Effects of Prenatal and Postnatal Dietary Polyunsaturated Fatty Acids on Retinal Fatty Acid Composition and Gene Expression in Adult Rat

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Abstract

Long chain polyunsaturated fatty acids (LC-PUFA) are the major determinants of the structure and function of retina, any impairment in their maternal and dietary supply might result in the defective retinal development, structure and function. The present study is aimed to investigate the effects of different pre- and postnatal diet varying only in their dietary n-6 and n-3 fatty acid content but not in the total energy on the molecular environment of retina. Female weaning Wistar-NIN rats received a diet enriched with either linoleic acid (LA) or alpha linolenic acid (ALA) or long chain n-3 PUFA (fish oil, FO) for 90 days, and mated with stock diet fed male rats. The first generation male pups of ALA and FO group were continued with the respective diet (ALA-ALA and FO-FO). Male pups delivered by LA fed mothers were divided into three groups; continued with either mother’s diet (LA-LA) or assigned a diet enriched with either ALA (LA-ALA) or LCn-3PUFA (LA-FO). The effect of this dietary regimen on the expression of genes involved in fatty acid metabolism and phototransduction; total fatty acid composition, was analyzed in the retina after 105 days of post weaning feeding. The data show that prenatal and post weaning feeding of LCn-3PUFA enriched diet upregulated the expression of genes involved in phototransduction and fatty acid metabolism; decreased the level of arachidonic acid in retina. These results suggest that feeding LCn-3PUFA both at prenatal and postnatal could be beneficial for the molecular environment of retina.

Keywords: Maternal diet; n-6 and n-3 fatty acid; LCn-3PUFA; Rat retina; Fatty acid composition

Introduction

Long chain polyunsaturated fatty acids (LC-PUFA) are derived from shorter chain fatty acids through several desaturation and elongation reactions. The desaturase and elongase enzyme system is shared by linoleic acid 18:2n-6 (LA) and α-linolenic acid 18:3n-3 (ALA) for the formation of respective long chain products of n-6 and n-3 family. In tissues such as retina and brain, LC-PUFA represent up to 30% of total fatty acids suggesting their crucial role in the normal functioning of these specialized tissues [1-4]. Humans depend mainly on diet for the precursors of LC-PUFA such as LA and ALA. However the LC-PUFA could also be supplied directly through diet; meat products and sea foods are major source of the common LC n-6 and LC n-3 PUFA namely arachidonic acid (AA) and docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). The major LC-PUFA present in retina are DHA (22:6n-3) and AA (20:4n-6), found primarily in neural and vascular cell membrane phospholipids [5]. Highest body concentration of DHA per unit weight is found in photoreceptor outer segments of retina. Decreased availability of DHA due to metabolic abnormalities or decreased intake is associated with alterations in structure and function of visual system. Several evidences associate the higher content of DHA in retina and proper photoreceptor function [6,7]. Reports suggest that DHA-enriched cell membranes can favor rhodopsin conformational changes and function [8-10]. DHA and EPA, influence eicosanoid metabolism by regulating the metabolites of LC n-6 PUFA which are involved in the angiogenic and proinflammatory pathways [11]. Metabolites of LC-PUFA are known to function as ligands to transcription factors of many genes that are involved in cellular differentiation and growth and metabolism of lipid, protein, and carbohydrate. Thus the composition of n-6 and n-3 PUFA in the cell and subcellular membranes determine the metabolic fate of the cell by modulating growth, metabolism, expression of macromolecules and function [12-14].

Several evidences have shown that perturbation in the nutritional status of mother, particularly in utero conditions, may program the fetus for the development of diseases in the adulthood [15,16]. Recent studies suggest that nutritional imbalances during pregnancy cause perturbation of prenatal development which in turn permanently alters the structure, function and metabolism of various tissues and organs [17]. Studies also indicate that an increased intake of LC n-3 PUFA during pregnancy is directly proportional to the length of gestation and birth size [18] and beneficial in maintaining the overall metabolic status of the offspring after growth [19]. Whereas n-3 PUFA deficiency during pregnancy predisposes the offspring to metabolic abnormalities such as hypertension in adult life [20] indicating that maternal PUFA status during pregnancy is critical for the normal development of the fetus. Reports also suggest that maternal intake of saturated fatty acids (SFA) and high fat diet adversely affect the fetal health by predisposing the offspring to insulin resistance [21] and metabolic syndrome [22] respectively.

