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Effects of diets enriched in linoleic acid and its peroxidation products on brain fatty acids, oxylipins, and aldehydes in mice


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ABSTRACT

Background: Linoleic acid (LA) is abundant in modern industrialized diets. Oxidized LA metabolites (OXLAMs) and reactive aldehydes, such as 4-hydroxy-2-nonenal (4-HNE), are present in heated vegetable oils and can be endogenously synthesized following consumption of dietary LA. OXLAMs have been implicated in cerebellar degeneration in chicks; 4-HNE is linked to neurodegenerative conditions in mammals. It is unknown whether increasing dietary LA or OXLAMs alters the levels of oxidized fatty acids (oxylipins), precursor fatty acids, or 4-HNE in mammalian brain.

Objectives: To determine the effects of increases in dietary OXLAMs and dietary LA on levels of fatty acids, oxylipins, and 4-HNE in mouse brain tissues.

Methods: Mice (n = 8 per group) were fed one of three controlled diets for 8 weeks: (1) a low LA diet, (2) a high LA diet, or (3) the low LA diet with added OXLAMs. Brain fatty acids, oxylipins, and 4-HNE were quantified in mouse cerebellum and cerebral cortex by gas chromatography-flame ionization detection, liquid chromatography-tandem mass spectrometry, and immunoblot, respectively.

Results: Increasing dietary LA significantly increased omega-6 fatty acids, decreased omega-3 fatty acids, and increased OXLAMs in brain. Dietary OXLAMs had minimal effects on oxidized lipids but did decrease both omega-6 and omega-3 fatty acids. Neither dietary LA nor OXLAMs altered 4-HNE levels.

Conclusion: Brain fatty acids are modulated by both dietary LA and OXLAMs, while brain OXLAMs are regulated by endogenous synthesis from LA, rather than incorporation of preformed OXLAMs.

1. Introduction

Linoleic acid (LA) is the most abundant polyunsaturated fatty acid in modern industrialized diets, accounting for approximately 3 to > 17% percent of energy (%E) intake in individuals worldwide [1–3]. Current LA intakes in industrialized populations are higher than historical and evolutionary norms of 2–3% E. The importance of LA to human health has, classically, been attributed to three primary functions: (i) its role as an “essential fatty acid” because small amounts of LA (about 0.5% E) in the diet are required for integrity of the epidermal water barrier [4]; (ii) its ability to reduce serum low density lipoprotein cholesterol when replacing dietary saturated fats [3,5]; and (iii) for being the precursor to arachidonic acid (AA); AA is enzymatically converted to peroxidation products with well-established bioactivities, including prostanooids and leukotrienes (reviewed in [3] and [6]).
Like AA, docosahexaenoic acid (DHA) and other polyunsaturated fatty acids, LA contains a 1,4 cis-cis pentadiene system and thus can serve as the substrate for enzymatic peroxidation to synthesize biochemically active oxygenated derivatives, collectively known as oxylipins. The subset of oxylipins derived from LA, which are known as oxidized linoleic acid metabolites (OXLAMs), include hydroperoxy-octadecadienoates (HpODEs), hydroxy-octadecadienoates (HODEs), oxo-octadecadienoates (oxo-ODEs), epoxy-octadecenoates (EpOMEs), dihydroxy-octadecenoates acids (DIOHMEs), hydroxy-epoxides [6], keto-epoxides [6], and trihydroxy-octadecenoates (TriHOMEs) [2]. HpODEs and lipid peroxides derived from other omega-6 fatty acids are also precursors for α,β unsaturated reactive aldehyde degradation products including 4-hydroxy-2-nonenal (4-HNE) [7]. Since mammals cannot synthesize LA de novo, the LA content of diet is likely to be a critical determinant of accumulation of LA, OXLAMs, and 4-HNE in many tissues [2,8]. HpODEs and other OXLAMs can also be formed non-enzymatically when vegetable oils rich in LA are cooked or otherwise heated [9–11]. A substantial portion of vegetable oils in industrialized populations, including those used in many processed and packaged foods, is heated prior to consumption; and these preformed OXLAMs could potentially be absorbed and incorporated into certain tissues including brain after consumption [12–14]. Therefore, the abundance of OXLAMs and 4-HNE in human and other mammalian tissues could potentially be affected by both consumption of non-oxidized LA, with subsequent conversion to OXLAMs in the body, and by consumption of preformed OXLAMs.

