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Dietary Linoleic Acid Lowering Reduces Lipopolysaccharide-Induced Increase in Brain Arachidonic Acid Metabolism

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Abstract

Linoleic acid (LA, 18:2n-6) is a precursor to arachidonic acid (AA, 20:4n-6), which can be converted by brain lipoxygenase and cyclooxygenase (COX) enzymes into various lipid mediators involved in the regulation of brain immunity. Brain AA metabolism is activated in rodents by the bacterial endotoxin, lipopolysaccharide (LPS). This study tested the hypothesis that dietary LA lowering, which limits plasma supply of AA to the brain, reduces LPS-induced upregulation in brain AA metabolism. Male Fischer CDF344 rats fed an adequate LA (5.2 % energy (en)) or low LA (0.4 % en) diet for 15 weeks were infused with LPS (250 ng/h) or vehicle into the fourth ventricle for 2 days using a mini-osmotic pump. The incorporation rate of intravenously infused unesterified ¹⁴C-AA into brain lipids, eicosanoids, and activities of phospholipase A₂ and COX-1 and 2 enzymes were measured. Dietary LA lowering reduced the LPS-induced increase in prostaglandin E₂ concentration and COX-2 activity ($P < 0.05$ by two-way ANOVA) without altering phospholipase activity. The ¹⁴C-AA incorporation rate into brain lipids was decreased by dietary LA lowering ($P < 0.05$ by two-way ANOVA). The present findings suggest that dietary LA lowering reduced LPS-induced increase in brain markers of AA metabolism. The clinical utility of LA lowering in brain disorders should be explored in future studies.

Keywords

Linoleic acid (LA); Lowering; Deficiency; Docosahexaenoic acid (DHA); Omega-6 (n-6); Omega-3 (n-3); Polyunsaturated fatty acids (PUFAs); Bacterial lipopolysaccharide; Neuroinflammation; Ventricle

Introduction

Neuroinflammation, present in aging and many progressive brain disorders, is associated with microglial activation, increased cytokine, and eicosanoid production and reduced

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neuronal viability [1–5]. In rodents, neuroinflammation can be induced by direct intracerebroventricular (i.c.v.) injection of bacterial lipopolysaccharide (LPS) derived from gram-negative bacteria into the brain [6–9]. Doing so has been reported to increase the metabolism of the omega-6 (n-6) poly-unsaturated fatty acid (PUFA), arachidonic acid (AA, 20:4n-6), without altering omega-3 (n-3) PUFA docosahexaenoic acid (DHA, 22:6n-3) metabolism [10, 11]. AA and DHA, which are enriched in brain membrane phospholipids, participate in neurotransmission [12, 13], gene transcription, and the regulation of brain immunity [14, 15]. They are synthesized in the liver from their nutritionally essential dietary precursors linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (α -LNA, 18:3n-3), respectively, or can be obtained directly from the diet [16–18].

AA and DHA are hydrolyzed from the stereospecifically numbered-2 position of brain membrane phospholipids by group IVA cytosolic calcium-dependent phospholipase A₂ (cPLA₂) and group VIA calcium-independent phospholipase A₂ (iPLA₂), respectively [19]. Unesterified AA can be converted non-enzymatically into pro-inflammatory lipid mediators such as 8-isoprostane, or enzymatically into cyclooxygenase (COX)-2-derived prostaglandin (PG)-E₂ or COX-1 derived thromboxane (TX)-B₂ [20–23]. Unesterified DHA can be converted by 5 or 12/15-lipoxygenase (LOX) into 17(S)-resolvin D1 (7S, 8R, 17S-trihydroxy-docosa-4Z,9E,11E,13Z,15E,19Z-hexaenoic acid) [24], which is further converted enzymatically into several anti-inflammatory lipid mediators [15, 25–27].

Compared to an n-6 PUFA adequate diet containing 27.6 % LA of total fatty acids, n-6 PUFA deprivation produced by lowering dietary LA composition to 2.3 % for 15 weeks [28], was reported to decrease messenger RNA (mRNA), protein and activity of AA-metabolizing cPLA₂ and COX-2, and to increase activity of DHA-metabolizing iPLA₂ [29]. DHA turnover within brain membrane phospholipids was increased [30], and AA rate of loss from brain was decreased [31]. N-6 PUFA deprivation also reduced the concentration of non-enzymatic AA-derived eicosanoids, PGF-2 α , 5-hydroxyeicosatetraenoic acid (HETE), 8-HETE, 11-HETE, 12-HETE, and 15-HETE, and increased concentration of non-enzymatic EPA-derived metabolites without altering that of DHA-derived mediators [31].

LPS administered i.c.v. to rats for 6 days at a rate of 0.5 or 250 ng/h was reported to increase brain PGE₂ and TXB₂ concentrations, activities of cPLA₂ and secretory phospholipase A₂ (sPLA₂), mRNA, and protein of cPLA₂ and phosphorylated cPLA₂ which represents the active form of cPLA₂ [10, 32–34]. In preliminary experiments involving the 6-day 250 ng/h LPS model, many of these effects were not reproduced. Thus, the first objective of this study was to determine the LPS dose (0.5 versus 250 ng/h) and timeframe (2 versus 6 days) that upregulated brain AA metabolism. The 2-day time point was chosen in view of a study which reported increased brain AA-derived isoprostanes between 10 and 72 h after i.c.v. LPS administration in mice [35]. As will be presented below, we found that 2-day LPS at 250 ng/h increased brain markers of AA metabolism.

The second objective was to test the hypothesis that decreasing dietary LA would reduce upregulated brain AA metabolism in this validated model of neuroinflammation (i.e., 2-day LPS i.c.v. at 250 ng/h), in view of the reported dampening effects of n-6 PUFA deprivation on brain AA metabolism [29, 31]. Rats were fed an adequate LA diet containing 27.6 % LA

of total fatty acids (equivalent to 5.2 % energy (en)) or a low LA diet containing 2.3 % LA of total fatty acids (0.4 % en) [36] for 15 weeks, and then infused with LPS (250 ng/h) i.c.v. into the fourth ventricle for 2 days using a mini-osmotic pump. Two days later, the incorporation rate of intravenously infused unesterified ^{14}C -AA into brain lipids, concentrations of PGE_2 , TXB_2 , 8-isoprostane and resolvin D1, and activity of AA and DHA metabolizing enzymes (cPLA₂, iPLA₂, sPLA₂, total COX and COX-2) were measured. DHA-metabolizing enzymes and resolvin D1 were measured to determine whether the low LA diet affected brain DHA metabolism in view of studies that reported increased brain DHA turnover in rats on a low LA diet compared to adequate LA controls [30, 31].