In recent years, the intake of SFA, n-6 PUFA and trans-fatty acids (TFA) has been increased whereas n-3 PUFA intake has decreased, which eventually resulted in increased ratio of n-6/n-3 PUFA in the diet. Studies on dietary fat intake among Indians showed that n-6 PUFA requirements are fully met owing to their high levels in cereal, millet and vegetable oils but n-3 PUFA intake is low and thus leading to higher n-6/n-3 ratio [23]. This change in dietary habits might be responsible for the increased prevalence of diet-related chronic diseases. In view of increasing interest in the role of LC-PUFA during...
fetal and early development, a study was conducted to understand the effects of diet varying only in the composition of the essential fatty acids during prenatal and postnatal period on metabolic programming of the offspring with respect to glucose metabolism, insulin sensitivity and tissue fatty acid profile. The dietary fats used in the study represented diets enriched with (a) 18:2n-6 (LA diet), (b) 18:3n-3 (ALA diet) and (c) LC n-3 PUFA (EPA and DHA diet). One of the findings of the study was that the maternal intake of ALA diet increased the levels of LC n-3 PUFA in the skeletal muscle of the offspring which is suggested to be beneficial in terms of increased insulin sensitivity [24]. Considering the importance of the study design and the role of LC-PUFA in the overall structure and function of the fetus and adult retina, we investigated the effect of prenatal and postnatal dietary n-6 and n-3 fatty acids on the retinal gene expression, retinal protein levels and retinal fatty acid profile in the adult rats as a part of the previous study [24].

Materials and Methods

Materials

Anti-glial fibrillary acidic protein (GFAP) antibody, anti-rabbit & anti-mouse IgG antibodies conjugated to peroxidase, TRI reagent and heptadecanoic acid were obtained from Sigma-Aldrich (St. Louis, MO). cDNA synthesis kit was procured from Invitrogen (Carlsbad, CA), IQTM SYBR Green supermix was from Bio-Rad (CA, USA), nitrocellulose membrane was of Millipore (Bedford, MA). Mouse monoclonal anti-rhodopsin antibody was from Chemicon (Temecula, CA). A chemiluminescence kit (Amersham Biosciences) was used for development and visualization of immunoreactive bands on Kodak X Omat AR Film. Authentic fatty acid standards were obtained from Nu-Chek (Elysian, MN).

Design of Animal experiment: The entire set of experiments of the present manuscript is a part of the previous study and the composition of the maternal and offspring diet, and fatty acid composition of diets were presented in detail in the previous report [24]. In brief, three week old male (n=12) and female (n=24) Wistar-NIN (WNIN) rats were obtained from National Centre for Laboratory Animals, Hyderabad, India and fed cereal-pulse based diet. While the male rats were fed on a stock colony diet, the female rats were randomly divided into three groups and fed the same stock diet enriched with either LA or ALA or LC n-3 PUFA (fish oil, FO) for 90 days (Table 1). Dietary carbohydrate provided 59% of energy while dietary protein and fat provided 20 and 21% of energy respectively.

Table 1: Feeding protocol of different groups of mothers and offspring and the amount of fatty acids added to diet along with source of these fatty acids.

