_2$	Phospholipase A_2
AD	Alzheimer disease
sPLA$_2$	Secretory Ca^{2+}-dependent PLA_2
cPLA$_2$	Intracellular cytosolic Ca^{2+}-dependent PLA_2
iPLA$_2$	Intracellular Ca^{2+}-independent PLA_2
PPAR	Peroxisome proliferator-activated receptors
DHA	Docosahexaenoic acid
FAME	Fatty acid methyl esters
AA	Arachidonic acid
NPD1	Neuroprotectin D1
SFA	Saturated fatty acids
LTP	Long-term potentiation

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✉ Wagner F. Gattaz
gattaz@usp.br

[1] Embrapa Dairy Cattle, Brazilian Agricultural Research Corporation, Juiz de Fora, Minas Gerais, Brazil

[2] Laboratory of Neurosciences-LIM27, Department and Institute of Psychiatry, Faculty of Medicine, University of São Paulo, São Paulo, Brazil

[3] Faculty of Pharmacy, Research Center for Innovative Health Sciences, Federal University of Juiz de Fora, Juiz de Fora, Minas Gerais, Brazil

Introduction

Alzheimer disease (AD) is the most frequent cause of dementia in the elderly, with both genetic and environmental factors being involved in its pathology (Barberger-Gateau et al. 2007). The economic and social impact of AD is becoming more critical as the population ages, increasing thus the need for therapeutic or preventive strategies to counteract or delay dementia onset. Cholinesterase inhibitors have shown symptomatic benefits in patients with mild to moderate AD, but to date there is no pharmacological treatment able to halt or slow the disease progression (Frisardi et al. 2010). There is growing evidence that nutrition plays an important role in the prevention of AD, and a great deal of attention has been paid to dietary fat and antioxidants (Butterfield et al. 2002; Luchsinger and Mayeux 2004). Most of the studies evaluating the potential benefits of dietary lipids on AD have focused on n-3 fatty acids. Overall, both epidemiological and animal studies suggest that the consumption of docosahexaenoic acid (DHA) is associated with a reduced risk of AD (Morris et al. 2003; Green et al. 2007), but results from controlled clinical trials have been less promising (Dangour and Uauy 2011). In addition to DHA and its n-3 counterparts, another group of bioactive fatty acids—the so-called conjugated linoleic acid (CLA)—has attracted the attention of the scientific community since the discovery of its anticarcinogenic activity nearly 30 years ago (Pariza 2004). CLA is a group of positional and geometric isomers of linoleic acid with conjugated double bonds which have shown potential health-promoting effects in both in vitro and animal studies (Bhattacharya et al. 2006).

Phospholipases A_2 (PLA_2) are a family of hydrolytic enzymes which cleave the membrane phospholipids at *sn*-2 position, resulting in lysophospholipids and free fatty acids, usually arachidonic acid, which is the precursor of eicosanoids and other active products. Both fatty acid and lysophospholipids are important mediators in the transmission and processing of neuronal signals (Piomelli 1993; Bazan et al. 1993) by modulating ion channels, acting as second messengers regulating gene expression, and altering the release and neurotransmitter uptake (Lautens et al. 1998).

The three main groups of PLA_2, intracellular, calcium independent ($iPLA_2$), intracellular, calcium dependent ($cPLA_2$), and extracellular, calcium dependent ($sPLA_2$), differ in several aspects (e.g. substrate specificity, requirement for calcium, cell localization, and mechanisms of action) (Dennis et al. 2011). Reduced PLA_2 activity was first reported in postmortem brains of patients with AD about two decades ago, and the reduction was correlated with the severity of dementia (Gattaz et al. 1995; Ross et al. 1998). Because PLA_2 influences amyloid formation and Tau pathology, it is assumed that the modulation of different PLA_2 groups in the brain may have implications for the disease (Schaeffer et al. 2010).

Most of the studies evaluating the immunomodulatory effects of CLA used a mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers in nearly equal amounts and focused on their effects on eicosanoids and cytokines synthesis (O'Shea et al. 2004). However, few studies have addressed the effect of these CLA isomers on activity and expression of PLA_2, with responses being reported in tissues other than brain (Eder et al. 2003; Stachowska et al. 2007). Interestingly, these two CLA isomers were shown to be actively incorporated and metabolized in rat brain tissue and in cultures of astrocytes (Fa et al. 2005). Based on the anti-inflammatory effects of CLA reported in previous studies, it has been hypothesized that CLA activity in the brain could positively impact neurological disorders such as AD (Fa et al. 2005), which is supported by the reduction in the levels of prostaglandin E_2 (PGE_2)—a strong pro-inflammatory eicosanoid—in brain tissue of mice fed synthetic CLA (Nakanishi et al. 2003).

