Protein restriction during pregnancy affects maternal liver lipid metabolism and fetal brain lipid composition in the rat

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Submitted 13 July 2009; accepted in final form 11 November 2009


First published November 17, 2009; doi:10.1152/ajpendo.00437.2009.—Suboptimal developmental environments program offspring to lifelong metabolic problems. The aim of this study was to determine the impact of protein restriction in pregnancy on maternal liver lipid metabolism at 19 days of gestation (dG) and its effect on fetal brain development. Control (C) and restricted (R) mothers were fed with isocaloric diets containing 20 and 10% of casein. At 19 dG, maternal blood and livers and fetal livers and brains were collected. Serum insulin and leptin levels were significantly higher than that observed in C rats (P < 0.01). In addition, protein restriction significantly reduced gene expression in maternal liver of desaturases and elongases. In fetus from R mothers, a low body weight was observed (C = 0.2 ± 0.04 vs. R = 0.9 ± 0.16 g, P < 0.01) and was inversely related to early growth retardation. Serum leptin in R mothers was significantly higher than that observed in C rats (C = 5 ± 0.1 vs. R = 7 ± 0.7 ng/ml, P < 0.05). In addition, protein restriction significantly reduced gene expression in maternal liver of desaturases and elongases and the concentration of arachidonic (AA) and docosahexaenoic (DHA) acids. In fetus from R mothers, a low body weight (C = 3 ± 0.3 vs. R = 2 ± 0.1 g, P < 0.05), as well as liver and brain lipids, including the content of DHA in the brain, was reduced. This study showed that protein restriction during pregnancy may negatively impact normal fetal brain development by changes in maternal lipid metabolism.

HUMAN EPIDEMIOLOGICAL (28, 31) and experimental animal studies (16, 23, 25) have shown that a suboptimal environment either in the womb or early in the neonatal life alters growth and predisposes individuals to lifelong health problems. Maternal dietary deficiencies in pregnancy result in multiple adverse outcomes in the offspring (3, 12). Fetal growth depends mostly on the amount and type of nutrients obtained from the mother. Therefore, the mother must adapt her metabolism to support this continuous draining of substrates. The effects of an altered intrauterine environment can be passed transgenerationally by epigenetic mechanisms involving changes in gene expression (41). During late gestation, maternal liver plays a central role in whole body lipid metabolism. Maternal triglycerides (TG) are not transported intact across the placenta, whereas free fatty acids (FFAs), including long-chain polyunsaturated fatty acids (LC-PUFAs), can be transported (32). Therefore, a deficient maternal FA intake, particularly essential FAs (EFAs), may have important consequences on fetal maturation and postnatal development.

Arachidonic acid (AA) and docosahexaenoic acid (DHA) constitute the major LC-PUFAs in brain tissue and are important structural components of the central nervous system. Low content of AA and DHA is associated with abnormal prenatal and postnatal development of retina and brain (24). These FAs transferred across the placenta are accumulated in brain during fetal development (34). AA and DHA are formed from the dietary linoleic acid (LA) and α-linolenic acid (LNA), respectively, by a series of alternating desaturation and elongation reactions to form LC-PUFAs (37). Maternal liver is probably the main source for fetal brain LC-PUFAs, since no measurable activity of fetal liver and placenta desaturases has been found (30). In the rat, downregulation of Δ6 desaturase (Δ6D) in the liver of maternal protein deficiency during pregnancy has been reported (9), and a reduction in maternal dietary protein intake in pregnancy resulted in a lower concentration of DHA in maternal liver and plasma and impaired accumulation of DHA into fetal brain phospholipids (5). In the sheep, maternal nutrient restriction (50% of regular food intake) from early to midgestation modifies the profiles of LC-PUFAs in fetal tissues (43). However, to our knowledge, little attention has been paid to studying the effects of nutrient restriction on maternal liver lipid metabolism during pregnancy. We hypothesized that maternal dietary protein restriction in the absence of any change, either qualitative or quantitative, in the fat content of the diet would lead to lowered DHA and AA concentrations in the maternal liver, the key site of LC-PUFA synthesis. Since the fetus has a low ability to synthesize these essential fats, we further hypothesized that fetal brain concentrations would also be reduced. Although brain development spans the fetal and neonatal period in both altricial species such as rodents and precocial species such as man, early development is a period marked by myelination in critical areas of the brain such as the cortex.
METHODS