OXLAMs and 4-HNE have been mechanistically linked to several pathological conditions including cardiovascular disease [15], steatohepatitis [16,17], neurodegenerative diseases [18], and chronic pain [6,19,20], reviewed in [3,15,21]. Consumption of heated vegetable oils rich in LA [22,23], or intravenous administration of HpODEs [24], produces cerebellar necrosis and axatia in chicks without damaging the cerebral cortex, indicating that OXLAMs could potentially have brain-region specific neurotoxic effects in some species. Plausible mechanisms exist whereby high exposure to OXLAMs could have neurotoxic effects in humans, including endothelial cell activation [25], generalized lipid and membrane peroxidation [26,27], mitochondrial dysfunction [28], and microglial activation [29–31]. 4-HNE, which forms chemical bonds with cysteine, lysine, and histidine residues [32] and has been implicated in protein misfolding and aggregation [33], is linked to the development or progression of neurofibrillary tangles and amyloid plaques that are characteristic of Alzheimer’s disease [33,34]. However, despite these plausible mechanisms, there is a lack of data to assess the effects of increasing dietary LA and dietary OXLAMs on the fatty acid, oxypill, and aldehyde compositions in mammalian brain.

In the present paper, we examine if high intakes of LA (from unheated corn oil), or exogenously produced OXLAMs (from heated corn oil), both characteristic of modern industrialized diets, impact mammalian brain biochemistry. Findings support the hypothesis that diets enriched in LA and OXLAMs alter cerebral and cerebellar lipid accumulation and peroxidation in mammals.

2. Methods

Wild type male C57BL/6 mice (n = 8 per group) were fed ad-libitum one of three controlled diets designed to contain 40% fat by weight in g/kg, for 8 weeks: (i) a low LA diet designed to contain 4%E as LA, (ii) a high LA diet designed to contain 17%E as LA or (iii) the low 4% E LA diet enriched with dietary OXLAMs from thermally-stressed corn oil. The three study diets were prepared by Dyets Inc. (Bethlehem, PA) using unheated and thermally stressed oils of known fatty acid composition. Despite being designed to contain the same amount of LA as the Low LA diet, the Low LA + OXLAMs diet was observed to contain substantially less LA on gas chromatography analysis. Fatty acid and OXLAM concentrations of the three diets are shown in Table 1. Fatty acid percent compositions are shown in Table S1. This animal protocol followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) and was approved by the University of California, San Diego Institutional Animal Care and Use Committee (protocol number S11200).

2.1. Preparation of heated corn oil

Thermally stressed corn oil was prepared by heating 2 kg of Crisco™ brand corn oil purchased from a local grocery store in a shallow cast iron pan in an oven at 115 °C for approximately 4 weeks, with daily stirring of the oil. Progress was monitored by 1H NMR (400 MHz) in deuterated chloroform by comparing the decrease in integration of the trans, conjugated dienes of HpODEs (5.40–5.58 ppm) and conjugated cis, trans and trans, trans dienes of HODEs (5.40–5.58 ppm) which remained unchanged (referenced to a sample taken from the corn oil immediately upon opening). Lipid peroxide composition was assessed by estimating the integration of newly formed peaks in regions that were sufficiently separated from potential interference of peaks arising from the vitamin E in the corn oil. These corresponded to the conjugated cis, trans, and trans, trans dienes of HpODEs (5.40–5.58 ppm, 5.86–6.11 ppm, smaller multiplets at 6.12–6.32 ppm) as described by Guillen et al. [35]. Based on LA composition of corn oil as 55%, in the 2KG sample there were 192 g of HpODEs present in the total oil mixture at reaction end as calculated from the NMR integration results. Further oxidation products were also observed as minor aldehyde peaks (9.40–9.56 ppm).

2.2. Fatty acid and oxylipin analysis of study diets

Dietary fatty acids in the food pellets were analyzed by gas chromatography (GC) with a flame ionization detector (FID) as previously reported [36]. Briefly, food pellets were crushed with pestle and mortar. 0.4 mL toluene, 3 mL methanol and 0.6 mL 8% HCl in methanol were added to 30 mg of powder after adding 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine as an internal standard. Samples were heated for 1 h at 90 °C. One mL of hexane and 1 mL water were added, and the samples were allowed to sit at room temperature for a few minutes. The upper hexane layer containing the fatty acid methyl esters was
transferred to a new tube containing 0.45 mL water, vortexed and centrifuged for 2 min at 13,000 rpm. The hexane layer was separated, dried under nitrogen, reconstituted in 0.2 mL hexane and subjected to gas-chromatography analysis. Fatty acid methyl esters were analyzed with a Clarus 500 GC system equipped with FID (Perkin Elmer, CA, USA) and a fused silica capillary column (DB-FFAP, 30 m, 0.25 mm i.d., 0.25 μm film thickness, Agilent, Santa Clara, CA, USA). The injector and detector temperatures were set to 240 °C and 300 °C, respectively. The oven temperature program was set at 80 °C for 2 min, increased to 185 °C at the rate of 10 °C/min, and to 240 °C at the rate of 5 °C/min, and held at 240 °C for 13 min. Helium was the carrier gas and was maintained at a flow rate of 1.3 mL/min.