Dairy products are the main source of CLA in the human diet (Lawson et al. 2001), and a pronounced increase in milk fat *cis*-9, *trans*-11 CLA content can be achieved by supplementing the diets of dairy cows with plant oils (Collomb et al. 2006). Butter naturally enriched in CLA was shown to reduce the risk of mammary cancer in rats (Ip et al. 1999) and improve the plasma lipoprotein profile in hamsters (Lock et al. 2005). However, to our knowledge, no study has addressed the neuroprotective potential of butter naturally enriched in CLA.

The objective of this study was to evaluate the effects of a *cis*-9, *trans*-11 CLA-enriched butter on the expression of *Pla*$_2$-encoding genes and enzymatic activity of PLA_2 groups in rat brain tissue. In the present study we analyzed the hippocampal mRNA expression of 19 out of 27 genes identified in the rat brain tissue as PLA_2 subtypes at the NCBI Gene database (http://www.ncbi.nlm.nih.gov/gene, June 2014). We also investigated whether changes in PLA_2 activity or gene expression induced by the CLA-enriched butter were associated with memory improvement.

Materials and methods

Animals and environment

Thirty ($n = 30$) male Wistar rats (aged 8–10 weeks and weighing 200–250 g) were obtained from the animal house from the University of Sao Paulo Medical School, São Paulo, Brazil. During the experimental period, animals

were housed in polypropylene cages ($n = 4$/cage) at 22 ± 2 °C with 12-h light/dark cycles, with free access to water.

Treatments

After 7 days of acclimatization, animals ($n = 30$) were assigned the following dietary treatments for 4 weeks: (1) control ($n = 10$): a commercial diet (NUVILAB CR1, NUVITAL®, Curitiba, Paraná, Brazil), (2) Low CLA diet ($n = 10$): diet containing a low CLA butter, and (3) High CLA diet ($n = 10$): diet containing an enriched-CLA butter. The commercial diet contained (per kg of product) as declared by the manufacturer: 22 % crude protein, 4 % fat, 8 % crude fiber, and 10 % ash. Low and high CLA butters were produced from dairy cows fed on diets containing either 0 or 4.5 % sunflower oil on a dry matter (DM) basis, respectively. Diet supplementation with plant oils has been shown to be an effective dietary strategy to increase the milk fat CLA content of dairy cows (Collomb et al. 2006). Low and high CLA butters were produced according to the procedure described elsewhere (Gonzalez et al. 2003) and were used as ingredients (210 g/kg of diet) of low and high CLA diets as shown in Table 1. Butters were melted in a water bath at 40 °C and mixed with other dietary ingredients to obtain a homogeneous mass which was used to produce handmade pellets. Samples of pellets were collected and analyzed for chemical composition and fatty acid profile (Table 2). All animals had the same intake of food and it was recorded daily throughout the study.

Behavioral procedures

Animals were trained on inhibitory avoidance tasks (Izquierdo et al. 2003; Rossato et al. 2006) 2 days before the beginning of the treatment period. Latency to step

Table 1 Feed composition of both low and high CLA diets

Ingredients[a]	g/kg
Casein	195.0
DL-Methionine	3.0
Sucrose	341.5
Corn starch	150.0
Butter[b]	210.0
Cellulose	50.0
Mineral mix (AIN-93G-MX)	35.0
Vitamin mix (AIN-93-VX)	10.0
Calcium carbonate	4.0
Antioxidant (BHT)	0.04

[a] TD.88137 (Harlan Teklad, Harlan Laboratories, Inc.)

