Effects of parental folate deficiency on the folate content, global DNA methylation, and expressions of FRα, IGF-2 and IGF-1R in the postnatal rat liver

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Abstract

We examined the effect of parental folate deficiency on the folate content, global DNA methylation, folate receptor-alpha (FRα), insulin-like-growth factor-2 (IGF-2) and -1 receptor (IGF-1R) in the liver and plasma homocysteine in the postnatal rat. Male and female rats were randomly fed a folic acid-deficient (paternal folate-deficient, PD and maternal folate-deficient, MD), or folic acid-supplemented diet (paternal folate-supplemented, PS and maternal-folate-supplemented, MS) for four weeks. They were mated and grouped accordingly: PSxMS, PSxMD, PDxMS, and PDxMD. Pups were killed on day 21 of lactation. The hepatic folate content was markedly reduced in the PDxMD and PSxMD and PDxMS as compared with the PSxMS group. The hepatic global DNA methylation was decreased in the PDxMS and PSxMD groups as much as in the PDxMD group, and all the three groups were significantly lower as compared to the PSxMS group. There were no significant differences in the hepatic FRα, IGF-2 and IGF-1R expressions among the groups. Positive correlations were found between the hepatic folate content and global DNA methylation and protein expressions of FRα, IGF-2 and IGF-1R, whereas an inverse correlation was found between hepatic folate content and plasma homocysteine level in the 3-week-old rat pup. The results of this study show that both paternal and maternal folate deficiency at mating can influence the folate content and global DNA methylation in the postnatal rat liver.

Key Words: Postnatal liver, folate content, global DNA methylation, FRα, IGF-2

Introduction

Folate is a water soluble B vitamin that encompasses both endogenous, naturally-occurring folates and synthetic (folic acid) form. It participates in various one-carbon transfer reactions, including nucleic acid synthesis that is important in embryonic and fetal development during the widespread cell division [1]. Folate is a methyl donor in the methylation cycle, which maintains adequate cellular levels of S-adenosylmethionine (SAM) for biological methylation reactions, including DNA methylation [2]. The byproduct of this reaction is homocysteine, and the sign of decreased activity in the methylation cycle results in elevation of plasma total homocysteine [3].

Among the proteins that play a major role in regulating prenatal growth and development of the fetus is the insulin-like growth factor-2 (IGF-2) [4], and its biological action is mediated by the insulin-like growth factor-type 1 receptor (IGF-1R) [5]. IGF-2 is a fetal growth factor wherein monoallelic expression from the paternal allele is manifested during gestation in all rat tissues [6-7]. IGF-2 becomes biallelic and parental imprinting conserved in the rat in most tissues [6-8], however, the expression from the paternal allele in the liver of a 3-day-old rat exceeds that from the maternal allele by three orders of magnitude [6]. In contrast to reports showing extinct expression of IGF-2 in postnatal life, recent studies have demonstrated that it is persistently present in the postnatal circulation. IGF-2 is found to be expressed in early postnatal rat tissues such as liver, serum, kidney and intestine [9-10], and human plasma [11], wherein expressions may signify a role in postnatal growth and metabolism [9]. The activity of the IGF-2 protein is mainly produced in the liver of the rat [12], which is also the main storage organ for folate taken up by hepatocytes [13] and contains the highest percent of body folate [14]. Among the several transporters, folate receptor-alpha (FRα) transports folate by receptor-mediated endocytosis, and is expressed on the membrane of epithelial tissues [15].

DNA methylation is a major epigenetic phenomenon regulating gene expression and genome integrity that are critical for genomic imprinting in the offspring [16-18]. Folic acid deficiency may prevent normal methylation of epigenetically regulated IGF-2 gene. Since its expression is regulated by DNA methylation, it may be vulnerable to abnormal methylation during development [19-20]. A number of studies have also demons-

trated that maternal supplementation with folic acid increased the methylation of the IGF-2 gene in the rat liver [21] and human blood offspring [22]. Although there is growing evidence that maternal nutritional status can alter the epigenetic state of the offspring genome [18,21-23], the influence of paternal folate nutrition on the genomic imprinting and thereby offspring growth has not yet been extensively studied. Paternal epigenetic alterations may lead to fetal mutations [24] and also results in poor embryonic development [25-27]. Not until recently, a few studies have demonstrated that paternal folate status plays an important role in the folate metabolism in the placenta [28], as well as in the expression of IGF-2 and global DNA methylation in the fetal brain [29]. This study therefore hypothesized that both paternal and maternal (parental) folate deficiency can influence the hepatic folate content, global DNA methylation, expressions of FRα, IGF-2 and IGF-1R, and plasma homocysteine level in the postnatal rat offspring.