It should be noted that the 5.2 % and 0.4 % en LA diets are often called the “n-6 PUFA adequate” or “n-6 PUFA deficient” diets in the literature [28–30]. However, since the 0.4 % en LA diet does not represent true n-6 PUFA deficiency (i.e., 0 % en LA) [37], we will refer to it as the “low LA” diet. The n-6 PUFA adequate diet containing 5.2 % en LA (27.6 % of total fatty acids) will be called the “adequate LA” diet, because it is comparable in fatty acid composition to the AIN-93M rodent diet (26 % LA of total fatty acids) [38].

We found that dietary LA lowering reduced LPS-induced increase in brain PGE_2 concentration and COX-2 activity, but did not change the concentration of DHA-derived resolvin D1 or activity of cPLA₂, iPLA₂, sPLA₂, or total COX. AA incorporation rate decreased independent of LPS treatment in rats on the low LA diet compared to adequate controls.

Materials and Methods

Experiments were conducted in accordance with the NIH “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health Publication Nos. 86–23) and were approved by the Animal Care and Use Committee of *Eunice Kennedy Shriver* National Institute of Child Health and Human Development.

Experiment to Establish the Optimum LPS Dose and Administration Time Frame

The goal of the first study was to establish the optimum LPS dose and administration period that would increase brain AA metabolism. Ninety-six 2-month-old male Fischer rats (CDF344) (Charles River Laboratories, Wilmington, MA, USA) were housed in an animal facility in which temperature, humidity, and light cycle (12-h light/dark) were regulated. The rats consumed the NIH-31 diet containing as a percent of total fatty acids, 20.1 % saturated fatty acids, 22.5 % monounsaturated fatty acids, 47.9 % LA, 5.1 % α -LNA, 0.02 % AA, 2.0 % EPA, and 2.3 % DHA [39].

One week after acclimatization, the rats were surgically implanted with an indwelling cannula attached to an osmotic mini-pump (Alzet® pump, 0.5 $\mu\text{l/h}$, 200 μl volume) that delivered artificial cerebrospinal fluid (ACSF) or LPS at a low (1 $\mu\text{g/ml}$ ACSF delivered at 0.5 ng/h) or high (0.5 mg/ml ACSF delivered at 250 ng/h) dose. The LPS came from *Escherichia coli*, serotype 055:B5 (Sigma-Aldrich, Saint Louis, MO, USA; purified by trichloroacetic acid extraction). The ACSF contained 140 mmol/l NaCl, 3.0 mmol/l KCL, 2.5 mmol/l CaCl_2 , 1.0 mmol/l MgCl_2 , and 1.2 mmol/l NaPO_4 at pH 7.4. Pumps were primed

the day before by flushing the cannula and connecting rubber tube (Plastics One, Roanoke, VA, USA) with ACSF or LPS, and then filling the pump, cannula and rubber tube with LPS or ACSF. The pump was connected to the cannula via the rubber tube. Repeated flushing was performed when necessary to get rid of air bubbles. The pump, tube, and cannula system were stored in 50 ml Falcon tubes containing ~30–35 ml of ACSF, and placed overnight in a water bath at 37 °C.

On the day of surgery, each rat was anesthetized with 50 mg/kg Nembutal, its forehead shaved and its body stationed on a stereotaxic. An incision was made on the scalp, to which lidocaine was applied. The cannula was implanted with the aid of the stereotaxic, 2.5-mm posterior to lambda on the anterior-posterior axis (i.e., the midline) and 7.0 mm below the dura after aligning bregma and lambda to the horizontal plane [6]. The wound was closed with sutures and cleaned with 70 % ethanol. Polysporin ointment was applied on top of the sutured wound to avoid infections. We confirmed that the implanted cannula coordinates targeted the fourth ventricle by injecting i.c.v. 0.1 % Evans Blue dye into the fourth ventricle of a separate batch of rats ($n = 3$) that were subsequently euthanized. Visual inspection of the dissected postmortem brains confirmed that the location of the dye was in the fourth ventricle.

After 2 or 6 days of ACSF or LPS infusion into the ventricles, half the rats from each group ($n = 48$) were anesthetized with 50 mg/kg Nembutal and subjected to high-energy microwave fixation to stop brain lipid metabolism (5.5 kW, 4.8 s; Cober Electronics, Norwalk, CT) [40, 41], and the other half ($n = 48$) were killed with CO₂ asphyxiation and their brains rapidly excised and frozen in isobutane chilled on dry ice. Samples were stored in –80 °C until they were analyzed.

Brains collected from the microwave-fixated rats were used for eicosanoid analysis and from the CO₂-killed rats were used for phospholipase activity measurements (procedures described below).

Dietary Study

It was established that 2-day, high-dose LPS (250 ng/h) increased markers of brain AA metabolism (Fig. 1). The effects of dietary n-6 PUFA deprivation on LPS-induced changes in brain AA metabolism were then tested using this 2-day LPS model.

Ninety-six 18–21-day-old male CDF344 rat pups (Charles River Laboratories, Wilmington, MA) weighing ~28 g, and their nursing surrogate mothers, were purchased from Charles River Laboratories (Portage, MI, USA). The pups were weaned upon arrival and randomly assigned to an adequate or low LA diet obtained from Dyets Inc. (Bethlehem, PA, USA; catalog #180780 for LA adequate diet and #180784 for low LA diet). The mothers were euthanized by CO₂ asphyxiation.

The diets contained (g/100 g) 20 protein, 60 carbohydrate, 10 fats, 4.95 cellulose, 3.5 salts, 1.0 vitamins, 0.3 L-cystine, 0.25 choline chloride, and 0.002 tert-butylhydroquinone. The diets were isocaloric and differed only in their fat composition. The LA-adequate diet contained 60.1 % saturated fatty acids, 7.7 % monounsaturated fatty acids, 27.6 % LA and

4.5 % α -LNA derived from hydrogenated coconut oil (6 g/100 g), safflower oil (3.23 g/100 g), and flaxseed oil (0.77 g/100 g) [28]. The low LA diet contained 85.5 % saturated fatty acids, 7.4 % monounsaturated fatty acids, 2.3 % LA, and 4.8 % α -LNA [28] derived from hydrogenated coconut oil (8.73 g/100 g), flaxseed oil (0.77 g/100 g), and olive oil (0.5 g/100 g) [28]. The percent energy provided by LA was 5.2 % en for the adequate LA diet and 0.4 % en for the low LA diet [36].

Rats were group-housed in a facility with regulated temperature (24 °C) and humidity (40–70 %), under a 12-h light/dark cycle. They were initially housed at six rats per cage until they reached a weight of approximately 100 g, after which they were separated into four per cage. Water and food were provided ad libitum. The food was changed every 3–4 days.