Care and Maintenance of Animals

All procedures were approved by the Animal Experimentation Ethics Committee of the Instituto Nacional de Ciencias Medicas y Nutricion, Salvador Zubiran (INNSZ). Details of maternal diet, breeding, and management of the experimental groups of offspring have been published in detail (42). Briefly, mothers were virgin female albino Wistar rats aged 11 ± 1 wk and weighing 240 ± 20 g, obtained from the INNSZ. Female rats with regular cycles were maintained on Purina 5001 rodent diet and under controlled lighting (lights on from 0700 to 1900 at 22–23°C). Eighteen female rats were mated overnight with proven male breeders, and the day on which spermatozoa were present in a vaginal smear was designated as the day of conception (day 0). Only rats that were pregnant within 5 days of introduction of the male were retained in the study. Pregnant rats were transferred to individual cages and allocated at random to one of two groups to be fed either a 20% casein [control diet (C); n = 7] or 10% casein isocaloric diet [restricted diet (R); n = 6] (42). Rats were weighed daily and during the study had free access to the experimental diet and water. Food was provided in the form of large, flat biscuits that were retained behind a grill through which the rats nibbled the food. The amount of food provided each day was weighed, as was the amount remaining after 24 h. Food intake was also measured in six female age-matched nonpregnant rats. At 19 days of gestation (dG) (19 was present in a vaginal smear was designated as the day of conception (day 0)), pregnant rats were rapidly euthanized by decapitation by 10.2 ± 0.3 mL/kg. The male were retained in the study. Pregnant rats were transferred to the male rats. Fetal brain and liver were isolated, weighed, and pooled per litter and immediately frozen in liquid nitrogen for further determination. The need to pool tissues from all fetuses in the litter precluded the ability to analyze outcomes according to offspring sex.

Biochemical Analyses

Carcass components. After being weighed (wet weight), each carcass was chopped into small pieces, placed in a tared beaker, and dried at 60°C to constant weight. The weight lost is considered to be body water. The dried carcasses were ground up, and aliquots were taken for lipid determination by the Soxhlet method (1). Leptin radioimmunoassay. Maternal serum leptin concentration was determined by radioimmunoassay (RIA) using a commercial rat kit from Linco Research (St. Charles, MO), cat. no. RL-83K, with detection limit 0.5 ng/mL, using 100-μL samples. Each serum sample was assayed in duplicate. The intra- and interassay coefficients of variations were <4 and <5%, respectively.

InulinRIA. Serum insulin concentration was determined by RIA using commercial rat kits from Linco Research, cat. no. RI-13K. Each serum sample was determined in duplicate. The intra- and interassay coefficients of variations were <4 and <6%, respectively.

Blood glucose measurement. Serum glucose concentration was determined spectrophotometrically using the enzymatic hexokinase method (Beckman Coulter, Fullerton, CA). Intra- and interassay coefficients of variations were <2 and <3%, respectively.

Lipid measurements. Serum TG, HDL, LDL, VLDL, and cholesterol were determined enzymatically with the Synchron CX auto analyzer (Beckman Coulter).

FA Analysis

Total lipids were extracted from maternal liver by Soxhlet method (1) and fetus brain and liver according to the method used by Bolch et al. (13), with some modifications. This sample was homogenized with 500 μL of 0.9% NaCl and 1 mL of chloroform-methanol (2:1). After that, extraction of FAs was carried out with the addition of chloroform (3 × 2 mL). The organic phase was pooled, and 120–150 μL of methanol was added until organic phase turned transparent, and then 1 g of Na2SO4 was added and vortexed. The organic phase was transferred in a new tube and evaporated under a stream of nitrogen.

Preparation of FA Methyl Esters

Two milliliters of methanol, 100 μL of toluene, and 40 μL of 2% methanolic sulfuric acid were added to the above residue and heated at 90°C for 2 h. After that, the tubes were placed on ice, and 1 mL of 5% NaCl was added. FA methyl esters (FAME) were extracted with

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<th>Table 2. Maternal biochemical parameters at 19 dG in rats fed control (20% protein) or restricted (10% protein) diet during pregnancy</th>
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<tr>
<td>Glucose, mg/dl</td>
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<td>Insulin, ng/mL</td>
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Results are means ± SE. *P < 0.05; †P < 0.01 vs. control.

