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Folate supplementation increases genomic DNA methylation in the liver of elder rats

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The availability of folate is implicated as a determinant of DNA methylation, a functionally important feature of DNA. Nevertheless, when this phenomenon has been examined in the rodent model, the effect has not always been observed. Several reasons have been postulated for the inconsistency between studies: the rodent is less dependent on folate as a methyl source than man; juvenile animals, which most studies use, are more resistant to folate depletion than old animals; methods to measure genomic DNA methylation might not be sensitive enough to detect differences. We therefore examined the relationship between folate and genomic DNA methylation in an elder rat model with a newly developed method that can measure genomic DNA methylation sensitively and precisely. Thirty-nine 1-year-old rats were divided into three groups and fed a diet containing 0, 4·5 or 18 μmol folate/kg (folate-deplete, -replete and -supplemented groups, respectively). Rats were killed at 8 and 20 weeks. At both time points, mean liver folate concentrations increased incrementally between the folate-deplete, -replete and -supplemented rats (P for trend $<0{\cdot}001$) and by 20 weeks hepatic DNA methylation also increased incrementally between the folate-deplete, -replete and -supplemented rats (P for trend$=0{\cdot}025$). At both time points folate-supplemented rats had significantly increased levels of DNA methylation compared with folate-deplete rats ($P<0{\cdot}05$). There was a strong correlation between hepatic folate concentration and genomic DNA methylation in the liver (r 0·48, $P=0{\cdot}004$). In the liver of this animal model, dietary folate over a wide range of intakes modulates genomic DNA methylation.

Folate: DNA methylation: Ageing: Liver: Rat

In vertebrate genomes, approximately 4 % of cytosine residues are modified post-synthetically to 5-methylcytosine. Most of these 5-methylcytosine residues are found in the CpG dinucleotide sequence within promoter regions. Maintenance of normal patterns of DNA methylation is important for cellular homeostasis since DNA methylation is thought to play a critical role in both the regulation of gene expression and the maintenance of gene integrity (Antequera *et al.* 1989; Razin & Cedar, 1991).

In some of its coenzymatic forms folate is a biological methyl donor, and thus it appears to be a determinant of genomic DNA methylation under certain circumstances. In human studies conducted in metabolic units, folate depletion has consistently been observed to produce genomic DNA hypomethylation in peripheral blood lymphocytes. Jacob *et al.* (1998) reported that marginally folate-deplete volunteers housed in a metabolic unit had diminished genomic DNA methylation. Subsequent repletion of folate status reversed the effect. Rampersaud *et al.* (2000) reported a similar effect in their metabolic unit study where a less restrictive diet (120 μg folate/d) was used.

In 1993 Balaghi *et al*. demonstrated similar effects in laboratory rodents; hepatic DNA from folate-deficient rats became genomically hypomethylated compared with DNA from control rats. However, when this phenomenon was re-examined in rat liver (Kim *et al.* 1995) or in other tissues such as the colon (Sohn *et al.* 2003), the effect was not always reproduced. Although the different degrees of folate depletion achieved in these studies have sometimes been cited to explain these discrepancies, other factors may be involved as well.

Ageing has also been observed to be associated with DNA hypomethylation. Studies in both man and laboratory rodents have repeatedly observed incremental decreases in genomic DNA methylation with ageing, and such effects have been observed in a wide variety of tissues (Mays-Hoopes *et al.* 1986; Wilson *et al.* 1987; Drinkwater *et al.* 1989). Even in cell culture, repeated passages (to the extent that it is a model of ageing) are associated with decreases in DNA methylation (Wilson & Jones, 1983; Holliday, 1986).

We therefore investigated the effect of folate status on genomic DNA methylation in a well-established rat model of chronic moderate folate depletion (Varela-Moreiras & Selhub, 1992; Kim *et al.* 1996) in elder rats. This model of chronic moderate deficiency is more clinically relevant than the short-term, severe deficiency described in previous studies (Choi *et al.* 1998). It is relevant to note that this long-term, moderate deficiency has not been found

Abbreviation: SAdoHcy, *S*-adenosylhomocysteine.

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in prior studies of juvenile adult rodents to be a sufficiently perturbing force to induce a significant degree of hypomethylation in hepatic DNA (Kim *et al.* 1995, 1996).

Experimental methods

Animals

The present study was approved by the Institutional Animal Care and Use Committee of the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University. Middle-aged (12 months old, *n* 39) male Sprague-Dawley rats (Zivic-Miller, Zelienople, PA, USA) were randomized to receive one of three diets identical with regard to amino acids but with different folate levels: (i) 0 μmol folate/kg (folate-deplete); (ii) 4.5 μmol/kg (basal requirement of folate); (iii) 18 μmol/kg (folate-supplemented state) (Dyets, Bethlehem, PA, USA). These amino acid defined diets constitute a standard means of predictably inducing deplete, replete and supplemented levels of folate status in rodents (Walzem & Clifford, 1988; Bills *et al.* 1991). By intent, a moderate, and not a severe, degree of folate depletion is produced in the deplete groups owing to the fact that no sulphonamide is added to their diets.