Surgical Procedures

After 15 weeks on a given diet, all 96 rats were surgically implanted with an indwelling cannula attached to an osmotic mini-pump (Alzet® pump). Thirty-two of these rats were used for [1 - 14 C] AA radiotracer infusion to measure brain AA metabolism, 32 for enzyme (PLA₂ and COX) activity assays and 32 for eicosanoid measurements.

Rats were implanted with an indwelling cannula aimed at the fourth ventricle, through which ACSF or high-dose LPS (0.5 mg/ml delivered at 250 ng/h) were delivered for 2 days as described above. After 2 days, the animals were anesthetized with isoflurane (induction with 5 % v/v in O₂ and maintenance at 2–3 % isoflurane) and polyethylene (PE 50) catheters were surgically inserted into the right femoral artery and vein (four rats per day, one from each of the four groups) [42]. The wound was closed with surgical clips and the rats were loosely wrapped, with their upper body remaining free, in a fast-setting plaster cast taped to a wooden block. Surgery for each rat lasted 20–25 min. Rats were allowed to recover from anesthesia for 3–4 h in a temperature-controlled environment maintained at 25 °C. Rectal temperature was maintained at 36.5–37.5 °C using a feedback-heating device and rectal thermometer. Arterial blood pressure and heart rate were measured with a blood pressure recorder (CyQ 103/302; Cybersense, Nicholasville, KY) prior to the beginning of infusion.

Infusion of [1 - 14 C] Arachidonic Acid—[1 - 14 C] AA (50 mCi/mmol, >98 % pure, Moravik Biochemicals, Brea, CA) was dissolved in saline containing 50 mg/ml fatty acid-free bovine serum albumin (Sigma) and the mixture was sonicated for 20 min at 37 °C. An unanesthetized rat was infused intravenously for 5 min with 1.3 ml HEPES-BSA buffer containing 170 μ Ci/kg of [1 - 14 C] AA at a rate of $0.223(1 + e^{-0.32t})$ ml/min (t in seconds) using a computer-controlled variable rate infusion pump (No. 22; Harvard Apparatus, South Natick, MA), to achieve a constant plasma-specific activity within 1 min [43]. Arterial blood samples were collected at 0, 15, 30, 45, 90, 180, 240, and 300 s during infusion to determine radioactivity of unesterified AA in plasma. Blood samples were centrifuged immediately at 18,000g for 30 s at room temperature to obtain plasma. Unlabeled (cold) AA concentration was determined from plasma collected at 300 s. Five min after starting infusion, the rat was anesthetized with sodium pentobarbital (50 mg/kg, i.v.) and subjected to head-focused microwave irradiation to stop brain lipid metabolism (5.5 kW, 4.8 s; Cober Electronics,

Norwalk, CT) [40, 41]. The brain was excised, sagittally dissected to obtain left and right hemispheres, and stored at $-80\text{ }^{\circ}\text{C}$ for further analysis.

Plasma and Brain Lipid Extraction and Separation—Total lipids were extracted from frozen plasma and from one cerebral hemisphere by the Folch method [44].

Unesterified heptadecanoic acid (17:0) was added as an internal standard to plasma prior to extraction. Total lipid extracts were separated by thin layer chromatography (TLC) on silica gel plates (Silica Gel 60 A^o TLC plates; Whatman, Clifton, NJ, USA). Unesterified fatty acids were separated using a mixture of heptane/diethyl ether/glacial acetic acid (60:40:3 by volume) [45], alongside cholesteryl ester, triacylglycerol, unesterified fatty acid, cholesterol, and phospholipid standards that were run in separate lanes to identify the lipids.

Phospholipid classes (choline glycerophospholipids (ChoGpl); phosphatidylserine (PtdSer); phosphatidylinositol (PtdIns); ethanolamine glycerophospholipids (EtnGpl)) were separated in chloroform/methanol/H₂O/glacial acetic acid (60:50:4:1 by volume) [46] and identified with unlabeled standards in separate lanes. The plates were sprayed with 0.03 % (*w/v*) 6-p-toluidine-2-naphthalene sulfonic acid (Acros, Fairlawn, NJ, USA) in 50 mM Tris buffer (pH 7.4), and the lipid bands were visualized under ultraviolet light. Each band was scraped, and the silica gel was used directly to quantify radioactivity by scintillation counting or to prepare fatty acid methyl esters (FAMES) (see in the following section).

Quantification of Radioactivity—Samples for measuring radioactivity were mixed with 5–10 ml of liquid scintillation cocktail (Ready SafeTM plus 1 % glacial acetic acid), and their radioactivity was determined using a liquid scintillation counter (2200CA, TRI-CARB®; Packard Instruments, Meriden, CT, USA).

FAME preparation—FAMES were formed by heating the TLC scrapes in 1 % H₂SO₄ in methanol at 70 °C for 3 h. Prior to methylation, appropriate quantities of di-17:0-PC were added as an internal standard for brain lipids. FAMES were extracted with 3 ml heptane, washed with 1.5 ml water, and reconstituted in 25–200 μl of isoctane.

Gas Chromatography Analysis—FAMES were separated on a SPTM-2330 fused silica capillary column (30 m × 0.25 mm inner diameter, 0.25-μm film thickness) (Supelco, Bellefonte, PA, USA), using gas chromatography (GC) with a flame ionization detector (Model 6890N; Agilent Technologies, Palo Alto, CA, USA) [39]. The initial temperature setting was 80 °C. Temperature was increased to 150 °C (10 °C/min) and 200 °C (6 °C/min), held at 200 °C for 10 min, and then increased to 240 °C (total run time of 38 min). Peaks were identified by the retention times of authentic FAME standards (Nu-Chek-Prep, Elysian, MN, USA). Fatty acid concentrations (nmol/g brain or nmol/ml plasma) were calculated by proportional comparison of the GC peak areas to that of the 17:0 internal standard.

Calculations—The model for determining *in vivo* kinetics of brain fatty acids in rats is described in detail elsewhere [47]. Unidirectional incorporation coefficients,

k_i^* (ml · s⁻¹ · g⁻¹) of [1-¹⁴C] AA, representing incorporation from plasma into stable brain lipid *i* (total lipid, phospholipid, triacylglycerol, cholesteryl ester) was calculated as follows:

$$k_i^* = \frac{C_{br,i}^*(T)}{\int_0^T C_{pl}^* dt} \quad (1)$$

$C_{br,i}^*(T)$ (nCi · g⁻¹) is radioactivity of brain lipid i at time $T = 5$ min (time of termination of experiment), t is time after starting infusion, and C_{pl}^* (nCi · ml⁻¹) is the plasma concentration of labeled unesterified AA during infusion. Integrals of plasma radioactivity were determined by trapezoidal integration. The synthesis of AA from its dietary precursor, LA, is minimal and represents less than 0.5 % of the plasma AA flux into brain [48]. Thus, net rates of incorporation of unlabeled unesterified AA from plasma into brain lipid i , $J_{in,i}$ (nmol · s⁻¹ · g⁻¹) due to entry from plasma, were calculated as follows:

$$J_{in,i} = k_i^* C_{pl} \quad (2)$$

C_{pl} (nmol · ml⁻¹) is the concentration of unlabeled unesterified AA in plasma.