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<th>Table 3. %Fatty acids in the maternal liver at 19 dG in rats fed control (20% protein) or restricted (10% protein) diet during pregnancy</th>
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<tr>
<td>C12:0 Lauric</td>
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<td>C14:0 Myristic</td>
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<td>C16:0 Palmitic</td>
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<td>C16:1 Palmitoleic</td>
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<td>C18:0 Stearic</td>
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<td>C18:1 Oleic</td>
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<td>C18:2 Linoleic</td>
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<td>C20:3 Arachidic</td>
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<td>C20:4 Arachidonic</td>
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<td>C20:5 Eicosapentaenoic</td>
</tr>
<tr>
<td>C22:6 Docosahexaenoic</td>
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Results are means ± SE; n = 5. ND, not detectable. *P < 0.05 vs. control.
hexane (3 × 2 ml), and mixture was centrifuged at 1,500 g for 1 min. The organic phase was pooled and evaporated under stream of nitrogen. Two hundred microliters of hexane was added to the dark-colored residue and then centrifuged at 1,500 g for 5 min. The clear solution was injected in the chromatograph. The FA analysis was carried out in an Agilent model 6850 gas chromatograph equipped with a flame ionization detector, and automatic split injection was carried out using an Agilent 6850 autosampler. The chromatographic column was an HP-INNOWax capillary column (30 m, 0.25 mm, 0.25 μm) (J & W Scientific). One hundred twenty-five micrograms of heptadecanoic acid as internal standard was added to 100 mg of tissue. A sample of 1 μl was injected in split mode (50:1) at 250°C. The carrier gas was helium with a constant linear velocity of 24 cm/s, and the interface temperature was kept at 280°C. The oven temperature was raised from 50 to 230°C (33). Identification of the FAME was based upon retention times obtained for methyl ester standards from Poly Science, and each one was expressed as percentage of total FA in the sample.

Isolation of Total RNA and Northern Blot Analysis

Total RNA was isolated from maternal liver of rats at 19 dG using the method of Chomczynski and Sacchi (8). Fifteen micrograms of RNA was electrophoresed in a 1% agarose gel containing 37% formaldehyde, transferred to a nylon membrane filter (Hybond-N Plus), and cross-linked with an ultraviolet cross-linker (Amersham). RNA integrity and location of the 28S and 18S ribosomal RNA bands were determined under ultraviolet light.

Analysis of mRNA Expression by Real-time Quantitative RT-PCR

For mRNA expression of stearoyl-CoA desaturase-1 (SCD-1), ΔSD and Δ6D by real-time RT-PCR, 300 ng of total RNA was subjected to reverse transcription and then amplified by PCR using Taqman Universal Master Mix (Applied Biosystems). Parallel nontemplate
control reactions were run in the absence of RNA to assess the degree on any nucleic acid contamination in the reaction mixture. TaqMan fluorogenic probes and oligonucleotide primers were obtained from Applied Biosystems. TaqMan PCR assays for each target gene were carried out by triplicate in 96-well optical plates with the ABI prism 7000 sequence detection system (PerkinElmer Applied Biosystems). PCR was performed using 1.4 μl of cDNA, 0.6 μl of TaqMan assay mix containing 200 nM sense and antisense primers and 100 nM TaqMan fluorogenic probe, 6 μl of TaqMan Universal PCR Master Mix, and 4 μl of H2O. The protocol used for PCR amplifications was as follows: one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and at 60°C for 1 min. The relative amount of each mRNA was calculated by using the comparative threshold cycle method (user bulletin no. 2; PerkinElmer Applied Biosystems). The probes and primers for rat genes were obtained from PerkinElmer Applied Biosystems (predeveloped TaqMan assay reagent control kits). The probes and primers for rat genes were obtained from PE Applied Biosystems (predeveloped TaqMan Assay Reagent Control kits). The assay numbers for each gene were Rn00594894-g1 (SCD-1), Rn00584915_m1 (Δ5D), and Rn00580220_m1 (Δ6D). Amplification was then performed by 45 cycles at 95°C for 15 s and at 60°C for 60 s. The cDNA quantity in each sample was normalized with the 18S. Real-time PCR was carried out in triplicate for each sample.