<table>
<thead>
<tr>
<th>Mothers (n)</th>
<th>LA diet (12)</th>
<th>ALA diet (6)</th>
<th>LC-n3 PUFA diet (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source and amount of fatty acids</td>
<td>Safflower seed oil and palm oil in the ratio of 4:5:5:5 that provided about 4 g LA and 0.14 g of ALA/ 100 g of diet with an n-6:n3 PUFA ratio of 28</td>
<td>Groundnut oil, palm oil and linseed oil in a 3:3:4 ratio provided 3.02 g LA and 1.19 g ALA/ 100 g with a n-6:n3 PUFA ratio of 2.5</td>
<td>Groundnut oil, palm oil and fish oil (4:5:1) provided 3.79 g LA, 0.15 g ALA and 0.2 g LC n-3 PUFA/ 100 g diet with an n-6:n3 ratio of 11</td>
</tr>
<tr>
<td>Duration</td>
<td>90 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mating</td>
<td>Two females + one male**</td>
<td>ALA diet</td>
<td>LC-n3 PUFA diet</td>
</tr>
<tr>
<td>Male pups (post weaning)</td>
<td>LA diet</td>
<td>ALA diet</td>
<td>LC-n3 PUFA diet</td>
</tr>
<tr>
<td>(n)</td>
<td>(7)</td>
<td>(7)</td>
<td>(7)</td>
</tr>
<tr>
<td>Duration</td>
<td>105 days</td>
<td></td>
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</tbody>
</table>

**Female rats received a diet that is similar to that of males wherein the fat source was replaced with respective oil blends.

| Table 2: Primer sequences and the amplicon size of the respective genes. |
| GENE | PRIMER SEQUENCE | AMPICICON SIZE |
| Hprt | F | TTG GGG CTG TAC TGC TTA C | 246 bp |
| | R | CTT TGC TCA CCT GCT GGA TTA C | |
| Rhodopsin | F | CTT CCT GAT CTC TGT GCT T | 285 bp |
| | R | ACA GTG TCT GCC CAG CAC TA | |
| Gnat 1 | F | TGA CGT GCA TCA TTT TCA A | 350 bp |
| | R | TTA AGC TCG AGC ACG TGC A | |
| Pde6B | F | AGC TGC CCA CTT TGT TTA A | 350 bp |
| | R | GCC AGA GTC CAT ACC CAG T | |
| Cone opsin | F | TGA GAT TTG ATG CTA AGC TGG | 350 bp |
| | R | TGG CCG TTC ATA AAG ACA TAG | |
| Gnat 2 | F | TCA AGA CAA CAG GGA TCA AC | 350 bp |
| | R | AAG AGA AGG ATG GAC GTA GC | |
| Gfap | F | TTT CTC CAA CCT CCA GAT GC | 350 bp |
| | R | AGC TTT AGG CCC TCA CAC TG | |
| Crx | F | GGA TGT GTA TGC AGC TGA G | 350 bp |
| | R | CCA CTG CTG TGG TAG GTG AA | |
| Elov2 | F | ACT AGC GCC TGT CTC TGT C | 313 bp |
| | R | TTA TTG AGC CTT CTC GTC CG | |
| Elov4 | F | ACA TAG AAT GAG CCG AAG CAG TC | 401 bp |
| | R | CGG ATG TGA ACA GGA GAG G | |
| 3.5 Desaturase | F | AAG GCC CTT GTA GAA AAG TAT A | 388 bp |
| | R | ACA AAA TGA TGT ACC AGG TGA TT | |
| 3.6 Desaturase | F | CTA TAT CCC TTT CTA TGG CAT CTT | 373 bp |
| | R | AGA CTT CTT CAG TGA ACT CAC AAT | |
After 90 days of feeding, the female rats were mated with males (2 females + 1 male) and the pregnant rats were continued on their respective diets throughout the gestation. After delivery, the litter size of each mother was equalized to 7 male pups and the mothers were continued on the respective experimental diets. Upon weaning, the male pups of LA-fed mother were randomly split into three groups; one group was continued on the same diet (LA-LA), the other two groups were assigned to either ALA (LA-ALA) or LC-n-3 PUFA (LA-FO) diets. Male pups born to mothers fed with ALA and FO were continued on the respective diet (ALA-ALA and FO-FO). All the procedures involved in animal experiments were conducted in accordance with guidelines and approval of institutional animal ethical committee. We adhered to the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research.

