Ability of Endogenous Folate from Soy Protein Isolate to Maintain Plasma Homocysteine and Hepatic DNA Methylation during Methyl Group Depletion in Rats

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Summary We investigated different means of achieving methyl depletion by feeding weanling rats modified AIN diets depleted of folate (FD), folate + choline (FCD), and folate + choline + methionine (FCMD), and examined the consequent effects on folate status, homocysteine levels, and methylation status. Control rats were fed a 12% protein diet consisting of either casein or soy protein isolate (SPI) and containing 2 mg/kg folate, 0.2% choline, and 0.4% methionine. After the rats had been on the diets for 4 and 8 weeks, whole blood folate concentration was measured and found to be significantly depleted in the folate deficient treatments compared with controls at 4 weeks ($p < 0.001$), this reduction being significantly greater ($p < 0.03$) in casein-fed rats (60%) than in SPI-fed rats (32%). The omission of choline and methionine from the diet had no further influence on whole blood folate. A significant inverse correlation was observed in the casein-fed rats after 8 weeks between mean plasma homocysteine concentration and decreasing methyl content of the diet ($r^2 = 0.978$, $p < 0.002$), an effect not seen in the corresponding SPI-fed rats. Hypomethylation of hepatic DNA evidenced by a reduction in 5-methylcytosine content was present in the casein rats fed FCD and FCMD relative to control ($p < 0.05$). No hepatic DNA methylation changes were observed in the SPI-fed rats. The results obtained in the present work demonstrate that a soy-based diet can compensate against methyl group depletion by maintaining plasma homocysteine levels and an adequate level of DNA methylation, a result we attribute to endogenous folate content.

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Dietary methyl group deficiency has been linked to the development of certain cancers (1, 2). The nutrients folate, methionine choline, and betaine are all capable of influencing the availability of labile methyl groups that subsequently play an important role in many processes, such as DNA synthesis and methylation. There is increasing evidence indicating that an abnormal DNA methylation pattern may play a role in the progression of human cancers (3, 4).

The objective of the present study was to investigate the effect of feeding rats a folate deficient diet (FD) alone and with superimposed choline and methionine deficiencies on a whole blood folate concentration and hepatic DNA methylation status. Other parameters related to folate metabolism that were studied included plasma homocysteine concentration. The progressive depletion of dietary methyl donors was designed to assess the degree of severity of methyl group depletion in these modified AIN diet preparations. These diets were based on the AIN-76A formula (5), but modified to contain either casein or soy protein isolate (SPI) as the base protein source supplying 12% protein and 20% sunflower seed oil. Soy was chosen as the protein source because of its ready availability and its supposed inhibitory effect on various types of cancers in animals (6).

MATERIALS AND METHODS

Animals/diets. A total of 48 weanling male Sprague-Dawley rats (70 g) were purchased from Animal Resource Centre, Murdoch University, Perth, Australia. The animals were housed in stainless steel wire-bottom cages, maintained in an air-conditioned environment 23°C with a 12-h light:12-h dark cycle. Rats at the age of 4 weeks were randomly divided into eight groups of six animals each and fed ad libitum experimental powdered diets and given free access to deionized water for eight weeks. Body weights were recorded weekly. Each rat was placed in a metabolic cage for 48 h in the seventh week on an experimental diet. Food intake and fecal and urine outputs were recorded.

The experimental diets were modified forms of the AIN (5) semipurified diet. They consisted of 12% protein (either casein (80% protein) or soy protein isolate (85% protein), Protein Technologies International, St. Louis, MO), 5% cellulose as dietary fiber, 35% starch, 20% sunflower seed oil as the fat source, 20% sucrose, 3.5% AIN-76A minerals, and 1% AIN-76A vitamins. The nutrient-replete diets contained 2 mg/kg folic acid, 0.2% choline, and 0.4% methionine. The diet lacking only folate was (FD); diets lacking folate and choline were (FCD); and the diets lacking folate, choline, and methionine were (FCMD).