Rats were housed individually in wire-bottomed stainless steel cages to minimize coprophagy. Body weights were recorded biweekly. Water was supplied *ad libitum*. The amount of diet supplied to each diet group was matched to the mean daily food consumption of the group with the least food consumption. At 8 weeks after initiation of the diets, five rats from each group were killed. At 20 weeks, at which time the animals were 17 months of age, eight rats from each group were killed. The time points of 8 and 20 weeks were chosen because we wished to examine the effects of chronic depletion and because our prior studies had demonstrated that a mild and moderate degree of depletion occurs at these two time points, respectively.

Rats were anaesthetized in a CO_2 breathing chamber and the abdomen was then opened. The liver was harvested and the animal then killed by cross-cutting the aorta. Aliquots of liver tissue were flash frozen in liquid N and subsequently stored at −70°C.

Measurement of folate concentration and S-adenosylhomocysteine

Hepatic folate concentrations were determined by a conventional microbiological microtitre plate assay using *Lactobacillus casei* after treatment with chicken pancreas conjugase (Tamura, 1990). Hepatic *S*-adenosylhomocysteine (SAdoHcy) concentrations were measured by HPLC with UV detection using the method described Fell *et al.* (1985).*DNA methylation*

Liquid chromatography–electrospray ionization MS (Hewlett Packard/Bruker, Billerica, MA, USA) was used to analyse genomic DNA methylation. A detailed method has been previously described and validated by us (Friso *et al.* 2002*a*,*b*).

Briefly, DNA was extracted by a conventional technique using a lysis buffer containing proteinase K followed by extraction with phenol, chloroform and isoamyl alcohol. Then 1 μg DNA was hydrolysed by sequential digestion with three enzymes (Crain, 1990): nuclease P1 (Roche Molecular Biochemicals, Mannheim, Germany); venom phosphodiesterase I (Sigma, St. Louis, MO, USA); alkaline phosphatase (Sigma). The hydrolysed DNA solution was delivered directly onto the analytical column (Supelco, Bellefonte, PA, USA) in isocratic mode. This allowed the separation of the four DNA bases as well as the identification of 5-methylcytosine. Electrospray ionization MS was performed in positive ion mode. Identification of cytosine and 5-methylcytosine was obtained by mass spectrometric analysis of chromatographic peaks. The isotopomers [$^{15}N_3$]2′-deoxycytidine and methyl-D3, ring-6-D1 5-methyl-2′-deoxycytidine (Cambridge Isotope Laboratories, Cambridge, MA, USA) were used as internal standards. DNA methylation status was defined as the percentage of 5-methylcytosine of the overall amount of cytosine and 5-methylcytosine.

Statistical analysis

Statistical significance for differences in quantitative variables was analysed by ANOVA followed by the *post hoc* Tukey test. Logarithmic transformation was performed on all skewed variables to normalize their distributions and statistical comparison was based on logarithmic-transformed data. *P* for trend was analysed by regression analysis. For genomic DNA methylation, the difference was examined by a two-tailed Student's *t* test. The level of significance was set at $P<0{\cdot}05$ for all analyses. Statistical analysis was performed using Systat 10 for Windows software (SPSS, Chicago, IL, USA).

Results

We reported some of the data on body weight in a previous paper that focused on the effects of age on colonic folate metabolism (Choi *et al.* 2003). Rats fed folate-deplete diets experienced a small, but significant, mean weight loss compared with folate-replete and -supplemented rats, starting at 10 weeks (data not shown). All rats survived the 20-week experiment except two elderly rats from the folate-deplete group. They died at 8 and 19 weeks.

Table 1 shows that mean liver folate concentrations increased in an incremental fashion between the folate-deplete, -replete and -supplemented rats (*P* for trend $<0{\cdot}001$). This confirms the ability of these diets to produce predictable levels of tissue folate. Furthermore, at both time points the mean liver folate concentrations of the folate-deplete group were significantly lower than the corresponding values of the folate-replete and -supplemented groups ($P<0{\cdot}05$, Table 1), demonstrating a significant degree of hepatic depletion of folate. In a similar but reciprocal fashion, hepatic SAdoHcy increased incrementally within each group as revealed by the comparison of folate-supplemented, -replete and -deplete rats (*P* for trend $<0{\cdot}001$, see Table 1). Both hepatic folate and SAdoHcy concentrations of the elder rats were within the same range of values observed in corresponding diet groups of younger adult rats reported in our previous studies (Kim *et al.* 1995, 1997).