Diet OXLAM concentrations were measured in 30 mg of a crushed food pellet dissolved in 200 μL ice-cold methanol containing 0.1% acetic acid and 0.1% BHT and spiked with 10 μL antioxidant mix and 10 μL surrogate standard containing 5 pmol of d11-12(12)-EpETrE, d11-14,15-DiHETrE, d4-6-keto-PGF1a, d4-9-HODE, d4-LTB4, d4-PE2, d4-TXB2, d6-20-HETE, and d8-5-HETE dissolved in methanol. The antioxidant solution contained three antioxidants mixed at a 1:1:1 ratio (v/v/v) consisting of 0.6 mg/mL ethylenediaminetetraacetic acid in water, 0.6 mg/mL BHT in methanol, and 0.6 mg/mL triphenylphosphine in water:methanol (1:1; v/v), that were filtered through a Millipore filter to remove solid particles. Samples were hydrolyzed and oxylipins extracted with solid phase extraction (SPE) and analyzed by LC-MS/MS as described below for the mouse brain tissue analysis.

2.3. Mouse tissue collection and fatty acid, oxylipin, and 4-HNE analyses

Mice were killed by CO2 overdose. Tissue samples were immediately collected, frozen on dry-ice chilled isobutane, and stored at −80 °C. Tissue oxylipins were analyzed as previously described [37]. Briefly, two hundred microliters of ice-cold methanol containing 0.1% acetic acid and 0.1% BHT was added to approximately 30 μL of tissue oxylipins and 10 μL surrogate standards as described above. The brain samples containing the extraction solvent, antioxidant mix, and surrogate standards were cooled in −80 °C freezer for 30 min and then homogenized for 2 min using a bead homogenizer. The homogenized samples were stored for 30 min at −80 °C freezer, followed by centrifugation at 13,000 rpm (15,870 g) in a 5424R microcentrifuge (Eppendorf) for 10 min at 0 °C. The supernatant was transferred to a new tube for the oxylipin analysis, while the pellet was kept at −80 °C for fatty acid analysis. The supernatant was hydrolyzed in equal volumes of 0.5 M sodium carbonate solution (26.5 mg per ml of 1:1 v/v methanol/water) at 60 °C for 30 min. The samples were acidified to pH 4 to 6 with 25 μL acetic acid and 1575 μL water was added to dissolve the resulting salts. The solution containing hydrolyzed oxidized lipids (oxylipins) was subjected to SPE. Samples were poured onto 60 mg Waters Oasis HLB 3 cc cartridges (Waters, Milford, MA, USA) pre-rinsed with one volume ethyl acetate and two volumes methanol and conditioned with two volumes of SPE buffer (5% methanol and 0.1% acetic acid in ultrapure water). The columns were rinsed twice with SPE buffer and subjected to a vacuum (≈ 15−20 psi) for 20 min. Oxylipins were eluted with 0.5 mL of methanol and 1.5 mL ethyl acetate, dried under nitrogen, reconstituted in 100 μL methanol and filtered by centrifugation in a Ultrafree-MC-VV centrifugal filter (0.1 μm; Millipore Sigma, MA, USA). Oxylipins were quantified on an Agilent 1290 Infinity UHPLC system coupled to a 6460 triple-quadrupole tandem mass spectrometer with electrospray ionization (Agilent Corporation, Palo Alto, CA, USA), as previously described [38]. The pellet kept for fatty acid analysis was reconstituted in 0.4 mL toluene and subjected to direct transesterification with methanolic HCl, using the same protocol described above for the diet analysis [36]. Samples were analyzed by GC using the column and temperature program described above. The DHA peak co-eluted with nervonic acid, based on the retention times of the authentication standards. However, nervonic acid is negligible in brain tissue [39].