Casein as a protein source contained 0.045 mg/kg endogenous folate, and soy protein isolate contained 1.28 mg/kg endogenous folate, when analyzed by the

Lactobacillus casei assay (Chemistry Centre, WA).

This experimental protocol was approved by the Animal Experimentation and Ethics Committee in the CSIRO, Division of Human Nutrition, before commencing the work.

Sample collection. After the rats were on experimental diets for 4 weeks, blood samples were collected via the tail vein under ether anesthesia. Blood was collected in EDTA tubes and immediately diluted 1:1 with fresh 1% ascorbic acid and stored at -80°C for folate determinations. The rats were killed by exsanguination under ether anesthesia after 8 weeks on experimental diets, blood was collected into EDTA tubes, and a portion was diluted with 1% ascorbic acid for folate analyses. The remaining blood was centrifuged at $1,000 \times g$ for 10 min, and plasma was collected and stored at -80°C for subsequent homocysteine measurements. Livers were excised, weighed, and frozen in liquid nitrogen and stored at -80°C for subsequent DNA extraction.

Analytical methods. Whole blood folate was determined by HPLC with fluorescence detection (7). Total plasma homocysteine was measured by HPLC by using the fluorometric method of Vester and Rasmussen (8).

The DNA from liver and colonic mucosal scrapings was isolated by using a High Pure PCR Template preparation kit (Boehringer Mannheim) followed by treatment with 10 mg/mL solution of RNase that had been preheated to 100°C for 15 min and cooled slowly to room temperature. The purified DNA was then hydrolyzed with formic acid, and the individual nucleobases were determined by using HPLC (9) to measure the level of DNA methylation. The percentage of methylation was calculated from the content of 5-methylcytosine over 5-methylcytosine plus cytosine content multiplied by 100.

Statistical analysis. The GraphPad Instat software package (version 2; San Diego, CA, USA) was used for statistical analysis of experimental data. The statistical significance of the mean values was tested by one-way analysis of variance with the criterion for significance taken at $p < 0.05$. The differences between the means were then subjected to the Tukey-Kramer multiple comparison test with significance obtained at $p < 0.05$.

RESULTS

The mean daily food consumption of the animals fed the different dietary treatments were as follows (mean $\pm$ SE): casein, 17.4 ± 0.7 g; casein/FD, 16.2 ± 0.8 g; casein/FCD, 17.8 ± 0.9 g; casein/FCMD, 18.0 ± 0.5 g; SPI, 14.5 ± 0.9 g; SPI/FD, 15.5 ± 0.4 g; SPI/FCD, 14.1 ± 0.5 g; SPI/FCMD, 14.0 ± 0.8 g. Depleting the experimental diets of folate, choline, and methionine had no significant effect on the food intake of the animals. However, the rats fed the casein background diets consumed significantly more food per day (3 g/d) ($p < 0.001$).

The casein-fed animals were significantly heavier than the SPI-fed animals at the time of death ($p < 0.001$). A dietary lack of folate and choline had no influence

Table 1. Effect of folate, choline, and methionine deficiencies on whole blood folate concentration and hematocrit in rats fed casein or SPI diets deficient in folate, choline, and methionine.