Measurement of Brain AA and DHA Metabolite Concentrations

Brain PGE₂, TXB₂, 8-isoprostane, and resolvin D1 concentrations were measured by enzyme-linked immunoassay (ELISA) following extraction from microwaved brain samples with hexane-/isopropanol (3:2). Half-brains were homogenized in a glass Tenbroeck homogenizer in hexane-isopropanol (18 ml/g brain) [49]. The homogenate was transferred to a glass centrifuge tube and the remaining pellet/sample was washed twice with four volumes of hexane-isopropanol solution and then pooled with the first homogenate in the centrifuge tube. The pooled extract was centrifuged at 1500 rpm for 5 min at room temperature, and the top layer was collected. The pellet was re-extracted twice in 5 ml hexane-isopropanol and the top-layer extracts were pooled. The pooled extracts were dried under nitrogen at 45 °C, resuspended in 3 ml hexane-isopropanol and stored at -80 °C.

Concentrations of AA and DHA-derived metabolites were determined with commercially available ELISA kits in accordance with the manufacturer's instructions. The PGE₂ kit was obtained from Oxford Biochemical (Oxford, MI) and the 8-isoprostane and resolvin D1 kits were from Cayman Chemicals (Ann Arbor, MI). The 3 ml hexane-isopropanol sample was thawed at room temperature and vortexed. One ml of the hexane-isopropanol containing the extracted metabolites was dried under nitrogen and resuspended in 500 µl of the respective kit buffer.

PLA₂ and COX Activities

Brain tissue was homogenized in three volumes of detergent-free buffer containing 10 mM HEPES (pH 7.5), 1 mM EDTA, 0.34 M sucrose, and protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA) using a glass-homogenizer apparatus chilled on ice. The samples were then centrifuged at 100,000g for 1 h at 4 °C, and the supernatant from each sample was transferred to five separate microcentrifuge tubes and stored in -80 °C until they

were analyzed. The extracts were separated this way to ensure that each enzyme activity measurement was done on unfrozen samples.

Protein was quantified by the Bradford assay (Bio-Rad). cPLA₂ and iPLA₂ activities were measured using a radioisotopic method described elsewhere [50] and sPLA₂, total COX, and COX-2 activities were measured with a commercial kit (Cayman Chemicals, Ann Arbor, MI, USA) according to the manufacturer's instructions.

In the LA dietary study, all enzyme activity assays were performed on eight rats per group, except for total COX and COX-2 activity. Total COX and COX-2 activity could not be measured in one rat from the low LA-LPS group because no cytosolic extract was left. COX-2 activity could not be measured in four out of eight samples from each of the adequate LA-ACSF and adequate LA-LPS groups also because no cytosolic extract was left. This is because, initially, four cytosolic extracts from the adequate LA group treated with ACSF or LPS were used to measure cPLA₂ activity in an attempt to confirm a study which reported increased cPLA₂ activity following 6 days of high-dose LPS treatment [32]. Upon failing to confirm the increase in cPLA₂ activity, the assay was repeated on all four groups in order to increase statistical power. As will be shown in the results section, cPLA₂ activity was not altered by LPS. Later in the study, one of the authors (AYT) hypothesized that LPS may alter COX-2 activity in view of in vivo evidence of increased brain COX-2 expression following peripheral administration of LPS [51]. The assay was performed on the remaining cytosolic extracts; thus, the sample size for COX-2 activity was four adequate LA-ACSF, four adequate LA-LPS, eight low LA-ACSF, and seven low LA-LPS rats.

For the COX activity assays in the LA dietary study, mean background absorbance obtained from 20 out of 24 samples was used to calculate activity, because there was not enough cytosolic extract in three samples, and background absorbance could not be reliably established with one sample due to tissue debris contamination that became evident in the last available aliquot from the sample.

Exclusion Criteria—Animals that died, bled extensively or uncontrollably, or had a misplaced vein or arterial line were euthanized with Nembutal (100 mg/kg) and not dissected or used in any measurements, in accordance with animal care procedures. In the LA dietary study, it was realized that 8 of the 32 rats allocated for [1-¹⁴C] AA infusion had received opioid treatment post-cannula implantation surgery (2 per group) by the animal care facility staff. These rats were therefore eliminated from the study, due to the reported effects of opioids on rat brain AA metabolism through the endocannabinoid system [52]. The study was continued with the remaining 24 animals, which did not receive any post-operative analgesia treatment. Data related to animal loss, subjects that fit the exclusion criteria or sample insufficiency for the dietary LA lowering study are reported in Supplementary Table 1.

Statistical Analysis—A two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test was used to determine the effect of treatment (ACSF versus LPS) and time (2 versus 6 days infusion) on weight loss and brain AA and DHA metabolites and enzymes.

A two-way ANOVA followed by Bonferroni's post hoc test was used to test the effect of treatment (ACSF versus LPS) and diet (n-6 PUFA adequate versus deficient) on brain ^{14}C -AA metabolism, AA, and DHA metabolite concentrations and enzyme activity. Statistical significance was set at $P < 0.05$.

Results

LPS Dose and Time Response Study

Within the batch euthanized by microwave fixation ($n = 46$), one rat from each of the 2-day ACSF, 2-day low-dose LPS, and 6-day low-dose LPS groups died of unknown causes. One rat from each of the 2- and 6-day ACSF groups also died of unknown causes from the batch that was euthanized by CO_2 ($n = 46$).

Figure 1 shows the change in body weight 2 or 6 days after cannula i.c.v. implantation surgery from baseline (i.e., before surgery) and brain PGE_2 , TXB_2 , and resolvin D1 concentrations and PLA_2 activities. There was a significant main effect of time and treatment, and interaction between time and treatment, on change in body weight from baseline (difference in weight post and pre surgery; Fig. 1a). Bonferroni's post hoc test showed that the reduction in body weight was significantly greater in the high-dose LPS group compared to the ACSF group at 6 days ($P < 0.001$).