[b] Low or CLA-enriched butter

Table 2 Chemical composition and fatty acid profile of low and high CLA diets

	Low CLA diet	High CLA diet
Dry matter (DM) content, %	93.4	93.3
Crude protein, % diet DM	17.6	17.5
Fat, % diet DM	17.7	18.1
Fiber, % diet DM	4.2	4.6
Fatty acid profile, g/100 g of total FA		
C4:0	3.60	3.13
C5:0	0.02	0.04
C6:0	2.01	1.35
C7:0	0.02	0.01
C8:0	1.10	0.63
C9:0	0.02	0.01
C10:0	2.07	1.08
C10:1 *cis*-9 + C11:0	0.28	0.11
C12:0	2.30	1.19
C12:1 *cis*-9	0.09	0.05
C13:0	0.07	0.02
C14:0 *iso*	0.15	0.11
C14:0	9.44	6.09
C15:0 *iso*	0.37	0.25
C15:0 *anteiso*	0.65	0.49
C14:1 *cis*-9	0.80	0.39
C15:0	1.39	1.02
C16:0 *iso*	0.29	0.21
C15:1 *cis*-9	0.01	0.01
C16:0	30.1	19.5
C17:0 *iso*	0.45	0.41
C17:0 *anteiso*	0.68	0.55
C16:1 *cis*-9	1.32	0.77
C17:0	0.65	0.63
C18:0 *iso*	0.06	0.07
C17:1 *cis*-9	0.29	0.20
C18:0	8.97	17.9
C18:1 *trans*-4	0.01	0.08
C18:1 *trans*-5	0.01	0.04
C18:1 *trans*-6 to 8	0.10	0.47
C18:1 *trans*-9	0.20	0.50
C18:1 *trans*-10	0.20	0.68
C18:1 *trans*-11	1.50	5.97
C18:1 *trans*-12	0.14	0.84
C18:1 *trans*-13/14	0.34	1.00
C18:1 *cis*-9 + *trans*-15	18.7	25.5
C18:1 *cis*-11	0.98	1.11
C18:1 *cis*-12	0.23	0.70
C18:1 *cis*-13	0.08	0.14
C18:1 *trans*-16	0.15	0.53
C18:1 *cis*-15	0.07	0.18
C18:2 *cis*-9, *trans*-12	0.08	0.11
C18:2 n-6	1.42	1.44

Table 2 continued

	Low CLA diet	High CLA diet
C20:0	0.14	0.17
C20:1 *cis*-9	0.13	0.11
C20:1 *cis*-11	0.04	0.05
C18:3 n-3	0.21	0.14
CLA *cis*-9, *trans*-11[a]	0.72	1.98
CLA *trans*-9, *cis*-11	0.02	0.04
C21:0	0.04	0.04
CLA *trans*-10, *cis*-12	0.01	0.02
C20:2 n-6	0.02	0.02
C22:0	0.07	0.10
C20:3 n-6	0.06	0.04
C20:4 n-6	0.14	0.08
C20:5 n-3[b]	0.08	0.06
C22:5 n-3	0.05	0.04
C22:6 n-3	nd	nd
n-6:n-3 ratio[c]	4.8:1	6.7:1

nd not detected

[a] Co-elutes with *trans*-7, *cis*-9 and *trans*-8, *cis*-10 isomers

[b] Co-elutes with C24:0 in our analytical conditions

[c] Calculated as follows: (Σ C18:2 n-6 + C20:2 n-6 + 20:3 n-6 + C20:4 n-6)/(Σ C18:3 n-3 + C20:5 n-3 + C22:5 n-3 + C22:6 n-3)

down onto the grid with all four paws was measured; upon stepping down the animals received a 0.8 mA, 4-s scrambled footshock and were immediately withdrawn from the training apparatus. This brief but high footshock guarantees the persistence of the inhibitory avoidance memory for the length of the treatment period (Izquierdo et al. 2003). In the test sessions, animals were placed again on the platform, but the footshock was omitted and step-down latency was used as a measure of memory retention. Retention test sessions were conducted after the treatment period.

Tissue collection

Once tested for memory retrieval, the animals were killed after total exsanguination under anesthesia with ketamine and xylazine (90 and 10 mg/kg, respectively). Hippocampi were dissected in a cooled phosphate-buffered saline solution (PBS) and stored at −80 °C. Tissue aliquots were mixed with 5 mmol/L Tris–HCI pH 7.4 and ground in a pot. Tissue homogenates were divided into two aliquots, which were used to evaluate the transcriptional levels of selected *Pla*$_2$ genes and enzymatic activity of PLA$_2$ groups. The experimental protocols used in the study conform to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health and were approved by the local Animal Care Committee (COBEA/UFJF).

RNA extraction and cDNA synthesis

Hippocampi were homogenized (1:10 w/v) in 5 mmol/L Tris–HCl buffer pH 7.4 and 35 % of the homogenate of each sample was used for RNA extraction with TRIZOL® (Life Technologies, Carlsbad, CA, USA) (Chomczynski and Sacchi 1987). The quality and quantity of all the RNA samples were determined using NanoDrop® (Nano-drop Technologies, Wilmington, DE, USA) and the integrity of the total RNA was checked by electrophoresis in 1 % agarose gels containing 1 mol/L of guanidine isothiocyanate. cDNA synthesis was performed at 42 °C for 90 min, using 1 μg of total RNA, 200 U of ImProm-II TM Reverse Transcriptase (Promega, USA), 0.5 μg oligo (dT) 12–18, 0.5 mmol/L dNTPs, 3 mmol/L $MgCl_2$ and 1× reaction buffer in a 20 μL reaction volume.

