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Metabolic conversion of intra-amniotically-injected deuterium-labeled essential fatty acids by fetal rats following maternal n-3 fatty acid deficiency

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ABSTRACT

Accumulation of polyunsaturated fatty acids (PUFA) in the fetal brain is accomplished predominantly via a highly selective flow of docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n-6, AA) through the placenta. Little is known regarding the endogenous capability of the fetus to generate its own DHA and AA from lower homologues such as linolenic (18:3n-3, ALA) and linoleic (18:2n-6, LA) acids, respectively. Deuterium-labeled d5-ALA and d5-LA at millimolar concentrations were injected directly into the amniotic fluid in order to investigate maternal-independent metabolic conversion of the stable isotopes in brain and liver of the fetus near delivery. After 48 h under adequate maternal diet, the levels of d5-ALA metabolites in the fetal brain and fetal liver were 45 ± 2.2 pmol/mg and 86 ± 4 pmol/mg of which 79% and 63.6% were comprised of d5-DHA. At this time point, incorporation of d5-LA metabolites was 103 ± 5 pmol/mg and 772 ± 46 pmol/mg for brain and liver, of which 50% and 30% were comprised of d5-AA. Following sustained maternal dietary ALA deficiency, the levels of total d5-ALA derived metabolites in the fetal brain and fetal liver were increased to 231 pmol/mg and 696 pmol/mg of which 71% and 26% were comprised of d5-DHA. From the time course and relative rates of d5-ALA precursor displacement by d5-DHA in cellular phosphoglycerides, it is concluded that the fetal rat brain can generate its own DHA from its d5-ALA precursors particularly under dietary stress.

1. Introduction

The selective accumulation of long chain polyunsaturated fatty acids (LC PUFA) in the fetal brain during the late gestation period and through early phases of postnatal life has been investigated both in humans [12,14,28,31,51] and animal models [6,7,21,45,46,48,53]. Evidently, the almost complete dependency of the fetus on maternal supplies of LC PUFA via the placenta [24,32,40] a process which has been termed biomagnification [13], suggests a rigorous regulation to support the requirements of these compounds for fetal growth in general, and for brain ontogeny, in particular [23,29,33].

At discrete periods during intrauterine life, docosahexaenoic acid (22:6n-3, DHA), a major LC PUFA constituent in the brain, may even surpass accumulation of arachidonic acid (20:4n-6, AA), the other major LC PUFA metabolite [21,22,35]. DHA accretion has been strongly correlated with acquisition of function in photoreceptor cells [3] and has been shown to play an essential role in regulation of neurogenesis in general and in synaptogenesis, in particular [2,10,16,29,44,52].

In addition to maternal supplies of ALA and its higher metabolite DHA, the capacity of fetal organs to synthesize the former into the latter has been a debatable issue particularly because of ineffective converting enzymes around birth both in the placenta and in the fetal organs [11,26,49,50]. Nevertheless, tracer amounts of radioactively labeled ALA and LA injected via the intracranial route into near term rat fetuses were rapidly desaturated, elongated and ultimately esterified into brain neutral lipids and phosphoglycerides (PG) indicating an effective metabolic capacity of the brain to generate DHA and AA [19]. ALA conversion during the perinatal period was also documented *in vivo* in other animal models [4,45,48] as well as in cell cultures derived from endothelial and astrocytic origins [5,36,54].

To evaluate the extent of endogenous conversion of ALA and LA by the fetus, we have now injected relatively large amounts, in the millimolar range, of the deuterium-labelled stable isotope precursors d5-ALA and d5-LA, into the amniotic compartment and investigated the kinetics of appearance of deuterium-labeled

Abbreviations: FA, fatty acid; PUFA, polyunsaturated fatty acid; AA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; DTA, docosatetraenoic acid; LA, linoleic acid; ALA, alpha-linolenic acid; CPG, choline phosphoglyceride; EPG, ethanolamine phosphoglyceride; SPG, serine phosphoglyceride; IPG, inositol phosphoglyceride; PG, phosphoglyceride; D5, deuterated

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metabolites in fetal brain and liver PG. The latter has been selected amongst other rat organs for being a major site of PUFA biosynthesis [42].

In addition, the ability to simulate n-3 PUFA dietary deficiency in the course of gestational life in the rat, was also instrumental in evaluating the contribution of these stable isotope precursors on the overall content of fetal brain and liver PUFA. When n-3 PUFA dietary deficiency was imposed on the pregnant dam, a substantial decrease of DHA was noticed over a limited time span in the developing fetal brain [46]. The n-3 PUFA dietary deficiency is a reversible process which can be rapidly rectified during the perinatal life upon suitable supplements of DHA and ALA [20,27].

The current report provides compelling evidence that a significant portion of PUFA can be generated by the fetal metabolic machinery involving independently both brain and liver tissue. The alternative route to placental transport of essential fatty acids should lead to a better understanding of fetal development during adverse conditions such as placental insufficiency.