4-HNE-modified protein adducts in one hemisphere of cerebral cortex were analyzed by immunoblot using Anti-4-HNE (1:1000, Abcam, Cambridge, UK) and the intensity of bands were quantified using ImageJ. Briefly, cortex tissues were homogenized in RIPA buffer (Cell Signaling, Danvers, MA, USA) containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). For immunoblot analysis 40 μg of protein lysate was resolved on Any kD™ Mini-PROTEAN® TGX™ Precast polyacrylamide gels (Biorad, Hercules, CA, USA), transferred to nitrocellulose membrane, blocked in 5% Blotting-grade Blocker (Biorad, Hercules, CA, USA), and incubated with...
anti-4-HNE antibody (1:1000, Abcam, Cambridge, UK) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10000, Genetex, Irvine, CA, USA) for normalization, followed by incubation with peroxidase-conjugated secondary antibody (Cell Signaling, Danvers, MA, USA). Protein bands were visualized with enhanced chemiluminescence reagent and digitized using a CCD camera (ChemiDoc®, Biorad, Hercules, CA, USA). Densitometric analysis was performed with ImageJ after background subtraction and normalization to GAPDH (1:10,000, Genetex, Irvine, CA, USA).

### 2.4. Data analysis and graphical representation

Statistical analyses were performed using Stata version 13.1. Nonparametric analyses were employed due to the presence of non-normal distributions. A Kruskal–Wallis test was used for between-group comparisons, and the Dunn’s test of multiple comparisons was used to compare the high LA group and Low LA + OXLAM group to the Low LA reference group. P-values for the multiple comparisons were Sidak-adjusted. Diet-induced changes in selected oxylipins and their precursor n-3 and n-6 fatty acids were graphed using boxplots with medians and interquartile ranges.

### 3. Results

Body weight in the Low LA + OXLAM diet group was significantly lower than the other two groups (median weights in grams were 39.2 g (IQR 34.1–42.2 g), 40.3 g (IQR 33.5–42.5 g), and 30.0 g (IQR 27.5–34.2 g) for the Low LA, High LA, and Low LA + OXLAM diets, respectively; p < 0.01).

#### 3.1. Effects of dietary LA and OXLAMs on brain n-6 fatty acids

Increasing dietary LA significantly increased the abundance of LA (18:2n-6), the LA elongation product eicosadienoic acid (EDA, 20:2n-6), and the LA elongation and desaturation product arachidonic acid (AA, 20:4n-6), in both cerebellum and cerebrum (Fig. 1) (Tables S2–S5). The Low LA + OXLAM diet group had lower abundance of LA and EDA in cerebellum and decreased LA in cerebral cortex but had no effect on arachidonic acid in either tissue.

#### 3.2. Effects of dietary LA and OXLAMs on brain n-3 fatty acids

Increasing dietary LA significantly decreased the abundance of both eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6-3) in cerebellum, and decreased the abundance of EPA but had no effect on DHA in cerebrum (Fig. 2) (Tables S2–S3). The Low LA + OXLAM diet group had lower abundance of EPA in both cerebellum and cerebrum, and decreased DHA in cerebral cortex, but had no effect on DHA in cerebellum.

#### 3.3. Effects of dietary LA and OXLAMs on brain oxylipins

Increasing dietary LA significantly increased the abundance of OXLAMs, including epoxy- and hydroxy-LA derivatives, in both cerebellum (Fig. 3) and cerebral cortex (Fig. 4), but had comparatively minor effects on oxylipins derived from AA, EPA or DHA. Increasing dietary OXLAMs decreased the abundance of 12-HETE in cerebellum and cerebral cortex, and decreased TxA2 in cerebral cortex, without altering any other oxylipins (Fig. 4) (Tables S4–S5).

#### 3.4. Effects of dietary LA and OXLAMs on brain 4-HNE

4-HNE, a secondary product of fatty acid peroxidation, was not significantly altered in cerebral cortex by increases in dietary LA or dietary OXLAMs (Fig. 5).

### 4. Discussion

Per capita mean dietary LA has increased markedly in industrialized populations over the past century [1]. Current LA intakes are much higher than historical and evolutionary norms of 2–3% of total fat intake in human populations [40]. The LA in concentrated oil is susceptible to exogenous oxidation when heated or during deodorization and refinement of liquid vegetable oils, or when processed foods containing high LA oils are heated [9,41]. Because a substantial portion of the dietary LA in industrialized populations is heated prior to consumption, modern populations have substantially increased intakes of both LA and preformed LA peroxidation products. Consumption of heated oils rich in LA without adequate vitamin E [22,23], or intravenous administration of HPDEs [24], can produce cerebellar necrosis and ataxia in chicks, suggesting that dietary OXLAMs might potentially have adverse effects in CNS tissues. However, it is not yet known whether eating non-oxidized or heated vegetable oils has...
biochemical consequences in mammalian brain tissues.