For elongase-2 and -5 (Elovl-2 and -5) by RT-PCR, relative mRNA levels of target genes and invariant transcript β-actin were determined using cDNA preparation for tissues and HC11 cells. Synthesized cDNA was mixed with LightCycler Fast Start DNA MasterPLUS SYBR Green I (Roche, Indianapolis, IN) and with various sets of gene-specific forward and reverse primers as follows and subjected to real-time PCR quantification using the Light Cycler 3.5 detection System (Roche): for Elovl-2, forward GGA AGA AAT ACC TCA CGC AG and reverse TGG CTT TTT TCG GTA TGT C; for Elovl-5, forward TCT AAC CTG CTG TCT CTC TA and reverse ATC TGG TGG TTG TTC TTA CG; for β-actin, forward TGG AAT CCT GTG

Fig. 3. Northern blot analysis of Δ5D and Δ6D (A) and relative abundance or mRNA/ribosomal 28S of maternal liver (B) at 19 dG of rats fed C (20% protein) or R (10% protein) diet during pregnancy. Means ± SE; n = 4.

Fig. 4. Maternal liver Δ5D gene expression as a function of serum insulin at 19 dG of rats fed C (20% protein) or R (10% protein) diet during pregnancy. Pearson correlation, P = 0.008 and r = -0.78.
Table 4. Fetal and placenta characteristics at 19 dG in rats fed control (20% protein) or restricted (10% protein) diet during pregnancy

<table>
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<tr>
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<th>Control (n = 7)</th>
<th>Restricted (n = 6)</th>
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<tr>
<td>No. of fetuses/dam</td>
<td>10.3 ± 0.9</td>
<td>9.2 ± 0.6</td>
</tr>
<tr>
<td>Placenta weight, g</td>
<td>0.42 ± 0.02</td>
<td>0.40 ± 0.02</td>
</tr>
<tr>
<td>Placenta diameter, mm</td>
<td>0.99 ± 0.05</td>
<td>0.96 ± 0.06</td>
</tr>
<tr>
<td>Fetal weight, g</td>
<td>3.1 ± 0.3</td>
<td>2.0 ± 0.1*</td>
</tr>
<tr>
<td>Liver weight, mg</td>
<td>175 ± 19</td>
<td>140 ± 3*</td>
</tr>
<tr>
<td>Liver lipids, mg/100 mg</td>
<td>35.8 ± 1.8</td>
<td>18.7 ± 2.7*</td>
</tr>
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Results are means ± SE. Fetuses or placentas were averaged per litter. *P < 0.05; †P < 0.001 vs. control.

GCA TCC ATG and reverse TAA AAC GCA GCT CAG TAA CAG. PCR amplification was performed in a total volume of 20 μl containing 2 μl of the cDNA sample, 40 pmol of each primer, and 4 μl of Master™ SYBR Green. For each reaction, the polymerase was activated by preincubation at 95°C for 10 min. Amplification was then performed by 35 cycles at 95°C for 10 s and at 60°C or 62°C for 7 s. All reactions were performed in triplicate. Relative amounts of mRNA were calculated using the comparative threshold cycle method (cycle method, LightCycler software version 4.0; Roche). All results were normalized to the housekeeping gene abundance of β-actin mRNA.

Statistical Analysis

All data are presented as means ± SE. Fetal body weight and liver and brain weights, as well as placenta diameter and weight, are the average of the whole litter. Statistical analysis was performed using unpaired Student’s t-test to compare C and R mothers. Real time RT-PCR results (mRNA gene/18S or β-actin normalized ratio) are expressed relative to C group. Correlations for maternal serum insulin and Δ5D liver gene expression and maternal serum insulin and fetal weight were calculated using a Pearson correlation. P < 0.05 was considered significant.

RESULTS

Maternal Parameters at 19 dG

At the beginning of pregnancy, both groups of pregnant rats, C and R, increased their food intake (C = 38.7 ± 3.0, R = 42.9 ± 2.0 g/day) compared with age-matched nonpregnant rats (22.8 ± 1.03 g/day). Food intake was rising during pregnancy, and there were no differences between C and R groups except at 19 dG, in which both groups decreased their food intake, and this drop was more evident in the R group (C = 18.9 ± 0.7, R = 16.4 ± 0.8 g/day, P < 0.05). Table 1 shows that, at 19 dG, rats fed a low-protein diet have similar body weights compared with C pregnant rats. However, the R group showed a significant decrease in fat body composition. In both groups, the liver weights were similar; however, a significant decrease in maternal liver lipids was observed in the R group.