Gene expression analyses by qPCR

Differential gene expression was evaluated by quantitative real-time PCR (qPCR), using SYBR Green PCR Master Mix in an ABI 7500 Sequence Detection System (Applied Biosystems, CA, USA). The qPCR experiment workflow and analysis are in compliance with the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al. 2009). Assessing gene expression stability of putative normalized genes was performed using geNorm program (http://medgen.ugent.be/~jvdesomp/genorm/) (Vandesompele et al. 2002) and the reference genes selected were used to normalize the amounts of cDNA used in each experiment. For each qPCR assay we used 7.5 ng cDNA equivalents and 3.75 pmol of each primer in 15 μL reactions. For each target gene we included a no-template control as well as two geNorm-selected reference genes. The amplification program consisted of an initial step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. qPCR data were captured using the software Sequence Detector System 1.3.1 (Applied Biosystems). Relative gene expression levels were normalized according to the geometric mean of selected reference genes (Vandesompele et al. 2002). Oligonucleotide primers used for PCR amplification of *Pla*$_2$ genes and reference genes from rat cDNA are shown in Supplemental Table 1.

Determination of PLA$_2$ activity by radioenzymatic assay

Protein concentration was determined using the Bio-Rad DC protein assay (Bio-Rad, USA), modified from a previously described procedure (Lowry et al. 1951). Aliquots of hippocampus homogenates were used to determine the activity of PLA$_2$ groups by radioenzymatic assays. Briefly,

the substrate used was L-α-1-palmitoyl-2-arachidonyl-phosphatidyl-choline labeled with [l-14C] in the arachidonyl tail at position sn-2 (14C-PC) (48 mCi/mmol specific activity, PerkinElmer, MA). Prior to the enzymatic reaction, a mixture of arachidonyl-1-14C-PC and toluol-ethanol-butylhydroxytoluol antioxidants (1:10, v/v) was evaporated under a nitrogen stream (0.075 µCi/sample), resuspended in 0.3 mg/mL BSA in ultrapure water, and homogenized by sonication. Total brain tissue homogenates were diluted to a final protein concentration of 1.5 mg/mL with 50 mmol/L Tris–HCl (pH 8.5 for $sPLA_2$ and $cPLA_2$ or pH 7.5 for $iPLA_2$). The assays contained 100 mmol/L Tris–HCl buffer (pH 8.5 or pH 7.5), 1 µmol/L (for $cPLA_2$ and $iPLA_2$) or 2 mmol/L $CaCl_2$ (for $sPLA_2$), 100 µmol/L BEL (Biomol, USA), 300 µg of protein from diluted homogenates, and 0.075 µCi arachidonyl-1-14C-PC. After 30 min of incubation at 37 °C, the reactions were interrupted by adding a mixture of HCl-isopropanol (1:12, v/v). The released [l-14C] AA was extracted and the radioactivity of 14C-AA was measured in a liquid scintillation counter (Tri-Carb 2100 TR; Packard, USA) for calculating PLA_2 activities (pmol × mg/protein × 1/min). All PLA_2 activity assays were performed in triplicate.

Analysis of low and high CLA diets

The chemical composition of the experimental diets was determined according to AOAC (2000) for dry matter (DM), protein and fat contents, and as previously described for fiber content (Van Soest et al. 1991). For fatty acid profile analysis, lipids were extracted using a 3:2 (v/v) mixture of hexane and isopropanol followed by a 67 g/L sodium sulfate solution (Hara and Radin 1978). The FAME were obtained by base-catalyzed transmethylation using a freshly prepared methylation reagent (0.4 mL of 5.4 mol/L sodium methoxide solution + 1.75 mL methanol) with modifications (Christie 1982; Chouinard et al. 1999). The mixture was neutralized with oxalic acid (1 g oxalic acid in 30 mL of diethyl ether) and calcium chloride was added to remove methanol residues. The FAME were determined by gas chromatography (model 6890N; Agilent Technologies) fitted with a flame-ionization detector and equipped with a CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm × 0.2 µm film thickness; Varian Inc., Mississauga, ON). Operating conditions included injector and detector temperatures both at 250 °C, H_2 as carrier gas (1 mL/min) and for the flame-ionization detector (35 mL/min), N_2 as makeup gas (30 mL/min), and purified air (286 mL/187 min). The initial temperature was 45 °C and held for 4 min, increased by 13 °C/min to 175 °C and held for 27 min, increased by 4 °C/min to 215 °C, and held for 35 min (Cruz-Hernandez et al. 2007). The FAME were identified by comparison with reference FAME standards (Sigma Aldrich®, Nu-Chek-Prep Inc. and Larodan Fine Chemicals), and minor cis/trans-C18:1 isomers were identified according to their order of elution reported under the same chromatographic conditions (Cruz-Hernandez et al. 2007).