2. Materials and methods

2.1. Maternal dietary manipulation

On the second day after conception, and until delivery, Sprague-Dawley dams were subjected to a custom diet based on the AIN-93G [43] containing either n-3 adequate or n-3 deficient FA mixtures, prepared by Dyets Inc (Bethlehem, PA). The diet was modified with respect to its fat components so as to yield a distribution of 77–81% saturated and 4% monounsaturated FA, 15% of 18:2n-6 and 3% of 18:3n-3 for the n-3 adequate diet, or 15% of 18:2n-6 and 0.05% 18:3n-3 for the n-3 deficient diet; no long chain (C20 and C22) FA were added to the fat mixture. The diets were practically the same as reported elsewhere [37]. The fat supplement comprised of 10% (by weight) and the FA composition of the various diets is given in Table 1.

Table 1
Nutrient composition of experimental diets.[a]

Ingredient[b]		Amount (g/kg of diet)
Protein (20%)		
Casein (vitamin free)		200
Carbohydrate (60%)		
Cornstarch		150
Dextrose		199.5
Maltose-dextrin		150
Sucrose		100
Others (10%)		
Cellulose		50
Mineral & Salt Mix		35
Vitamin Mix		10
L-Cystine		3
Choline Bitartrate		2.5
TBHQ		0.02
Fat (10%)		100
	n-3 Adq Diet	n-3 Def Diet
HCO	77.5	81
Safflower Oil	17.7w	19
Flaxseed Oil	4.8	-
Fatty acid composition (wt% of total fatty acids)		
Saturates	77.2	80.85
Monounsaturates	4.3	3.92
18:2n-6	15.3	15.13
18:3n-3	3.12	0.04

[a] The experimental diets were a modification of the AIN-93G.
[b] TBHQ, *tert*-butylhydroquinone. HCO, hydrogenated coconut oil.

Rats were regularly housed with a maximum of three to a cage, and food pellets fed ad libitum and changed every second day. Dams developed macroscopically indistinguishable from each other and showed no particular differences with respect to number of fetuses, body weight, viability or time of delivery.

2.2. Surgery and stable isotope administration

All animal procedures were carried out in accordance with the National Institutes of Health animal care and welfare guidelines, and the protocol was approved by the NIAAA Animal Care and Use Committee. The animal holding facility had a 12 h (7 AM to 7 PM) light cycle, a temperature of 22 °C and a relative humidity of about 55%. Pregnant Sprague-Dawley female rats (Taconic, Germantown, NY; approx 250 g body weight) at 18 days gestation (21 days term) were anesthetized by isoflurane (Baxter Healthcare Corp., Deerfield, IL) according to NIH guidelines for survival rodent surgery. An abdominal midline incision was performed and the two uterine horns were exposed and kept moist throughout the surgery. A mixture of 2.3 mg each of d5-LA and d5-ALA (5–6 μl total delivery vol) was injected into the amniotic cavity (approx 0.8 ml internal volume) of each of 3–4 embryos aligned on each side of the horn using a syringe (29 gauge from Hamilton, Reno, NV). Isotopes were purchased from Cambridge Isotope Laboratories (Andover, MA). After injection, uteri were returned to the abdominal cavity and peritoneal and skin incisions closed with appropriate surgical sutures. By 10 min following surgery, all dams were awake and able to move in the cage. At designated time intervals including 1, 8, 24, 48 and 72 h after surgery, dams maintained on their designated diets, were killed by cervical dislocation, the fetuses delivered and promptly killed. Fetal brain and liver tissues were immediately collected, frozen on dry ice and stored at −80 °C until analysis.

2.3. Total lipid extraction

Frozen whole brain and liver tissues from individual fetuses were weighted and homogenized frozen in the presence of 7 vol of hexane: isopropanol (3:2 by vol, [25]) containing 5 mg% of butylated hydroxytoluene and 3 μg of 22:3n-3 ethyl ester as internal standard using a Polytron homogenizer. The organic layer containing the lipid extract was separated from the residual tissue by low speed centrifugation and stored at −80 °C until further processing.

2.4. Separation and isolation of lipid classes

Unless otherwise stated, equal volumes of lipid extracts from fetuses injected in one horn were pooled and assigned as single experimental points for statistical purpose and per each dam two such pooled extracts were obtained. Combined total lipid extract aliquots (0.2–0.25 ml) were separated by thin-layer chromatography (TLC) on silica gel G plates (Analtech, Inc Newark, DE). Major phosphoglyceride (PG) including choline-(CPG), ethanolamine-(EPG), serine-(SPG), and inositol-(IPG) classes were separated in one direction using a mixture of chloroform/methanol/40% methylamine (130/70/30 by vol). Spots were visualized by spraying with 50 μg% dichlorofluorescein in ethanol and the stained PG bands scraped off into Kimax tubes for direct transmethylation.

2.5. Non-labeled fatty acid analysis by gas chromatography (GC)

Each PG band scraped from the TLC plate was collected and dissolved in 1 ml of 14% BF_3-methanol (Alltech, Deerfield, IL) and 0.5 ml of hexane containing a known amount of 22:3n-3 ethyl ester as internal standard for quantitative estimation of FA in each individual phospholipid obtained after TLC separation. Test tubes were tightly sealed under nitrogen and incubated for 1 h at 105 °C. After cooling, 1 ml of water was added followed by 2.5 ml hexane. After vigorous mixing, tubes were centrifuged for 5 min at 1700x g and the upper layer