Group	Whole blood folate		Hematocrit (8 weeks) (% red cells)	Plasma homocysteine (8 weeks) (μ M)
	(4 weeks)	(8 weeks) (ng/mL)		
Casein (cas)	613 $\pm$ 43 ^a	584 $\pm$ 23 ^a	41.9 $\pm$ 0.40 ^a	1.29 $\pm$ 0.07 ^d
cas/FD	226 $\pm$ 25 ^c	186 $\pm$ 8 ^d	41.0 $\pm$ 0.37 ^a	3.64 $\pm$ 0.18 ^c
cas/FCD	254 $\pm$ 27 ^{bc}	172 $\pm$ 4 ^d	40.8 $\pm$ 0.40 ^a	8.75 $\pm$ 1.02 ^b
cas/FCMD	273 $\pm$ 28 ^{bc}	195 $\pm$ 7 ^d	38.5 $\pm$ 0.42 ^b	11.34 $\pm$ 0.70 ^a
SPI	583 $\pm$ 42 ^a	612 $\pm$ 22 ^a	41.2 $\pm$ 0.40 ^a	1.26 $\pm$ 0.07 ^d
SPI/FD	404 $\pm$ 18 ^b	300 $\pm$ 4 ^c	40.4 $\pm$ 0.56 ^{ab}	1.62 $\pm$ 0.14 ^{cd}
SPI/FCD	406 $\pm$ 26 ^b	409 $\pm$ 27 ^b	41.2 $\pm$ 0.40 ^a	2.58 $\pm$ 0.26 ^{cd}
SPI/FCMD	384 $\pm$ 36 ^b	437 $\pm$ 17 ^b	40.7 $\pm$ 0.49 ^a	1.81 $\pm$ 0.25 ^{cd}

Values are means $\pm$ SE for six rats. Values within each column with different superscript letters are significantly different ($p < 0.05$).

FD, folate-deficient; FCD-folate, choline-deficient; FCMD, folate-, choline-, methionine-deficient.

on the growth of the experimental animals. A lack of dietary methionine resulted in significantly reduced weight gains in both casein- and SPI-fed animals. This was more evident with the SPI treatment (33% fall) compared with the casein treatments (10% fall).

Table 1 shows the effect of feeding rats methyl-deficient experimental diets on whole blood folate concentration after 4 and 8 weeks. After 4 weeks, a significant depletion of folate concentrations was evident. In the casein-fed animals, folate concentrations fell 60%, and in the SPI-fed animals a fall of 32% was observed when compared with their control treatments. Feeding the diets for a further 4 weeks (total of 8 weeks) resulted in only minor additional reductions in folate concentrations. Depleting the diets progressively with choline and methionine on top of the folate deficit seemed to have no additional effect on the folate status in the whole blood.

Hematocrit values (Table 1) showed that the rats fed the casein/FCMD diet had a significantly ($p < 0.05$) lower hematocrit than those fed all other diets, except the SPI/FD diet. However, despite a halving of folate levels, changes in hematocrit were small.

Plasma homocysteine concentrations (Table 1) were significantly elevated ($p < 0.0001$) in the casein rats that were fed diets depleted of methyl groups. A significant negative correlation was observed in the casein-fed rats between plasma homocysteine concentration and decreasing methyl content of the diet ($r^2 = 0.978$, $p < 0.002$). Neither a significant correlation nor an elevation of plasma homocysteine

Table 2. Percentage of 5-methylcytosine in hepatic DNA.

Group	% 5-methylcytosine
Casein (cas)	3.703 ± 0.030^a
cas/FD	3.304 ± 0.191^{ab}
cas/FCD	2.934 ± 0.141^b
cas/FCMD	2.900 ± 0.151^b
SPI	3.347 ± 0.132^{ab}
SPI/FD	3.411 ± 0.169^{ab}
SPI/FCD	3.397 ± 0.100^{ab}
SPI/FCMD	3.206 ± 0.100^b

Values are means $\pm$ SE for six rats. Values in the same column not sharing a common superscript letter are significantly different.

SPI, soy protein isolate; FD, folate-deficient; FCD, folate-, choline-deficient; FCMD, folate-, choline-, methionine-deficient.

was observed with the corresponding SPI treatments.

The percentage of 5-methylcytosine content in hepatic DNA is shown in Table 2. Hypomethylation of DNA, that is, a decrease in the level of 5-methylcytosine, was observed in the casein groups with lowered folate status. This observation was significant ($p < 0.05$) in the casein treatments depleted of FCD and FCMD with over a fall in methylation of more than 20%. No significant change was noted in methylation relative to control in the SPI-fed rats.