Two-way ANOVA showed a significant effect of LPS ($P = 0.01$) on PGE_2 concentration, which was significantly higher in the high-dose LPS group compared to ACSF at 2 days ($P < 0.05$ by Bonferroni's post hoc test; 1B). TXB_2 concentration was reduced in all groups at 6 days compared to 2 days ($P < 0.01$ for main effect of time; 1C). Resolvin D1 (1D) and PLA_2 activities (1E to G) did not significantly change. Based on the significant increase in brain PGE_2 concentration after 2 days of high-dose LPS, the remaining dietary studies were done using the 2-day high-dose LPS model.

Dietary Studies

Surgery Outcome

Supplementary Table 1 summarizes the sample size for each experiment and includes all rats that were lost due to surgery, opioid injection, or insufficient sample. All 96 rats placed on the adequate (5.2 % en) or low (0.4 % en) LA diets for 15 weeks were implanted with indwelling cannulas that delivered ACSF or LPS into the fourth ventricle for 2 days via an osmotic pump. Of the 96 rats, a subset of 32 was allocated for enzyme activity assays, 32 for eicosanoid measurements and 32 for $[1-^{14}\text{C}]$ AA infusion. One low LA-ACSF rat from the subset of rats allocated for eicosanoid measurements died of unknown causes after the cannula implantation surgery.

As indicated in the "Materials and Methods" section, 8 of 32 rats allocated for $[1-^{14}\text{C}]$ AA infusion study were not analyzed because they were erroneously injected with opioids by the animal care facility staff. Of the remaining 24 rats, two adequate LA-LPS rats were euthanized because they bled at the site of the surgery incision. One low LA-ACSF rat was euthanized during the femoral artery/vein implantation because of a torn blood vessel which

led to uncontrollable bleeding. One low LA-LPS rat did not lose consciousness after infusing Nembutal through the vein line following tracer infusion, suggesting that the catheter was detached. This rat was not subjected to high-energy microwave-fixation and was euthanized with Nembutal. The total number of rats was 6, 5, 3, and 6 adequate LA-ACSF, adequate LA-LPS, low LA-ACSF, and low LA-LPS, respectively (Supplementary Table 1).

Physiological Parameters

Two-way ANOVA showed no statistically significant difference in body weight before or after i.c.v. surgery, change in body weight, body temperature, blood pressure, or heart rate between the groups (Table 1).

Plasma Unesterified Fatty Acid Concentrations

There was a main effect of diet on plasma unesterified LA, AA, n-6 DPA, and EPA ($P < 0.0001$, Table 2). LA, AA, and n-6 DPA were reduced by more than twofold in low LA rats compared to adequate LA rats, irrespective of LPS or ACSF treatment, whereas EPA was increased by approximately twofold.

Brain ^{14}C -AA Incorporation Kinetics

Plasma steady-state was achieved within 1.5 min following ^{14}C -AA infusion (Fig. 2). Two-way ANOVA showed that the area under the curve for plasma ^{14}C -AA concentration over time did not differ significantly among the groups (Table 1), suggesting no treatment or diet effect on input function.

There was a significant effect of LPS treatment on brain total lipid and ChoGpl k^* ($P < 0.01$), which was elevated by 13 % in adequate LA-LPS relative to adequate LA-ACSF controls, and 21–24 % in low LA-LPS relative to low LA-ACSF (Table 3). The low LA diet significantly increased PtdIns k^* by 7–10 % (Table 3). Post hoc comparison by Bonferroni's test revealed no significant differences between the means.

The incorporation rate, J_{in} , into total lipids and phospholipid subfractions was significantly reduced by 1.5–2.7-fold in rats on the low LA diet compared to adequate LA rats (Table 3). No significant differences between the means were seen with Bonferroni's post hoc test.

Brain Eicosanoid and Docosanoid Concentrations

As shown in Supplementary Fig. 1, one sample in the adequate LA-ACSF group was up to 5.2 times higher than the group mean for PGE₂, 8-isoprostane and resolvin D1, likely due to ischemia caused by incomplete microwave-fixation [53]. Grubb's outlier test revealed that this sample was an outlier, so it was removed from the statistical analysis.

As shown in Table 4, two-way repeated measures ANOVA showed a statistically significant main effect of LPS treatment on PGE₂ concentration. Post hoc analysis with Bonferroni's test showed that LPS significantly increased PGE₂ concentration in adequate LA rats compared to ACSF adequate LA controls ($P < 0.01$), but not in low LA rats ($P > 0.05$).

There was no significant effect of diet or LPS on 8-isoprostane, TXB₂ or resolvin D1 concentrations.

Because the removal of outliers can increase the risk of type I statistical errors, we normalized the data via a log transformation which included the outlier rat. As shown in Supplementary Table 2, two-way ANOVA yielded the same results —LPS increased log PGE₂ in the adequate LA group but not the low LA group. No significant changes were observed in log-transformed 8-isoprostane, TXB₂, or resolvin D1 concentrations.

Brain Enzyme Activity

Table 4 also shows cPLA₂, iPLA₂, sPLA₂, total COX and COX-2 activity. A two-way ANOVA showed no significant effect of diet or LPS treatment on cPLA₂ (3-A), sPLA₂ (3-C), or total COX activity (3-D).

A two-way ANOVA showed a significant effect of diet on brain iPLA₂ activity (3-B), but no effect of LPS treatment. Post hoc analysis with Bonferroni's test showed no significant differences among the groups.

There was a significant diet and LPS interaction on COX-2 activity (3-E). Post hoc analysis with Bonferroni's test showed that COX-2 activity was significantly increased by 4.7-fold following LPS compared to ACSF treatment in rats on the adequate LA diet ($P < 0.05$), but not the low LA diet ($P > 0.05$).

Discussion

This study validated a 2-day LPS model involving upregulated brain AA metabolism and showed that in this model, 15 weeks of LA lowering from 5.2 % to 0.4 % energy blocked the LPS-induced increase in brain PGE₂ concentration and COX-2 activity observed in rats on the adequate (5.2 % en) LA diet. LPS significantly increased the AA incorporation coefficient, k^* , by 13–24 % in total lipids and ChoGpls. Dietary LA lowering increased k^* in PtdIns, and decreased the incorporation rate, J_{in} , into total lipid and phospholipid subfractions. Dietary LA lowering also decreased plasma unesterified LA, AA, and n-6 DPA, and increased EPA concentrations, as reported [30].

Previous studies reported that 6-day i.c.v. administration of 0.5 or 250 ng/h LPS into the fourth ventricle increased cPLA₂ or sPLA₂ activity and PGE₂ or TXB₂ concentrations [10, 32, 33]. This was not entirely confirmed in the present study, when the same LPS doses were given (i.c.v.) for 6 days (Fig. 1). PGE₂ concentration increased only in the group that received high-dose LPS (250 ng/h) for 2 days (Fig. 1), which was reproduced in the second dietary experiment (Table 4). The cause of this discrepancy is not clear, although batch-to-batch variability in the response of rats to LPS is possible. Notably, the present experiments used the same rat strain and vendor and LPS source used in previous studies [32, 34].