Materials and Methods

Animals and diets

Five-week-old male and female Sprague Dawley (SD) rats were obtained from Joongang Shilheom Dongmool (Seoul, Korea), and were maintained at standard laboratory conditions under artificial 12 h light/dark cycle and an ambient temperature of 22-24℃. Rats were acclimated for a week on a non-purified diet, and then randomly assigned to two experimental diets for 4 weeks ad libitum: 0 mg/kg of folic acid (paternal folate-deficient, PD and maternal folate-deficient, MD), or 8 mg/kg of folic acid (paternal folate-supplemented, PS and maternal folate-supplemented, MS). Afterwards they were mated and allocated to four groups namely PSxMS, PSxMD, PDxMS, and PDxMD. Mating was confirmed by observation of a vaginal plug and recorded as gestation day 0. Wood shavings were provided to each dam housed in maternal acrylic cages. Throughout the study period, body weight was measured (once a week) and food intake (twice a week) of rats was recorded. The day of birth was identified as postnatal day 0. Suckling pups were kept with their mothers while dams were continued on their assigned experimental diets. On day 21 of lactation, pups were sacrificed by exsanguination through heart puncture. The blood samples were kept for laboratory determinations and carcasses were dissected to obtain liver samples. All the collected samples were stored at -80℃ until use. All experiments were carried out in accordance to the protocols approved by the Institutional Animal Care and Use Committee of Ewha Womans University (Approval No. 2009-2-1).

Hepatic folate assay

Folate content in the postnatal rat liver was assessed using Lactobacillus casei (ATCC 7469) in 96-well microplates. Samples were homogenized with 0.1 M potassium phosphate (KPO_4) buffer containing 1% ascorbic acid, and centrifuged twice (15,000 rpm for 15 min at 4℃). The supernatant was aspirated and mixed with serum folate conjugase (1:1 ratio), 0.1M potassium phosphate (KPO_4) buffer containing 1% ascorbic acid. The reaction mixtures were incubated in the heat block (8 h at 37℃) to hydrolyze polyglutamyl folates to monoglutamyl folates. Standard solution preparation was prepared using 0.01 g of [6RS]-5-formyltetrahydrofolate (Calcium salt, Sigma-Aldrich, St. Louis, MO, USA) dissolved in 10 ml deionized distilled water with pH adjusted to 7.0 by 0.1N-hydrochloric acid (Duksan Pure Chemicals co., LTD.) and 0.1N-sodium hydroxide (Duksan Pure Chemicals, co., LTD.).

Hepatic global DNA methylation quantification

Genomic DNA from the hepatic tissue samples was extracted using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). Extracted DNA samples were buffer-diluted and quantified to 50 ng (input DNA amount) using BioSpec-nano (Shimadzu, Columbia, MD, USA). The methylation analysis was performed in duplicate aliquots using anti-methylated cytosine antibody-based Methylflash™ Methylated DNA Methylation Quantification Kit (Epigentek Group Inc., New York, NY, USA). The absolute quantification of global methylation occurs by the covalent addition of a methyl group at the 5-carbon of the cytosine ring by DNA methyltransferases, resulting in 5-methylcytosine. The level of methylated DNA was read proportional to the spectroscopic end point (optical density, OD) intensity on an ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm. Standard curve was generated with mean OD values plotted against the amount of calibrators, using 6 concentration points. The slope (OD/ng) of the standard curve (using 5 concentration points) was determined using linear regression.

Plasma homocysteine concentration determination

Blood samples were centrifuged (3,000 rpm for 15 mins at 4℃) to separate plasma from the blood of the 3-week-old rat. Plasma homocysteine level was determined by means of a solid-phase enzyme immunoassay using Axis™ Homocysteine EIA kit (Abbott Laboratories, Mississauga, Ontario, Canada) with complete set of reagents included. Standard curve was generated with mean OD values plotted against the calibrators (S-adenosyl-L-homocysteine) at the 6 concentration points. A four parameter logistic curve fit was used for the calibration curve and calculation of unknown samples.