Statistical analysis

Data from PLA_2 activity and gene expression assays were analyzed by one-way ANOVA followed by post hoc Tukey test, and results were expressed as mean ± SEM. The behavioral data were expressed as median ± interquartile range and required non-parametric statistics (Duncan multiple range test). Correlations between PLA_2 activities and memory were calculated using the Spearman's coefficient (r_s). Statistical analysis was performed using SPSS version 17.0 and two-tailed probabilities were considered significant at $P < 0.05$.

Results

The hippocampal mRNA levels were increased ($P < 0.001$) in rats fed the high CLA diet when compared to both control and low CLA diet for five of the 19 *Pla*$_2$ genes investigated: *iPla*$_2$*g6γ*; *cPla*$_2$*g4a*, *sPla*$_2$*g3*, *sPla*$_2$*g1b,* and *sPla*$_2$*g12a* (Fig. 1). Furthermore, the expression of the *sPla*$_2$*g12a* gene was also increased ($P = 0.024$) by the low CLA diet when compared to control (Fig. 1e). The mRNA levels of the remaining *Pla*$_2$ genes were unaltered by both low- and high-CLA diets (data not shown).

Compared to control, the hippocampal activity of some PLA_2 groups was increased by the high-CLA diet or both low- and high-CLA diets. The $iPLA_2$ activity (Fig. 2a) was increased by 22.5 % ($P = 0.023$) and 55.0 % ($P < 0.001$), whereas $cPLA_2$ activity (Fig. 2b) was increased by 28.6 % ($P = 0.014$) and 71.4 % ($P < 0.001$) in rats fed low- and high-CLA diets, respectively. Consistent with the mRNA results, $sPLA_2$ activity was significantly increased (74 %, $P < 0.001$) in the hippocampus of rats fed the high CLA diet when compared to control (Fig. 2c). Increased ($P < 0.001$) hippocampal activity of all PLA_2 groups was observed in rats fed the high CLA diet as compared to those fed the low CLA diet (Fig. 2).

The increase in the PLA_2 activities correlated significantly with memory enhancement, as indicated by the improved performance in the step-down inhibitory avoidance task after 4 weeks of treatment ($r_s = 0.69$ for $iPLA_2$, $P < 0.001$; $r_s = 0.81$ for $cPLA_2$, $P < 0.001$; and $r_s = 0.69$ for $sPLA_2$, $P < 0.001$).