In this study, the bregma and lambda skull coordinates were aligned to the horizontal plane prior to cannula implantation into the fourth ventricle. It is not clear whether the same alignment procedures were done in previous reports. However, in an exploratory study in which the cannulas were purposely mis-implanted by inserting them 1–2 mm past the fourth

ventricle, no significant changes in PLA₂ activities or eicosanoid concentrations were found in LPS-treated rats (250 ng/h for 6 days) compared to ACSF controls (Taha and Blanchard, unpublished). The transient effect of LPS on brain AA metabolism is in general agreement with Montine et al. who reported transient increases in AA-derived isoprostanes that peaked at 24 h and returned to baseline by 72 h after a single i.c.v. dose of LPS in mice [35].

The transient increase in brain AA metabolism at 2 but not 6 days reflects tolerance to LPS. Transcription of the pattern recognition receptor, CD14, as well as inhibitory factor kappa (marker of NF-kappa B activity) and IL-1 β , was reported to increase 1 h after a single i.c.v. injection of LPS and to progressively decline 3 and 6 h post-injection [8].

Dietary LA lowering did not reduce baseline cPLA₂ or COX-2 activity, or increase iPLA₂ activity compared to LA-adequate rats as reported by Kim et al. [29]. This is likely because the rats in this study were surgically implanted (i.c.v.) with a cannula 2 days prior to sacrifice. A brain surgery represents a trauma-like event, which could have altered baseline enzyme activity levels and masked dietary changes. In agreement with this suggestion, two studies reported increased brain cytokines following hippocampal surgical trauma [54] or minor abdominal injury [55]. PLA₂ and COX-2 enzymes were not measured in these studies, although they were reported to increase in rat primary astrocytes in response to cytokines [56].

Compared to ACSF controls, LPS significantly increased k^* by 13–24 % in total lipids and ChoGpls irrespective of diet, suggesting increased brain AA metabolism in association with LPS-induced inflammation. This is in general agreement with previous reports which showed increased brain; total lipid or phospholipid k^* following 6-day i.c.v. LPS at 0.5 or 250 ng/h [10, 33]. The increase in k^* is likely caused by increased production of cytokines, which were reported to increase following direct LPS injection into the hippocampus, cortex, or substantia nigra [7]. LPS, given intraperitoneally to rats also increased pro-inflammatory PGE₂ concentration in hypothalamus [57].

The significant 7–10 % increase in AA k^* within PtdIns in low LA compared to adequate LA rats represents an adaptive response to reduced plasma unesterified AA availability to the brain. This is in agreement with the reported reduction in rat brain AA loss and prolonged half-life from phospholipids following 15 weeks of n-6 PUFA deprivation [35], and the 1.5–2.7-fold reduction in J_{in} within brain phospholipids (Table 3). A similar increase in rat brain phospholipid k^* for DHA was reported when circulating DHA and incorporation rate (J_{in}) were reduced by chronic n-3 PUFA deprivation [58, 59].

Compared to ACSF, LPS increased PGE₂ concentration and COX-2 activity in the adequate LA group, but not the low LA group, suggesting an anti-inflammatory effect of the low LA diet. LPS administration in vitro or in vivo induces transcription or activity of COX-2, which converts unesterified AA into PGH₂, the metabolic precursor to PGE₂ [51, 60]. The 1.5–2.7-fold reduction in unesterified AA J_{in} in rats on the low LA diet likely explains its protective effects, since reduced AA availability to the brain may be substrate limiting for COX-2. The decrease in AA J_{in} reflects the reported reduction in brain AA total lipid and phospholipid

concentrations of rats fed a low LA diet compared to an adequate LA diet [28, 30]. It is not known, however, whether brain AA concentrations are affected by LPS treatment.

LPS did not alter brain concentration of LOX-derived resolvin D1 in either the dose and time response study (Fig. 1) or the dietary LA study (Table 4), consistent with other reports which showed no change in brain DHA metabolism following 6-day low- or high-dose LPS treatment [11, 32]. Studies reported increased formation of non-enzymatic DHA metabolites (F₄-neuroprostane) in mouse cerebrum within 12 h after acute LPS injection into the left lateral ventricle [35, 61], suggesting that DHA may play a role in regulating some aspects of the LPS-induced inflammatory response [62]. The role of F₄-neuroprostane and other DHA-derived mediators in regulating brain immunity should be explored in future studies [15].

The low sample size of the ¹⁴C-AA incorporation and COX-2 activity studies increases the likelihood of type I or II statistical errors [63], so the data should be interpreted with caution. Despite our intent to have eight rats per group, several rats had to be euthanized due to excessive bleeding, unintentional opioid injection by the animal care facility staff or sample insufficiency. Future experiments should factor unexpected animal or sample losses in the sample size calculation.

This study demonstrates that dietary LA lowering can be used to target upregulated brain AA metabolism, which has been associated with progressive neurodegenerative disorders [1–3, 5]. Supporting this suggestion is evidence of reduced headache frequency in chronic migraine patients who reduced their LA intake from 7 to 2 % energy and increased EPA and DHA intake to 1.5 g/day for 12 weeks [64]. In these subjects, the frequency of headaches was positively associated with total LA concentration in plasma [65].

In summary, dietary LA lowering reduced LPS-induced upregulation in brain markers of AA metabolism. The translational relevance of LA lowering in humans on brain disorders involving upregulated brain AA metabolism merits testing in future studies. Positron-emitting tomography could be used to image changes in brain AA metabolism in relation to dietary efficacy and disease progression [5].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

α-LNA	Alpha-linolenic acid
AA	Arachidonic acid

ACSF	Artificial cerebrospinal fluid
ANOVA	Analysis of variance
cPLA₂	Calcium-dependent phospholipase A ₂
COX	Cyclooxygenase
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
en	Energy
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunoassay
EPA	Eicosapentaenoic acid
FAMES	Fatty acid methyl esters
GC	Gas-chromatography
HETE	Hydroxy-eicosatetraenoic acid
i.c.v.	Intracerebroventricular
iPLA₂	Calcium-independent phospholipase A ₂
LA	Linoleic acid
PG	Prostaglandin
PUFA	Polyunsaturated fatty acid
sPLA₂	Secretory phospholipase A ₂
TX	Thromboxane