At the end of the experimental period (105 days), the adult rats were sacrificed by CO2 asphyxiation. Eye balls were dissected out immediately and cut into two hemispheres to remove the lens and vitreous body. Care was taken to collect the retina intact from the concave regions of the eye ball adjacent to the optical nerve; retinas from two eyes were pooled and snap-frozen in liquid nitrogen and stored at -70°C for further analysis. Retinas of three individual animals from each group were divided into two portions and used for RNA extractions and western blotting. Retinas of the remaining four animals were pooled individually and used for the fatty acid analysis.

**RNA extraction and quantitative real-time PCR:** Total RNA was isolated from retinal tissue using TRI reagent and reverse transcription was performed with 5 µg total RNA in a 40 µl reaction volume using cdNA synthesis kit according to manufacturer's instructions. Quantitative real-time PCR was performed using 20 ng of cdNA of each sample in triplicates in a reaction mixture consisting of 10 µl of 2X IQTM SYBR Green supermix, 250 nM of each forward and reverse primers of target gene in a total reaction volume of 20 µl with a thermocycler (IQ Multicolour iCycler; Bio-Rad). The expression of genes involved in phototransduction like rhodopsin (Rho), MW-cone opsin (Opsin), rod and cone transducins (Gnat1 & Gnat2), and genes involved in fatty acid metabolism like Δ5 & Δ6 desaturases, elongases (Elov2, Elov4) were analyzed. Hypoxanthine phosphoribosyltransferase (Hgprt) was also amplified from the samples of different dietary groups as house keeping control. The primer sequences and the amplicon sizes are given in Table 2. The target gene expression values were normalized to house keeping control gene (Hgprt) according to comparative Ct-value method for relative quantification [25-27]. To confirm the specificity of amplified products, the PCR products were subjected to melting curve analysis.

**Protein expression by immunodetection:** Retina of individual animal was homogenized in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate and 0.1% triton-X-100. The homogenate was centrifuged at 8000 rpm for 10 minutes and supernatant was used as total retinal lysate. Equal amounts of protein from retinal lysate was separated on 10% SDS–polyacrylamide gel electrophoresis using petroleum ether. FAME were analyzed by gas chromatography using a capillary column (30 m x 0.2 µm film thickness, 0.25 mm internal diameter). Individual fatty acids were identified using authentic standards. Heptadecanoic acid (17:0) was used as internal standard [24].

**Statistical analysis:** Statistical analysis was done using the SPSS statistical program (SPSS, Inc, Chicago, IL) and the data were presented as mean along with their standard errors. Data were evaluated by One-way ANOVA and ANCOVA by following least significant difference (LSD) post hoc tests. Log transformation of the data was done before One-way ANOVA and ANCOVA to stabilize variations. Similar results were observed in regression analysis also after controlling for other groups. Differences were considered significant at p<0.05.

**Results**

**Gene expression studies**

The relative fold expression of candidate genes in different dietary fatty acids at pre and postnatal stages. The relative mRNA abundance was normalized to Hgprt and presented in arbitrary units. **Panel A:** Rhodopsin & rod transducin (Gnat1); **Panel B:** Cone transducin (Gnat2), cone opsin medium wavelength (Opsin) & glial fibrillary acidic protein (GFAP). The values are presented as mean ± SE of six independent observations. Different superscripts on the bars indicate values are statistically significantly different from other groups (p<0.05).