Here we showed that increasing dietary LA specifically increased LA peroxidation products in cerebellum and cerebrum, without substantially affecting oxylipins derived from AA, EPA or DHA. By contrast, the consumption of preformed LA peroxidation products (OXLAMs) had minimal effect on the concentrations of brain oxylipins. Neither dietary LA nor dietary OXLAMs altered cerebral 4-HNE levels. These collective findings suggest that the altered mammalian brain lipid oxidation observed after LA feeding is driven by either endogenous enzymatic LA peroxidation, or endogenous free-radical mediated LA peroxidation, rather than by absorption of preformed LA oxidation products present in the diet. Specific LA peroxidation products that were increased by high LA diet in the brain—including HODEs, EpOMEs, and DiHOMEs—have shown diverse bioactivities in preclinical models, and have been implicated in the pathogenesis of conditions outside of the CNS that are characterized by inflammation and oxidative stress, including cardiovascular disease [3,15], non-alcoholic steatohepatitis [16,17], acute respiratory distress syndrome [42–44] asthma [45,46], and chronic pain [6,19,20], reviewed in [3,15,21]. The present finding that the body weight of the Low LA + OXLAM diet group was significantly lower than the other two groups, suggests that dietary OXLAMs could potentially have unfavorable effects on growth in mammals. However, future studies are needed to determine whether weight reducing effects of heated oils are due to decreased food intake, partial displacement of the energy provided by fatty acids with non-caloric oxidized lipids, or to other effects on metabolism.

Consistent with previous reports [8,19,47], results of the present study indicate that increasing dietary non-oxidized LA shifts the balance of brain precursor polyunsaturated fatty acids away from the n-3 family (EPA and DHA) towards the n-6 family (increases in LA, eicosadienoic acid, and AA). However, unlike LA, the observed diet-induced changes in EPA, DHA, and AA did not translate to changes in their oxylipin derivatives. Interestingly, the Low LA + OXLAMs diet appeared to significantly decrease the abundance of n-3 DHA and EPA in CNS tissues, despite containing less dietary LA than the Low LA diet alone. DHA and EPA are proposed to play critical structural and

Fig. 3. Effects of increases in dietary LA and dietary OXLAMs on cerebellar oxylipins. N = 8 tissue samples per group. Y-axis ranges differ in each graph. Box plots are based on the Tukey method. P-values with asterisks are derived from Dunn’s test of multiple comparisons using rank sums, comparing the Low LA reference group vs each of the other two groups. These P-values are also Sidak-adjusted. P-values with section signs are derived from the Kruskal-Wallis nonparametric rank-sum test across all three groups. LA, linoleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Cerebellum
functional roles in CNS tissues [48,49]. Thus, future studies are warranted to determine the effects of high intakes of thermally stressed high LA oils on developmental, behavioral, or cognitive endpoints.

Since heated oils are major source of dietary OXLAMs in modern diets, the use of heated corn oil is a strength of the present study. However, to gain a better understanding of the effects of specific components in heated oils, future studies should consider adding specific oxidation products (e.g. HpODEs) and degradation products (e.g. aldehydes) that are present in heated high LA oils to study diets. Since inclusion of oxidized oils may also alter the taste and texture of food pellets to influence food consumption, future studies should consider gavaging study oils to optimize the controlled nature of study diets. Future dietary OXLAM studies should consider using more tightly controlled diets that isolate OXLAMs as a controlled variable (keeping non-oxidized LA constant), to isolate the specific effects of individual OXLAMs.

In summary, the present study fills an important gap by establishing proof of principle in a mammalian model that diets enriched in LA and OXLAMs alter cerebral and cerebellar lipid accumulation and peroxidation. Mammalian brain OXLAM concentrations appear to be regulated by endogenous synthesis from dietary LA, rather than direct incorporation of preformed OXLAMs that are in the diet. Future studies are warranted to investigate whether and how diets enriched in LA and OXLAMs impact behavior, neurodevelopment, or neurodegeneration.

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Fig. 4. Effects of increases in dietary LA and dietary OXLAMs on cerebral cortex oxylipins.
N = 8 tissue samples per group. Y-axis scales differ in each graph. Box plots are based on the Tukey method. P-values are derived from Dunn’s test of multiple comparisons using rank sums, reporting the Low LA reference group vs each of the other two groups. P-values are Sidak adjusted. LA, linoleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.
The authors have no competing interests to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2018.07.007.

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