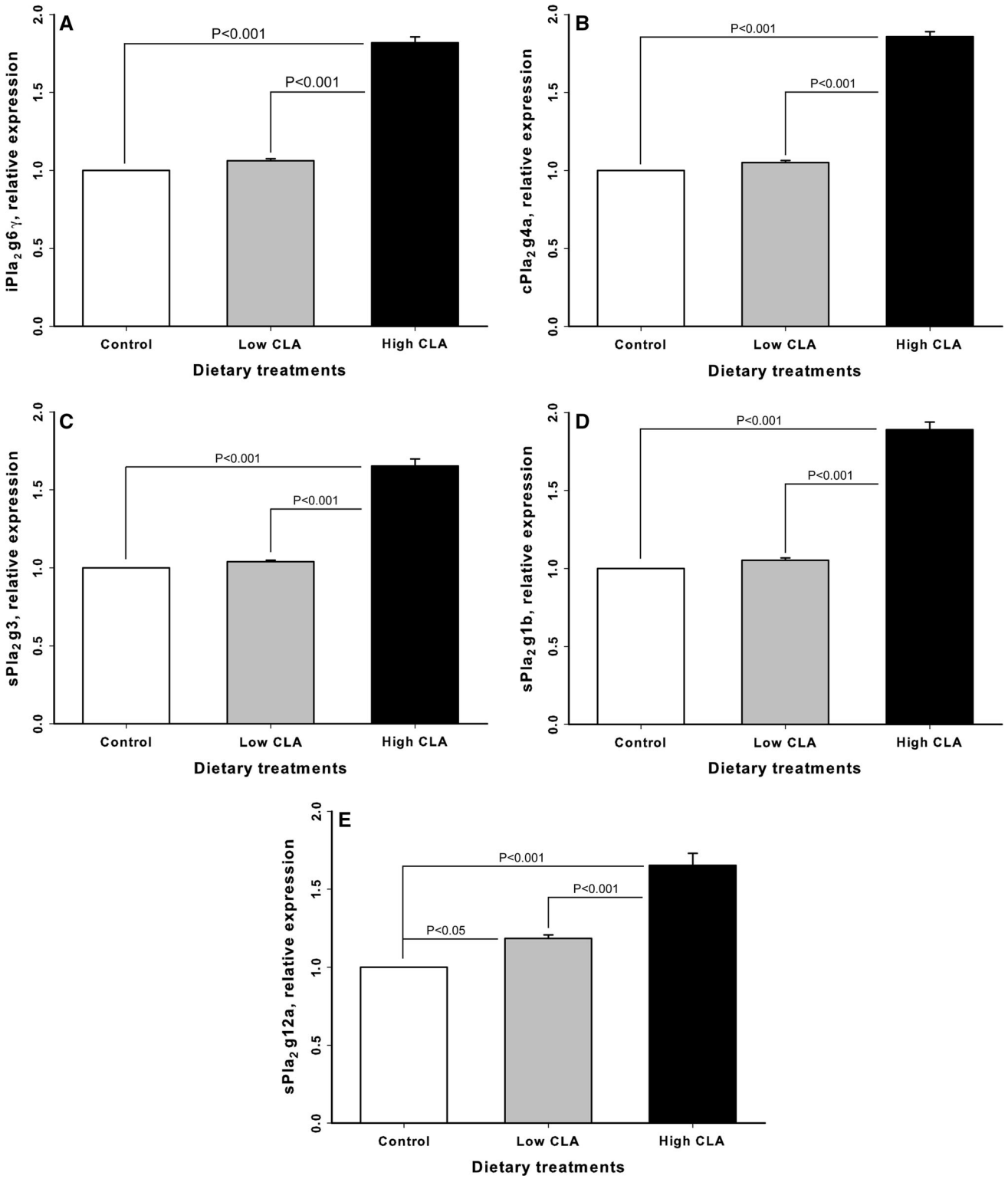

Fig. 1 Effects of low and high CLA diets on the expression of *Pla*$_2$-encoding genes (**a** *iPla*$_2$*g6gamma*, **b** *cPla*$_2$*g4a*, **c** *sPla*$_2$*g3*, **d** *sPla*$_2$*g1b*, **e** *sPla*$_2$*g12a*) in rat hippocampi. Results are expressed as mean ± SEM ($n = 10$/group). Statistical analysis was performed by one-way ANOVA followed by post hoc Tukey test