By 20 weeks, the incremental increase in hepatic DNA methylation between the folate-deplete, -replete and -supplemented groups (Fig. 1) was sufficiently prominent to be a significant trend across the entire range of folate status ($P=0{\cdot}025$). At both 8 and 20 weeks, folate-supplemented rats had significantly increased hepatic DNA methylation compared with the folate-deplete group ($P<0{\cdot}05$). A moderately strong, and highly significant, correlation between hepatic folate concentration and genomic DNA methylation (r 0·48, $P=0{\cdot}004$) was observed (Fig. 2).

Table 1. Liver folate and *S*-adenosylhomocysteine (S-AdoHcy)
(Values are means and standard deviations)

		Folate (nmol/g)		S-AdoHcy (nmol/g)	
Group	*n*	Mean	SD	Mean	SD
8-week folate-deplete	5	3·0[a]	0·9	14·0[a]	3·4
8-week folate-replete	5	15·0[b]	2·0	6·9[b]	1·9
8-week folate-supplemented	5	20·2[c]	3·2	5·4[b]	1·5
P for trend		<0·001		<0·001	
20-week folate-deplete	6	1·8[a]	0·7	15·0[a]	7·1
20-week folate-replete	8	15·9[b]	5·2	6·2[b]	2·0
20-week folate-supplemented	8	22·0[b]	8·4	4·4[b]	2·4
P for trend		<0·001		< 0·001	

[a,b,c]Mean values within a column with unlike superscript letters were significantly different ($P<0·05$ by ANOVA followed by the *post hoc* Tukey multiple comparison test).

Discussion

Compared with human studies (Jacob *et al.* 1998; Rampersaud *et al.* 2000), prior rodent studies of isolated dietary folate deficiency have generally failed to induce genomic DNA hypomethylation (Kim *et al.* 1995, 1997) with the exception of one study (Balaghi *et al.* 1993), even though folate depletion has been shown consistently to induce a marked increase in hepatic SAdoHcy, a compound well known to inhibit methylation reactions (De Cabo *et al.* 1995). It is worth noting that the rodent liver possesses a particularly active alternative pathway by which homocysteine can be remethylated (via the conversion of betaine to dimethylglycine), which may compensate for diminished folate availability and therefore explain, in part, the resistance of the rat liver to develop genomic hypomethylation. Furthermore, discrepancies between prior rodent studies might be ascribable to the different levels of severity or duration of the folate-deficient state that have been studied. There has also been speculation that previous methods to measure genomic DNA methylation might not be sensitive enough to detect the alteration induced by folate depletion due to the imprecision of measurement (Friso *et al.* 2002*b*).

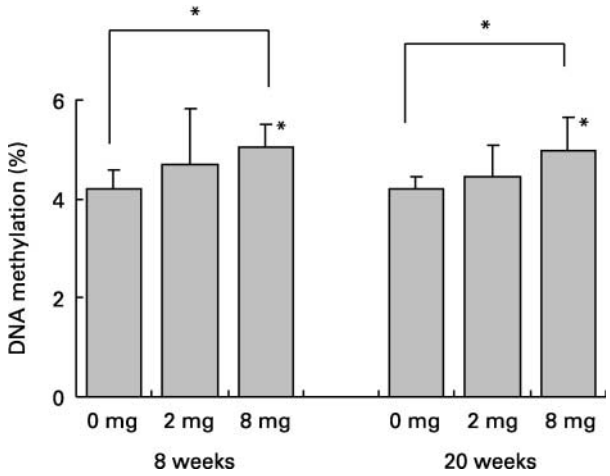

Fig. 1. Hepatic DNA methylation at different levels of dietary folate. By 8 and 20 weeks, hepatic DNA methylation showed a stepwise increase from the folate-deplete group to the folate-replete group to the folate-supplemented group (*P* for trend=0·08 and 0·025, respectively). Mean values of DNA methylation (folate-supplemented rats *v.* folate-deplete rats) were significantly different at both 8 weeks (five animals per group) and 20 weeks (eight animals per group): *$P<0·05$.

In the present study, using an elder rat model of chronic folate deficiency and a new, highly precise liquid chromatography/MS method, folate supplementation at four times the basal requirement of the rat (Reeves *et al.* 1993) significantly increased genomic DNA methylation compared with folate-deplete status in the liver. It is worth emphasizing that folate-deplete rats did not develop significantly lower levels of genomic DNA methylation compared with folate-replete rats, underscoring the importance of examining this phenomenon over a wide range of folate intakes. DNA methylation in the liver of the elder rats also displayed dependence on dietary folate in a significant manner as early as 8 weeks on the diet, an effect that was even more evident at 20 weeks.