Analysis of hepatic FR α, IGF-2 and IGF-1R protein expressions by Western blot technique

Liver samples were equally weighed and homogenized with EDTA-containing RIPA Cell Lysis Buffer (pH 7.5) (GenDepot,

Barker, TX, USA) and 1% of ProteoBlock Protease Inhibitor Cocktail (Fermentas, Burlington, Canada). Lysate samples were quantified with the exact protein concentration (µg/µl) using Pierce bicichoninic acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Using a 40% Acrylamide/Bis Solution, 12% and 15% gels were casted for electrophoresis using a 10-well electrophoresis cell (BioRad Laboratories, Hercules, CA, USA). Protein samples (40 µg) were electro-transferred to a nitrocellulose transfer membrane by means of semi-dry transfer (BioRad Laboratories, Hercules, CA, USA). The membrane was blocked for 2 h with phosphate-buffered saline (PBS)-skim milk (5%) solution and incubated overnight (18 h) at 4℃ in primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA and Abcam PLC, Cambridge, UK). Beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to normalize the band intensities. The bands were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Statistical analysis

All measurements were performed in duplicate. Data were analyzed using SAS Institute Inc., Cary, NC, USA and values are expressed as mean ± standard error of the mean (SEM). Statistical differences between the two experimental groups were evaluated by the t-test, while differences among the four groups were evaluated by mixed procedure, and preceded by Duncan's test for multiple comparisons. Coefficient of correlation of hepatic folate content with hepatic global DNA methylation, FRα, IGF-2 and IGF-1R protein expressions, as well as plasma homocysteine level, were evaluated using Pearson's correlation. A *P* value < 0.05 was considered statistically significant.

Results

Food intake, body weight, and litter size

The mean body weights at baseline and at mating, and the average food intake in male rats and the mean body weight at

Table 1. The mean body weight and food intake of parental male and female rats in the folic acid-supplemented and folic acid-deficient groups

	Folic acid-supplemented	Folic acid-deficient	*P* value
Male	(n = 8)	(n = 6)	
Body weight (g)			
Initial	126.0 ± 1.6	125.0 ± 2.7	NS[1]
At mating	392.3 ± 8.5	386.5 ± 9.2	NS
Food intake (g/day)	26.2 ± 0.7	25.9 ± 0.6	NS
Female	(n = 7)	(n = 7)	
Body weight (g)			
Initial	103.5 ± 1.7	103.4 ± 1.9	NS
At mating	244.3 ± 10.7	241.8 ± 7.5	NS
Day 20 of gestation	398.6 ± 7.9	376.5 ± 6.9	NS
Day 21 of lactation	299.5 ± 10.4	282.6 ± 10.4	NS
Food intake (g/day)			
At mating	18.4 ± 0.7	20.0 ± 1.2	NS
Day 20 of gestation	26.0 ± 1.1	28.2 ± 0.2	NS
Day 21 of lactation	32.2 ± 0.6	32.9 ± 0.7	NS

[1] Not significantly different between folic acid-supplemented and folic acid-deficient groups

initial, mating, day 20 of gestation and day 21 of lactation in female rats were all not significantly different between the folic acid-supplemented (FS) and folic acid-deficient (FD) groups (Table 1). The litter size was not significantly different in all groups, and the mean body weights and organ weights of the 3-week-old pups were similar among the PSxMS, PSxMD, PDxMS and PDxMD groups (Table 2).

Hepatic folate, plasma homocysteine, and hepatic global DNA methylation and FR α, IGF-2 and IGF-1R expressions

The hepatic folate content was markedly reduced in the PDxMD (-73.7%) and PSxMD (-69.2%) and PDxMS (-55.0%) groups as compared to the PSxMS group (*P* < 0.0001). The homocysteine levels were significantly elevated in the PDxMD (269.5%) and PSxMD (76.8%) groups as compared with the PSxMS group (*P* < 0.0001). On the other hand, there was a significant decrease in the global DNA methylation in the

Table 2. The litter size, mean body weight and organ weights of 3-week-old rat pups

	PSxMS[1] (n = 10)	PSxMD (n = 10)	PDxMS (n = 10)	PDxMD (n = 10)	*P*-value
Litter size	10.0 ± 1.7	10.6 ± 0.9	10.8 ± 0.9	11.0 ± 1.0	NS[2]
Pup weight (g)	36.9 ± 2.4	31.0 ± 2.0	33.0 ± 2.2	35.3 ± 2.9	NS
Organ weights (mg)					
Whole brain	1,339.3 ± 48.0	1,360.7 ± 39.62	1,328.6 ± 42.9	1,421.8 ± 56.6	NS
Liver	1,422.1 ± 150.8	1,182.5 ± 125.8	1,214.4 ± 135.6	1,689.4 ± 176.6	NS
Thymus	138.9 ± 11.7	103.0 ± 11.1	96.7 ± 11.7	112.0 ± 11.1	NS
Kidney	409.1 ± 39.2	361.7 ± 32.9	395.5 ± 35.4	437.1 ± 45.8	NS
Spleen	128.4 ± 12.9	92.3 ± 11.1	115.3 ± 11.8	117.0 ± 14.7	NS