DISCUSSION

This study showed that folate status can be significantly reduced after only four weeks of feeding a semipurified diet depleted of the B-vitamin folate. The degree of folate depletion is within the range reported by other researchers (10, 11) and would be regarded as a moderate folate depletion because hematocrits were affected only marginally. The failure to produce significant hematological change is consistent with the knowledge that the rat is resistant to such change relative to some other species studied (12).

The level of folate depletion achieved was significantly greater in the casein-fed rats consuming methyl-deficient diets than in the corresponding SPI-fed rats. This most likely reflects the much higher amount of endogenous folate present within the soy protein isolate (1.28 mg/kg compared with 0.045 mg/kg for casein). Although the sulfur amino acid content of the diets was balanced, the casein fed rats had a much greater body weight gain over the 8-week period compared with the SPI-fed rats. This indicates that casein is a superior quality protein to SPI for rat growth and is most likely attributable to a higher content of the essential amino acids isoleucine and threonine (13).

Homocysteine concentration in the plasma was significantly elevated in the

casein treatments, which were fed deficient levels of folate, and in the treatments with choline and methionine deficiencies superimposed. Interestingly, homocysteine levels were not increased in any of the SPI treatments. Plasma homocysteine levels have been shown by other researchers to be inversely correlated with intracellular folate because of the requirement of folate and its coenzymes (11). The remethylation of homocysteine to methionine is dependent on two main pathways. The first requires folate and its coenzymes with vitamin B-12 containing 5-methyl-tetrahydrofolate: homocysteine methyltransferase catalyzing the reaction (14). The other pathway in which homocysteine is remethylated uses choline. Here choline is oxidized to betaine, which serves as the methyl donor in a reaction catalyzed by betaine: homocysteine methyltransferase (15). The occurrence of hyperhomocysteinemia in the casein rats depleted of dietary methyl groups suggests that a functional or biochemical deficiency is present within these treatments and that a disruption in one or both of the above pathways has occurred. Since plasma homocysteine levels were not significantly elevated in the SPI treatments fed depleted methyl groups, even though blood folate levels were depleted, it suggests that endogenous factor(s) are within SPI preventing a functional deficiency of folate. The most obvious factor is folate; it was present in SPI at more than 28 times the concentration of the casein. The elevation of plasma homocysteine observed in the casein-fed rats depleted of folate, choline, and methionine may also be of importance in terms of risk of cardiovascular disease. Elevated levels of plasma homocysteine have been identified as being an independent risk factor for premature vascular disease (16).

A disruption of the transmethylation cycle in the liver resulted in hypomethylation of DNA in the casein-fed rats. This decrease in methylation was not observed in the corresponding SPI-treated rats, which also had a lesser degree of folate depletion. Altered DNA methylation has been proposed as an early step in the multistep process of tumorigenesis, and changes in DNA methylation can lead to altered regulation of gene expression (4). The induction of DNA hypomethylation could then be a mechanism by which diminished folate status acts to enhance carcinogenesis (17). Global and gene-specific hypomethylation of hepatic DNA has been observed in rats fed methyl-deficient diets (18, 19). The data presented here confirm that a dietary folate deficit alone was unable to alter DNA methylation significantly within the time frame studied. When choline and methionine deficits from the diet were superimposed on a folate deficit, however, the induction of hypomethylation of hepatic DNA was observed.

In summary, the present study has demonstrated that diets depleted of folate can lower folate status. Deficiencies of choline and methionine superimposed on a dietary folate deficit do not seem to influence blood folate status. Plasma homocysteine levels rose sharply in casein rats fed methyl-depleted diets, an observation not seen in SPI fed animals. Feeding an SPI diet to the rats was also allowed an adequate level of DNA methylation to be maintained during methyl group depletion. The type of dietary protein is important in influencing these metabolic parameters, which is probably a reflection of the amount of endogenous

folate present within the protein source.

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