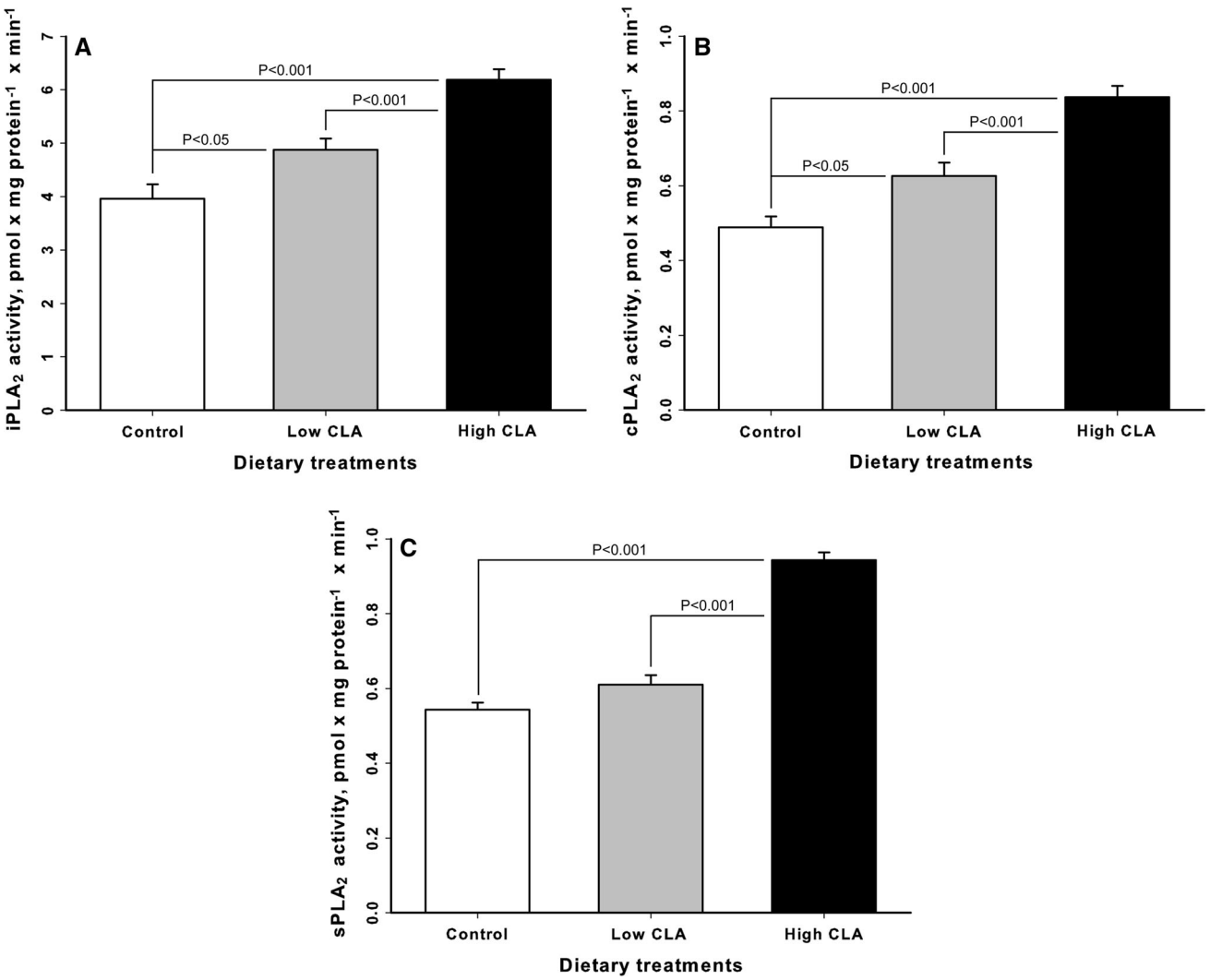

Fig. 2 Effects of low and high CLA diets on the enzymatic activity of PLA_2 groups (**a** $iPLA_2$; **b** $cPLA_2$ and **c** $sPLA_2$) in rat hippocampi. Results are expressed as mean ± SEM ($n = 10$/group). Statistical analysis was performed using one-way ANOVA followed by post hoc Tukey test

Discussion

The immunomodulatory properties of CLA have been demonstrated in a number of studies (O'Shea et al. 2004); however, only a few of them have addressed the effect of CLA on activity and expression of PLA_2, with alterations in PLA_2 being measured in tissues other than brain (Eder et al. 2003; Stachowska et al. 2007). As CLA isomers were shown to be actively incorporated and metabolized in cultures of astrocytes (Fa et al. 2005), we hypothesized that feeding CLA through its main natural source (i.e. dairy fat) could affect the activity and expression of *Pla*$_2$-encoding genes in rat brain tissue. As shown in the Figs. 1 and 2, rats fed the high CLA diet had higher hippocampal mRNA levels for five of the 19 *Pla*$_2$ genes evaluated, which was associated with increased enzymatic activity of their respective PLA_2 groups. In contrast, reduced total PLA_2 activity and $sPLA_2$ mRNA levels were observed in human macrophages (Stachowska et al. 2007) and aortic endothelial cells (Eder et al. 2003) incubated with *cis*-9, *trans*-11 CLA (the main CLA isomer in dairy fat), suggesting a tissue-specific response of PLA_2 expression and activity.

Reduced enzymatic activities of total PLA_2, $cPLA_2$, and $iPLA_2$ have been reported in the postmortem cortex, hippocampus, and cerebrospinal fluid of subjects with AD (Gattaz et al. 1995, 1996; Ross et al. 1998; Talbot et al. 2000; Smesny et al. 2008). Furthermore, the reduction in the activity of total PLA_2 in the brains of individuals with AD reported above (Gattaz et al. 1995; Ross et al. 1998) were correlated with the severity of dementia as well as the density of senile plaques and neurofibrillary tangles, which are the neuropathological hallmarks of AD (Selkoe 2003). However, it should be noticed that one study reported

increased PLA_2 activity in the cerebrospinal fluid of AD patients (Chalbot et al. 2010). Moreover, one study reported that the reduction of arachidonic acid in the brain improved cognitive deficits in hAPP J20 transgenic mice (Sanchez-Mejia et al. 2008). However, in the latter no PLA_2 activity was measured. Nevertheless, these discrepant findings suggest caution in the interpretation of the relationship between phospholipids metabolism and cognition.