[1] PSxMS, paternal supplemented x maternal supplemented; PSxMD, paternal-supplemented x maternal deficient; PDxMS, paternal-deficient x maternal supplemented, and PDxMD, paternal deficient x maternal deficient
[2] Not significantly different among the four groups

Table 3. The mean liver folate content, global DNA methylation, protein expressions of FRα, IGF-2 and IGF-1R expressions and plasma homocysteine level of the 3-week-old rat pups

	PSxMS[1] (n = 10)	PSxMD (n = 10)	PDxMS (n = 10)	PDxMD (n = 10)	*P*-value
Hepatic Folate content (nmol/g)	$3.36 \pm 0.16^{a,2)}$	1.00 ± 0.14^{c}	1.50 ± 0.15^{b}	0.87 ± 0.19^{c}	$P < 0.0001$
Plasma Homocysteine level (umol/L)	6.27 ± 2.34^{a}	10.73 ± 1.91^{b}	6.06 ± 2.08^{a}	23.60 ± 2.77^{c}	$P < 0.01$
Hepatic global DNA methylation (%)	4.72 ± 0.20^{a}	3.20 ± 0.18^{b}	2.85 ± 0.18^{b}	$2.\ 77 \pm 0.23^{b}$	$P < 0.001$
Hepatic protein expression					
FRα	0.76 ± 0.06	0.66 ± 0.06	0.59 ± 0.06	0.54 ± 0.06	$P = 0.0849$
IGF-2	0.51 ± 0.06	0.40 ± 0.05	0.40 ± 0.06	0.26 ± 0.06	$P = 0.0877$
IGF-1R	0.91 ± 0.05	0.88 ± 0.04	0.80 ± 0.04	0.72 ± 0.06	$P = 0.0905$

[1] PSxMS, paternal supplemented x maternal supplemented; PSxMD, paternal supplemented x maternal deficient; PDxMS, paternal deficient x maternal supplemented, and PDxMD, paternal deficient x maternal deficient

[2] Different superscript letters indicate the comparison with significant differences.

Table 4. Coefficients of correlation between hepatic folate content and plasma homocysteine level, hepatic global DNA methylation, and protein expressions of FRα, IGF-2 and IGF-1R in the postnatal rat

Hepatic folate content	$r^{1)}$
Plasma homocysteine level	$-0.520^{**2)}$
Hepatic global DNA methylation	0.756^{**}
Hepatic FRα expression	0.342^{*}
Hepatic IGF-2 expression	0.456^{**}
Hepatic IGF-1R expression	0.406^{**}

[1] Pearson's correlation coefficient
[2] $* P < 0.05$, $** P < 0.01$

PDxMD (-40.5%), PDxMS (-37.9%) and PSxMD (-31.0%) groups than in the PSxMS group ($P < 0.0001$) (Table 3). There hepatic expressions of FRα, IGF-2 and IGF-1R were all not significantly different. However, significant positive correlations were found between the hepatic folate content and global DNA methylation ($P < 0.01$), and protein expressions of FRα ($P < 0.05$), IGF-2 ($P < 0.01$) and IGF-1R ($P < 0.01$), whereas an inverse correlation was found between hepatic folate content and plasma homocysteine level in the 3-week-old rat pup (Table 4).

Discussion

We investigated the effect of paternal and maternal folate status on the folate content, global DNA methylation, FRα, IGF-2 and IGF-1R expressions in the liver during the early postnatal life, a period at which the organ is undergoing extensive functional maturation. In this study, we found that parental folate deficiency (PDxMD) resulted in a significant decrease in hepatic folate content, global DNA methylation and a significant elevation in the plasma homocysteine of the postnatal rat, as compared to the control group (PSxMS). The impact of both parent's folate deficiency was greater than either one, indicating that not only maternal folate nutrition during gestation and lactation but paternal folate status at mating can also influence the folate and DNA methylation status of the offspring.