Biochemical Maternal Serum Parameters at 19 dG in Rats Fed Protein-Restricted Diet

There were no differences in glucose, TG, HDL, VLDL, and cholesterol serum levels between C and R (Table 2). However, serum leptin levels were significantly higher in the R than in the C group. Serum insulin concentrations were 3.6 times higher in the R group compared with C mothers (Table 2).

Percentage of FAs in Maternal Liver at 19 dG

In the R group, the percentage of stearic acid in maternal liver was higher compared with C. In contrast, oleic acid, arachidic acid, AA, and DHA percentages were lower in the R group (Table 3).

Maternal Liver Desaturase and Elongase Gene Expression at 19 dG

No differences in SCD-1 mRNA relative abundance between C and R groups were observed (Fig. 1), but there was a significant decrease in Δ5D, Δ6D, and Elovl-2 and -5 mRNAs in the R group (Fig. 2). By Northern blots (Fig. 3), maternal liver Δ6D mRNA was in both groups significantly more abundant than Δ5D mRNA, and as expected, there was a significant negative correlation (r = −0.78, P < 0.001) between maternal insulin serum concentrations and liver Δ5D mRNA (Fig. 4).

Fetal Characteristics

Fetal weight and placenta weight and diameter at 19 dG are summarized in Table 4. Mean fetal weight was significantly lower in the R group than in the C group, with no differences in number of fetuses per dam and in placenta weight and diameter. There were also no changes in brain weight; however, color and texture were different, brains from the R group were darker (many dark spots) compared with C brains (Fig. 5D), and the texture for C brains was tougher than R. In contrast, liver weight, fetal liver, and brain lipids were lower in the R group (Fig. 5). The percentage of DHA from total FA in fetal brain was significantly lower in the R group (Fig. 5). In
addition, there was a negative correlation between fetal body weight and maternal serum insulin concentration at 19 dG ($r = -0.78$, $P < 0.01$; Fig. 6).

**DISCUSSION**

Many investigators have examined the effect of maternal malnutrition during pregnancy and lactation on the offspring; however, few studies have taken into consideration the impact of protein restriction on maternal lipid metabolism and its implication in fetal brain formation during gestation. Early malnutrition affects susceptibility to chronic diseases in adulthood (2), and maternal low-protein diet can program enzyme activity in offspring (26). However, previous studies indicated that offspring from rats fed a low-protein diet during pregnancy had higher susceptibility to develop chronic diseases (40, 41), and their mothers underwent metabolic adaptations to maintain an adequate fetal development. During the first half of normal pregnancy, progressive accumulation of maternal fat depots occurs due to increased adipose tissue lipogenesis and glyceroneogenesis (27). In this study, although there were not differences in maternal body and liver weights, there was a significant decrease in maternal body and liver lipid content as well as on fetal body weight; similar results have been reported with severe protein restriction (6%) (21).

Numerous animal studies have investigated the effect of maternal low-protein diet on glucose metabolism in the offspring (12, 40); however, little is known on the maternal responses derived from this condition. Herein, no differences in maternal serum glucose and TG concentrations were seen in C and R rats, which may be the result of an increased insulin-mediated glucose uptake by peripheral tissues and by the ability of insulin to suppress hepatic glucose output. Hyperinsulinemia is a common finding in pregnancy (12). Insulin resistance is responsible for both decline in adipose tissue lipoprotein lipase and enhanced adipose tissue lipolytic activities. This condition results in high TG synthesis by increasing FA and glycerol uptake by the liver. In this study, although protein restriction during gestation did not affect maternal serum glucose levels, high insulin serum concentrations were observed, suggesting metabolic adaptations in glucose metabolism at the end of pregnancy. In addition, high insulin concentrations at 19 dG in R rats were negatively correlated to fetal weight. These results were in agreement with those reported previously in pregnant women (38).

Leptin is a hormone that regulates food intake and energy expenditure. Serum leptin concentrations usually increase from...
the middle to the end of pregnancy (10, 11). During this period, the high levels of leptin do not mediate a great inhibitory effect on food intake (36). In this study, leptin serum concentrations were on the range of pregnant rats (3–7 ng/ml). However, fasting serum leptin concentrations in R rats were higher than those observed in C pregnant rats; despite the lower body fat in this group, leptin in the R mothers may alter the energy balance in contribution to fetal development.