The increase in the activity of $cPLA_2$ and $iPLA_2$ groups in brain tissue, as observed in the hippocampus of rats fed both low and high-CLA diets in the present study suggest a beneficial impact on AD pathology. This is supported by animal studies showing that, conversely, the selective inhibition of $iPLA_2$ or of both $iPLA_2$ and $cPLA_2$ in the brain impaired spatial learning in mice (Fujita et al. 2000) and memory acquisition and retrieval of a contextual learning task in rats (Schaeffer and Gattaz 2005, 2007), respectively.

The catalytic activity of PLA_2 is responsible for the production of free fatty acids, particularly arachidonic acid (AA), and long-term potentiation (LTP) Ca^{2+} sensitive induction in hippocampus slices of rat brains has been shown to be facilitated by the addition of AA (Nishizaki et al. 1999). Depending upon the interaction of AA products with different G-protein-coupled receptors, AA products may have both neuroprotective and neurotoxic effects. We found in a series of experiments that an increase in PLA_2 activity, which increases AA products, improves memory (Fattahi and Mirshafiey 2014; Schaeffer and Gattaz 2005, 2007; Schaeffer et al. 2009, 2010, 2011), although contradictory findings were also reported (Sanchez-Mejia et al. 2008; Chalbot et al. 2010).

In the present study, mRNA levels for the *iPla*$_2$*g6*γ isoform (which belongs to the VI $iPLA_2$ group) were increased in the hippocampus of rats fed the high-CLA diet (Fig. 1a), which suggests that an increased DHA release from brain phospholipids may have occurred, as there is evidence from cell culture and rodent studies that the VI $iPLA_2$ group is involved in cleaving DHA from brain phospholipids (Green et al. 2008). Free DHA can be converted through 15-lipoxygenase enzyme (15-LOX) into neuroprotectin D1 (NPD1), a lipid mediator that exhibits potent neuroprotective properties (Lukiw and Bazan 2010).

Accordingly, the addition of CLA to mouse cortical neurons cultured with glutamate increased the expression of Bcl-2 (Hunt et al. 2010), an antiapoptotic gene shown to be up-regulated by NPD1 in human brain cells (Lukiw and Bazan 2010). Thus, it is possible that a higher availability of free DHA induced by increased hippocampal *iPla*$_2$*g6*γ levels contributed, through its conversion to NPD1, to the improved memory observed in rats fed the high-CLA diet. However, this hypothesis warrants further investigation as the hippocampal levels of free DHA and NPD1 were not measured in the present study; furthermore, DHA was not detected in the high CLA diet and, although brain cells and in particular astrocytes have the capacity to synthesize DHA from C18:3 n-3 (Williard et al. 2001), it seems to occur to a limited extent (Demar et al. 2005). As observed for *iPla*$_2$*g6*γ, the hippocampal $cPLA_2$-IVA levels also increased in rats fed the high CLA diet, which may also contribute to memory improvement since $cPLA_2\alpha$ appears to play an important role in LTP and maintenance of synaptic membranes under normal physiological conditions (Farooqui et al. 2006; Qu et al. 2013).

The mechanisms by which CLA modulates the activity and gene expression of PLA_2 groups in brain tissue are still unclear, but recent evidence suggests that it may involve the activation of peroxisome proliferator-activated receptors (PPARs), a class of transcription factors which are regulated by steroids and lipid metabolites (Kummer and Heneka 2008). Agonists of PPARα and PPARγ have been reported to possess anti-inflammatory activity in astrocytes (Xu and Drew 2007; Bernardo and Minghetti 2008), and there is evidence that PPARγ is activated by CLA in animal models and clinical studies (Reynolds and Roche 2010). Interestingly, a recent in vitro study (Sergeeva et al. 2010) showed that the expression of $cPLA_2$ and $sPLA_2$ was inhibited by PPARα and PPARγ agonists in naïve astrocytes, but was increased by PPARγ activation in lipopolysaccharide (LPS)-stimulated astrocytes. Thus, if the anti-inflammatory properties of CLA reported in several studies are mediated by the activation of PPARγ, the resulting effects on the activity and gene expression of PLA_2 groups in brain tissue seem to depend on the inflammatory status of the tissue.

In conclusion, feeding rats with a *cis*-9, *trans*-11 CLA-enriched diet resulted in increased hippocampal mRNA levels for specific PLA_2 isoforms and higher enzymatic activity of all PLA_2 groups, which was closely correlated to memory improvement. Further studies in transgenic animal models of AD and in humans are needed to confirm the potential of CLA-enriched dairy products as a complementary strategy to delay or alleviate memory decline associated with AD.

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