Maternal folate deficiency (PSxMD) caused a decrease in hepatic folate content and DNA methylation, and an increase in the plasma homocysteine level. During gestation, placental transport of folate is dependent on maternal folate concentration, as evidenced by a positive association between maternal plasma, cord plasma and placental folate concentrations [30-31]. It is said that during lactation, the folate content of milk is ensured by sequestering folate from the maternal blood plasma into the mammary gland and it might facilitate the absorption of milk in the suckling animal [32]. This direct acquisition of folate nutrient from the folate supplemented-mother during gestation and suckling stage of the offspring may have given them a chance to recover (folate restoration period) despite periconceptional paternal folate deficiency. Poor folate status during pregnancy can lead to elevated maternal plasma levels of homocysteine [31], which is consequently a primary predictor of blood homocysteine in the developing fetus [33]. The results from the homocysteine level assessment may reflect a direct influence of the offspring's hepatic folate content secondary to paternal or maternal folate status, such that any folate deficiency-induced homocysteine elevation may have already occurred at birth and until postnatal day 20. Likewise, the maternal folate-deficient group in the plasma homocysteine level was elevated than the paternal folate-deficient group, while the latter had a recovery (folate restoration period) to the same level as in the control group (PSxMS). As expected, a strong negative correlation between hepatic folate content and plasma homocysteine level in the postnatal rat was found. Given the influence of folate-mediated one carbon metabolism, pups from the parental folate-supplemented group (PSxMS) were able to metabolize homocysteine more efficiently as compared to the parental folate-deficient group (PDxMD).

Surprisingly, paternal folate deficiency (PDxMS) caused a decrease in the hepatic folate content and global DNA methylation. In our study, even though the paternal folate-deficient group (PDxMS) had higher folate content as compared to the maternal folate-deficient group (PSxMD), it has not fully recovered (despite the folate restoration period) to the same level as in the control group (PSxMS). It is possible that paternal folate deficiency leads to epigenetic alternations in the sperm DNA. DNA methylation, as an early nutritional effect, can result in

a permanent defect of epigenetic regulation at the time of fertilization, and the resulting zygote is in possession of DNA wherein genes are expressed equally from the paternally inherited and maternally inherited alleles [34]. It can be speculated that paternal or maternal-induced DNA aberration can independently affect global DNA methylation in the developing liver of the offspring. In this study, both paternal (PDxMS) and maternal folate deficient groups (PSxMD) had a significant impact on the global DNA methylation status of the pup liver, to the same extent as in the deficiency of both parents (PDxMD). Despite adequate folate status from one parent, a folate deficiency from any of the parent can reduce the global DNA methylation status to as much as the reduction in the parental folate deficiency in the liver of the rat pup. It should also be noted that the global DNA methylation status was not improved in the maternal folate-supplemented group (PDxMS), despite the folate-recovery period. Paternal folate status at mating may have already influenced the epigenetic mechanisms involved at the time of conception.

No significant differences were found in the hepatic FRα, IGF-2 and IGF-1R expressions. A potential limitation of our study is the small sampling size that might have conferred insufficient power to show statistical significance. From our results, the sample size affected the size of the standard error, and doubling the sample size could have reached a statistically significant results. Further investigations requiring large sample size in the analyses of data is needed to understand and correct for any small sample bias.

In conclusion, parental folate deficiency resulted in a significant decrease in hepatic folate content, global DNA methylation, and a significant elevation in the plasma homocysteine of the postnatal rat. Maternal folate deficiency directly influenced the folate content of the 3-week-old offspring as much as the influence of the deficiency of both (parental), and is consequently reflected in the elevated plasma homocysteine level. Paternal or maternal folate deficiency can affect the global DNA methylation status to as much as the reduction in the parental folate deficiency in the liver of the rat pup. No significant differences were found in the hepatic FRα, IGF-2 and IGF-1R expressions. However, significant positive correlations were observed between the hepatic folate content and global DNA methylation, FRα, IGF-2 and IGF-1R expressions in the liver of the rat offspring. Our findings suggest that paternal folate status at mating must be as important as maternal folate status before conception and all throughout the pregnancy and lactation period in the DNA methylation of the offspring, and may consequently impact early postnatal life.

To our knowledge, this is the first study to demonstrate the independent effects of paternal and maternal folate deficiency on the folate content and global DNA methylation status in the liver of the postnatal rat. Further studies are needed to elucidate possible mechanisms involving folate nutrition and paternally-induced epigenetic modifications.

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