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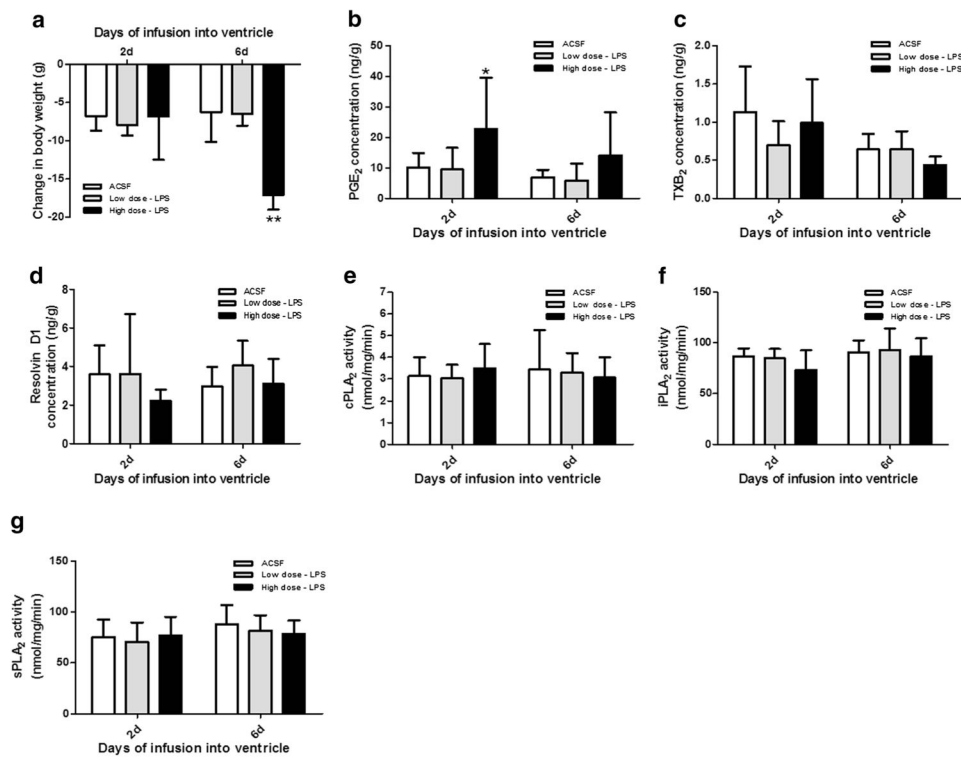


Fig. 1. Change in body weight after surgery (a), brain PGE₂ (b), TXB₂ (c) and resolvin D1 (d) concentrations, and cPLA₂ (e), iPLA₂ (f) and sPLA₂ (g) activities. Rats were treated for 2 or 6 days with artificial cerebrospinal fluid (ACSF), low-dose LPS (0.5 ng/h) or high-dose LPS (250 ng/h) via i.c.v. administration using a mini- osmotic pump. Data are mean \pm SD of $n = 7-8$ rats per group. Data were analyzed by 2-way analysis of variance followed by Bonferroni's post hoc test. Asterisk (*) indicates significant difference compared to ACSF

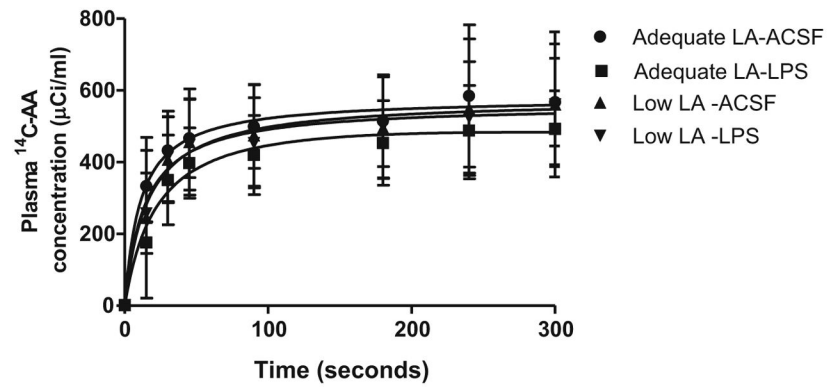


Fig. 2. Plasma ¹⁴C-AA concentration (µCi/ml) over the 300-s infusion period. Data are mean ± SD of $n = 6$ adequate LA-ACSF, five adequate LA-LPS, three low LA-ACSF, and six low LA-LPS. LA contributed 5.2 % and 0.4 % of calories in the adequate and low LA diets, respectively. The area under the curve reported in the results section did not significantly differ among the groups by 2-way analysis of variance followed by Bonferroni's post hoc test

Table 1

Physiological parameters before and 2 days after i.c.v. cannula implantation surgery

	Adequate LA-ACSF <i>n</i> = 6	Adequate LA-LPS <i>n</i> = 5	Low LA-ACSF <i>n</i> = 3	Low LA-LPS <i>n</i> = 6	Treatment	Diet	Interaction
Body weight before i.c.v. surgery	385 ± 34	388 ± 17	367 ± 23	393 ± 32	NS	NS	NS
Body weight 2-days after i.c.v. surgery	369 ± 33	373 ± 17	355 ± 29	377 ± 30	NS	NS	NS
Change in body weight	-17 ± 7	-15 ± 4	-12 ± 9	-16 ± 3	NS	NS	NS
Rectal temperature	37.3 ± 0.4	37.1 ± 0.4	37.0 ± 0.3	37.1 ± 0.3	NS	NS	NS
Systolic blood pressure	158 ± 19	159 ± 28	167 ± 22	171 ± 28	NS	NS	NS
Diastolic blood pressure	99 ± 14	107 ± 19	103 ± 17	112 ± 11	NS	NS	NS
Heart rate	452 ± 26	455 ± 44	460 ± 14	457 ± 16	NS	NS	NS

Data are mean ± SD of *n* = 3–6 per group. Data were analyzed by 2-way analysis of variance. LA contributed 5.2 and 0.4 % of calories in adequate and low LA diets, respectively

Table 2

Plasma unesterified PUFA concentrations (nmol/ml)

	Adequate LA-ACSF <i>n</i> = 6	Adequate LA-LPS <i>n</i> = 5	Low LA-ACSF <i>n</i> = 3	Low LA-LPS <i>n</i> = 6	Treatment	Diet	Interaction
n-6 PUFA							
LA	246.5 ± 55.1	250.5 ± 51.1	31.6 ± 9.5	31.2 ± 9.0	NS	<0.0001	NS
AA	43.0 ± 7.9	40.4 ± 5.6	19.5 ± 6.1	18.8 ± 2.4	NS	<0.0001	NS
n-6 DPA	6.9 ± 1.9	7.8 ± 1.8	0.1 ± 0.1	0.5 ± 1.0	NS	<0.0001	NS
n-3 PUFA							
α-LNA	32.4 ± 7.6	32.2 ± 7.0	25.1 ± 12.5	26.1 ± 8.6	NS	NS	NS
EPA	3.4 ± 0.9	2.6 ± 0.8	7.5 ± 2.9	5.8 ± 1.0	NS	<0.0001	NS
DHA	13.8 ± 3.3	15.6 ± 2.4	17.9 ± 3.8	18.3 ± 4.5	NS	NS	NS