**Figure 1:** Relative expression of retinal genes in adult WNIN rats fed varying dietary fatty acids at pre and postnatal stages. The relative mRNA abundance was normalized to Hgprt and presented in arbitrary units. **Panel A:** Rhodopsin & rod transducin (Gnat1); **Panel B:** Cone transducin (Gnat2), cone opsin medium wavelength (Opsin) & glial fibrillary acidic protein (GFAP). The values are presented as mean ± SE of six independent observations. Different superscripts on the bars indicate values are statistically significantly different from other groups (p<0.05).
experimental groups was determined considering the expression of Hgprt as internal control which is unaltered due to different dietary treatment. Mean relative expression levels were calculated from three independent samples as fold change over Hgprt and presented as arbitrary units. The LA-LA wherein the mother and offspring met the n-6 PUFA requirement fully but not the n-3 PUFA [18] was taken as reference group for comparing the effects of other groups enriched with different dietary PUFA. In general, feeding LC n-3 PUFA enriched diet in the form of fish oil during pregnancy and post weaning period (FO-FO) significantly upregulated the expression of a number of genes in the retina. For example, the rod specific genes namely Rhodopsin and Gnat1; cone cell marker gene Cone Opsin were upregulated by 1.8 folds, 2.5 folds and 2.3 folds respectively in FO-FO group animals when compared to LA-ALA animals (Figure 1A & 1B). Feeding LC n-3 PUFA throughout (pre and postnatal period, FO-FO) upregulated the genes involved in elongation of fatty acids >C22 (Elovl2, 5 folds) and very long chain fatty acids >C26 (Elovl4, 3.3 folds). While the increased expression of Elovl2 is statistically not significant, increase in Elovl4 is statistically significant. The Elovl4 gene is upregulated to the same extent even in the offspring of LA animals switched to post weaning


Figure 1: Retinal fatty acid composition

Figure 2: Relative expression of retinal genes in adult WNIN rats fed varying dietary fatty acids at pre and postnatal stages. The relative mRNA abundance was normalized to Hgprt and presented in arbitrary units. Panel A: Cone-rod homeobox gene (Crx) & phosphodiesterase 6B (Pde6B); Panel B: Elongases (Elovl4 & Elovl2); Panel C: Δ5- & Δ6-desaturases. The values are presented as mean ± SE of six independent observations. Different superscripts on the bars indicate values are statistically significantly different from other groups (p< 0.05).

Figure 3: Immunodetection of glial fibrillary acidic protein (GFAP) & rhodopsin in the retina of WNIN rats fed with different dietary fatty acids. The abundance of individual protein were quantified, normalized with the levels of β-actin and presented in arbitrary units considering respective protein expression in PD-Ia group as one unit. Panel A: Representative immunoblot of rhodopsin, GFAP and β-actin; Panel B: The values are presented as mean ± SE of three independent observations. Different superscripts on the bars indicate values are statistically significantly different from other groups (p< 0.05).

Figure 4: Protein expression

Figure 5: Retinal fatty acid composition

The data on retinal fatty acid composition showed no gross variations except for few notable changes. The percentage of saturated fatty acids is similar among all the groups. However, the level of AA (C20:4) is significantly (~17% of total AA) decreased in the FO-FO adult rats when compared to LA-LA animals (Table 3). This decrease
in the AA levels in the FO-FO fed animals reflected in almost ∼16% decrease in the total LA + AA levels in the retinal lipids. Further there was an increased ratio (1.32 fold) of total LC-n-3 PUFA (DPA + DHA) to LA + AA (biologically active n-6 PUFA) in the FO-FO fed animals (Table 3).

**Discussion**

Humans depend on diet as a major source for the LA and ALA, which are further elongated to LC-PUFA of respective fatty acid classes metabolically. Both LA and ALA and their LC-PUFA derivatives are required during normal fetal development to support the synthesis of structural lipids of brain and retinal tissue [28]. Diets in developed and developing countries are generally abundant of n-6 PUFA but poor in n-3 PUFA. Several evidences suggest that humans evolved on a diet with n-6/n-3 ratio of ∼1 whereas now this ratio is about 15 in many parts of world including India [29-31]. The dietary ratio of n-6/n-3 is potentially important because the ratios of these fatty acids in the tissues are determined largely by their ratios in the diet.[32,33] The excessive amount of n-6 PUFA and a very high n-6/n-3 ratio is reported to promote the pathogenesis of many diseases including cardiovascular,[ 34] cancer, [35] inflammatory and autoimmune diseases, whereas increased levels of n-3 PUFA (or a low n-6/n-3 ratio) is suggested to be beneficial [36-38]. Therefore, the present study was aimed to investigate the effect of maternal and post weaning (i.e. pre and postnatal) supply of diet enriched with (a) n-6 PUFA (LA) (b) n-3 PUFA (ALA) and (c) LC n-3 PUFA (EPA + DHA) with varying n-6/n-3 ratio on gene expression and fatty acid composition of adult rat retina.