One-carbon metabolism is a network of interrelated biochemical reactions in which a one-carbon unit from a donor compound is transferred to tetrahydrofolate for subsequent transfer to other compounds. Folate coenzymes in mammalian tissues thereby act as acceptors or donors of one-carbon units in a variety of reactions involved in amino acid and nucleotide metabolism (Mackenzie, 1984). Within the scope of this function is the synthesis of *S*-adenosylmethionine, a universal methyl donor for several biological methylation reactions. Methionine, the precursor of *S*-adenosylmethionine, is regenerated from homocysteine by methionine synthase in a reaction wherein 5-methyltetrahydrofolate serves

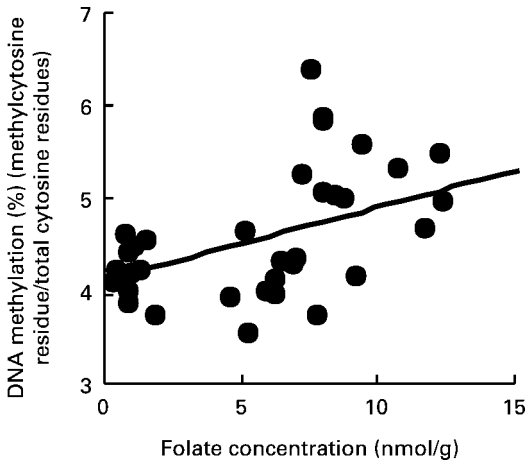

Fig. 2. Correlation between hepatic folate concentration and genomic DNA methylation in the rat liver at 8 and 20 weeks (*n* 3). A significant correlation was observed (*r* 0·48, *P*=0·004).

both as a cofactor and a substrate. Reduced availability of 5-methyltetrahydrofolate, the main circulating form of folate, decreases the biosynthesis of *S*-adenosylmethionine, limiting the availability of methyl groups for methylation reactions, and increases the cellular concentration of SAdoHcy, which is a direct inhibitor of methylation reactions. Studies to date suggest that it is the rise in SAdoHcy rather than the decease in *S*-adenosylmethionine that is the more critical determinant of DNA methylation (Yi *et al.* 2000).

Recent observations in a cystathionine-β-synthase knockout mouse model (Caudill *et al.* 2001; Choumenkovitch *et al.* 2002) as well as in a human study (Yi *et al.* 2000) have indicated that an elevation in SAdoHcy is sufficient to produce DNA hypomethylation, although the effect is tissue-specific. In the present study, elevations in hepatic SAdoHcy concentration accompanied folate depletion and, as would be expected, SAdoHcy decreased stepwise across the dietary folate levels from depletion to supplementation. This stepwise decrease in SAdoHcy was accompanied by a stepwise increase in genomic DNA methylation status. Therefore, the decrement in hepatic SAdoHcy produced by folate supplementation in this study was probably sufficient to reduce the inhibitory effect of SAdoHcy on genomic DNA methylation.

The identification of CpG methylation as a mechanism of epigenetic inheritance has led to the speculation that it might be involved in the ageing process (Holliday, 1987), because genomic DNA hypomethylation has also been observed in elder tissues *in vivo* (Mays-Hoopes *et al.* 1986; Wilson *et al.* 1987; Drinkwater *et al.* 1989). Since some studies indicate that hypomethylation is a consistent feature of proliferating cells (Hoal-van Helden & van Helden, 1989; Goodman & Counts, 1993) and increased proliferation is a characteristic of some elder tissues (Ronucci *et al.* 1988), this provides a possible explanation for this phenomenon. Whether folate depletion might exacerbate this age-related loss of methyl group is unclear, but this possibility is an attractive explanation of how diminished folate status and age might act synergistically to enhance the risk of carcinogenesis.

It is therefore not surprising that folate supplementation increased genomic DNA methylation in the elder rat liver, because folate has been shown in both animal and human studies to have a substantial impact on DNA methylation (Friso & Choi, 2002) and ageing enhances the susceptibility of folate depletion (Choi *et al.* 2003). We speculate that folate supplementation diminishes the tendency towards DNA hypomethylation that is produced by the synergy of ageing and lesser amounts of folate in the diet, both of which are important risk factors for carcinogenesis.

In conclusion, dietary folate over a wide range of intakes modulates genomic DNA methylation in the elder rat liver. In particular, folate supplementation at four times the basal requirement significantly increases genomic DNA methylation compared with folate-deplete status. Our ability to detect such an effect was probably related to the age of the animals used and/or the heightened accuracy of the method utilized to measure methylation.

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