Protein restriction significantly affected the production of maternal liver LC-PUFAs. There are several potential ways in which low protein (and hence, amino acid) intake in the maternal diet may affect maternal liver PUFA synthesis. The most likely is an effect on availability of amino acids for enzyme synthesis. Enzyme activity could also be impacted by altered histone acetylation and methylation resulting from changes such as decreased one-carbon cycle components. Indeed, maternal liver contained an increased stearic to oleic acid ratio. Although a nonsignificant difference in hepatic SCD-1 mRNA relative abundance, an enzyme that catalyzes oleic acid formation from stearic acid, which is the rate-limiting step for cellular synthesis of monosaturated FA from saturated FA, was observed in R rats compared with controls, alterations in the expression of SCD-1 cannot be ruled out and deserve further investigation.

In this study, maternal protein restriction resulted in a negative effect, particularly on the capacity of maternal liver to desaturate LA and LNA. A significant decrease in maternal hepatic AA and DHA was observed in the R group, which correlated with low expression levels of Δ5D, Δ6D, Elovl-2, and Elovl-5. In line with this observation, Mercuri et al. (22) showed that maternal severe protein restriction (5% protein) during gestation decreased Δ6D activity in the maternal liver, and they suggested that this could affect the normal supply of LC-PUFAs for the normal fetus growth and tissue development. In fact, in the present study, mild protein restriction during pregnancy reduced significantly the concentration of DHA in maternal liver and fetal brain. Before birth, most if not all fetal FAs originate from the maternal circulation (17). After birth, milk, maternal liver (29), and diet later in life (17) represent the main sources of FAs. Whether these maternal liver changes in lipid metabolism could be expressed transgenerationally cannot be ascertained from the results presented in this study. Ozanne et al. (26) demonstrated in 3-mo-old offspring from R pregnant mothers a decrease in hepatic Δ5D activity and DHA concentrations, suggesting the establishment of a particular gene expression pattern, such as that involving metabolic lipid enzymes, as seen in this study.

The last period of intrauterine life in the rat (17–21 dG) is demarcated by an increase in body and brain weight together with an active neurogenesis stage. During this period, a rapid accumulation of DHA, unparalleled to other FAs, takes place (35). The supply of EFA and LC-PUFAs is critical for normal development of the fetus (6, 15, 20, 24). Indeed, nutritional status of the mother during gestation has been positively associated with fetal growth. In general, reduced maternal nutritional status, particularly regarding EFAs, has been correlated with reduced neonatal growth and head circumference in humans (7). In fact, at birth, AA status in preterm infants has been correlated with body weight (18, 19), and it has been proposed that it is related to intrauterine growth rather than to postnatal growth (39).

The brain is one of the richest organs in lipids (4). Our results showed that a protein-restricted diet during gestation significantly reduced fetal body weight and liver weight, with no significant changes in fetal brain weight. However, the content of fetal brain lipids, particularly DHA, was significantly lower compared with C rats. This last finding correlates with data reported previously by Burdge et al. (5) in which DHA concentration in fetal brain at 20 dG from 50% of protein restriction was lower compared with controls. As described in Methods, the need to pool tissue from all fetuses in a litter removes the possibility of an analysis by fetal sex. We are currently studying the effect of the observed changes in brain composition on offspring behavioral phenotype.

In conclusion, there is evidence that small changes in maternal dietary fat intake modify the composition of brain membranes during fetal growth; however, few studies deal with the negative impact of maternal protein restriction during gestation on fetal brain development. The present study showed that moderated protein restriction during pregnancy significantly reduced the concentration of maternal liver AA and DHA and maternal liver desaturase and elongase gene expression. These results may negatively impact fetal development, including the brain (Fig. 7).

GRANTS

C. J. Bautista is a graduate student from Doctorado en Ciencias Biomedicas, Universidad Nacional Autónoma de Mexico. This work was partially supported by Consejo Nacional de Ciencia y Tecnología (48839), Mexico, and the National Institute of Child Health and Human Development (HD-21350).

DISCLOSURES

No conflicts of interest are declared by the author(s).

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