Data are mean ± SD of *n* = 3–6 per group. Data were analyzed by 2-way analysis of variance followed by Bonferroni's post hoc test. LA contributed 5.2 and 0.4 % of calories in adequate and low LA diets, respectively

PUFA polyunsaturated fatty acids, LA linoleic acid, AA arachidonic acid, n-6 DPA n-6 docosapentaenoic acid, α-LNA α-linolenic acid, EPA eicosapentaenoic acid, DHA docosahexaenoic acid

¹⁴C-AA incorporation into brain lipid compartments following 2-day ACSF or LPS infusion into the 4th ventricle of rats on a low or adequate LA diet

Table 3

	Adequate LA-ACSF n = 6	Adequate LA-LPS n = 5	Low LA-ACSF n = 3	Low LA-LPS n = 6	Treatment	Diet	Interaction
Plasma AUC	149,974 ± 38,254	126,319 ± 32,607	142,510 ± 47,316	140,059 ± 40,338	NS	NS	NS
<i>k</i> *							
Total lipids	27.0 ± 3.4	31.2 ± 4.3	29.1 ± 5.1	32.9 ± 3.9	0.0488	NS	NS
ChoGpl	7.9 ± 0.9	9.6 ± 1.5	8.9 ± 0.6	11.0 ± 1.6	0.0060	NS	NS
PtdIns	6.7 ± 0.6	7.4 ± 1.3	9.2 ± 1.2	9.8 ± 1.5	NS	0.0004	NS
PtdSer	1.6 ± 0.4	1.7 ± 0.5	1.3 ± 0.1	1.5 ± 0.2	NS	NS	NS
EtnGpl	2.4 ± 0.5	2.5 ± 0.6	2.0 ± 0.3	2.4 ± 0.4	NS	NS	NS
<i>J_m</i>							
Total lipids	1143 ± 146	1242 ± 55	587 ± 288	549 ± 88	NS	<0.0001	NS
ChoGpl	335 ± 33	381 ± 28	175 ± 66	183 ± 29	NS	<0.0001	NS
PtdIns	283 ± 38	293 ± 22	184 ± 82	163 ± 28	NS	<0.0001	NS
PtdSer	65 ± 12	67 ± 14	25 ± 10	25 ± 3	NS	<0.0001	NS
EtnGpl	100 ± 8	99 ± 14	40 ± 17	40 ± 7	NS	<0.0001	NS

Data are mean ± SD of n = 3-6 per group. Data were analyzed by 2-way analysis of variance followed by Bonferroni's post hoc test. LA contributed 5.2 and 0.4 % of calories in adequate and low LA diets, respectively

AUC area under the curve, *ChoGpl* choline glycerophospholipid, *PtdIns* phosphatidylinositol, *PtdSer* phosphatidylserine, *EtnGpl* ethanolamine glycerophospholipid

Brain enzyme activity, and eicosanoid and docosanoid concentrations following 2-day ACSF or LPS infusion into the 4th ventricle of rats on a low or adequate LA diet

Table 4

	Adequate LA-ACSF <i>n</i> = 8	Adequate LA-LPS <i>n</i> = 8	Low LA-ACSF <i>n</i> = 8	Low LA-LPS <i>n</i> = 8	Treatment	Diet	Interaction
cPLA ₂	4.5 ± 1.4	6.6 ± 5.3	4.7 ± 1.7	4.0 ± 1.2	NS	NS	NS
iPLA ₂	32.6 ± 9.6	35.7 ± 7.8	32.1 ± 7.6	24.6 ± 5.8	NS	0.0435	NS
sPLA ₂	66.4 ± 17.6	73.4 ± 12.8	74.8 ± 10.1 (<i>n</i> = 7) ^a	65.1 ± 17.9	NS	NS	NS
	<i>n</i> = 6 ^a	<i>n</i> = 7 ^a	<i>n</i> = 7 ^a	<i>n</i> = 6 ^{a,b}			
Total COX	213.2 ± 65.1	291.7 ± 103.1	258.8 ± 53.7	392.0 ± 363.9	NS	NS	NS
	<i>n</i> = 4 ^b	<i>n</i> = 4 ^b	<i>n</i> = 7 ^b	<i>n</i> = 7 ^b			
COX-2	222.7 ± 157.8	1035.7 ± 1055.6*	351.1 ± 209.7	279.8 ± 198.9	NS	NS	0.0465
	<i>n</i> = 7 ^c	<i>n</i> = 8	<i>n</i> = 7 ^d	<i>n</i> = 8			
PGE ₂	0.7 ± 0.4	2.9 ± 1.5***	1.0 ± 0.4	1.4 ± 0.6	0.005	NS	0.009
8-isoprostane	0.9 ± 0.4	1.3 ± 0.8	0.8 ± 0.5	1.0 ± 0.2	NS	NS	NS
Resolvin D1	3.4 ± 3.0	2.0 ± 1.0	2.6 ± 2.4	2.8 ± 1.8	NS	NS	NS
	<i>n</i> = 8	<i>n</i> = 8	<i>n</i> = 7 ^d	<i>n</i> = 8			
TXB ₂	1.3 ± 0.3	1.4 ± 0.5	1.9 ± 1.6	2.2 ± 1.5	NS	NS	NS

Data are mean ± SD of *n* = 4–8 per group. Data were analyzed by 2-way analysis of variance followed by Bonferroni's post hoc test.

Asterisk (***) indicates significant difference at *P* < 0.001 level compared to adequate LA-ACSF. LA contributed 5.2 and 0.4 % of calories in adequate and low LA diets, respectively. Original sample size was eight per group; footnotes indicate reason for loss of samples

cPLA₂ calcium-dependent phospholipase A₂, iPLA₂ calcium-independent phospholipase A₂, sPLA₂ secretory phospholipase A₂, COX cyclooxygenase

^a *n* less than eight due to negative activity values that were not included in the mean calculation

^b *n* less than eight because no cytosolic extract was left

^c *n* = 7 because Grubb's test identified one outlier value of 9.38, 8.55, and 10.22 for PGE₂, 8-isoprostane, and Resolvin D1 in adequate LA-ACSF rats, due to incomplete microwave fixation

^d *n* = 7 because one rat died after brain surgery due to unknown causes