Expression of many of the retinal genes tested that are critical for the retinal structure and functions are upregulated by pre and postnatal feeding of LC n-3 PUFA. The rod specific genes such as Rho, Gnat1, cone specific gene such as Opsin, the genes involved in the fatty acid conservation mechanism of retina. Studies demonstrated that n-3 PUFA and the increase was also prominent in the other dietary groups when compared to LA-LA. Studies indicate that n-3 PUFA deficiency may cause decreased rhodopsin expression and thereby decrease the retinal sensitivity to light [39]. However, in the present study we demonstrate that by increasing the n-3 PUFA levels in the diet through ALA and LC n-3 PUFA, the rhodopsin expression could be increased. However, expression of a stress response protein, GFAP which is usually upregulated during pathological conditions such as in retinal atrophy, was not altered by different dietary fatty acid regimen.

The direct effects of dietary ALA on insulin sensitivity and increasing the levels of EPA and DHA in non-neural tissues has been shown earlier [40]. Our previous study with the same design also showed that maternal supplementation of n-3 PUFA through ALA and FO could improve the n-3 PUFA status in muscle phospholipids [24]. However, in the present study the maternal and post natal feeding of LA and ALA rich diet did not really influence either the levels of total SFA, total n-3 PUFA or DHA. Despite the higher amount of LA in the diet the amount of 18:2n-6 in the retina of LA-LA diet fed animals did not reflect the dietary levels. Moreover, ALA is the direct precursor for the LC n-3 PUFA in retina and the levels of which were also not altered in the retina in respective groups (LA-ALA and ALA-ALA) suggesting that the retina has a specific and robust mechanism to preserve and maintain the fatty acids profile even under the extreme conditions of deficiency [41].

Studies indicate that decreasing the levels of AA, which is the precursor for the inflammatory metabolites, could alleviate the harmful effects of neuro-inflammation and other age-related neural degeneration [42,43]. However, studies reporting a decrease in AA levels in retinal tissues are limited which can be attributed to unique fatty acid conservation mechanism of retina. Studies demonstrated that adequate n-3 PUFA in the diet could marginally (approximately 15%) decrease the levels of AA in F2 generation rats [44]. In the present study the intake of LC n-3 PUFA rich diet resulted in 17% decrease in the total AA levels in the retinal tissues of the animals of the F1 generation which is statistically significant when compared to the other groups. However, it is important to note that postnatal feeding of LC n-3 PUFA or ALA to the offspring born to LA fed mothers does not seem to be effective in decreasing the levels of AA. This observation in turn clearly suggests that both prenatal and postnatal feeding of LC n-3 PUFA might be needed to achieve a better n-6/n-3 ratio. Further it is also
interesting to note that LC n-3 PUFA fed animals showed significant decrease in the levels of AA in retina which in turn reflected a higher ratio of total LC n-3 PUFA (DPA + DHA) / biologically active n-6 PUFA (LA +AA). This ratio in FO-FO fed animals is 2:6 compared to LA-LA fed animals where the ratio is 2:0, further indicates that higher n-3/n-6 ratio would be beneficial to retinal structure and function. The present data is limited for the detection of fatty acid with the chain length up to C22. Therefore, it is not clear whether the up regulation of Elolv4 & Elolv2 reflected in the retinal fatty acides of chain length >C22, since these elongates act on fatty acides with chain length >C22.

In conclusion, the present study showed that feeding n-3 LC-PUFA is beneficial in terms of retinal gene expression and fatty acid composition only when it is supplied throughout pregnancy, lactation and post weaning period.

Acknowledgements

Acknowledgements

References


