ORIGINAL ARTICLE

Lack of effect of diet-induced hypomethylation on endothelium-dependent relaxation in rats

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Summary
Background: Endothelial dysfunction is a key process in atherosclerosis. Hypomethylation is one of the postulated mechanisms involved in atherogenesis and is mainly secondary to a decrease in essential factors such as, folate and vitamin B12 for the biosynthesis of *S*-adenosylmethionine (SAM), the main methyl-group donor for methylation reactions.
Aim: To investigate in an animal model, whether hypomethylation, secondary to folate or vitamin B12 deficiency, affects endothelium-dependent relaxation (EDR) induced by acetylcholine (ACh).
Methods: Adult male Wistar rats were divided into 4 groups of 12 rats each: folate and B12 deficiency (FB12D 0 mg folate/kg, 0 μg/kg B12), folate deficiency (FD 0 mg folate/kg and 50 μg/kg B12), B12 deficiency (B12D: 8 mg/kg folate and 0 μg/kg B12 and control diet (CD)). After eight weeks the animals were killed and thoracic aorta and liver removed. Serum concentration of homocysteine, folate and vitamin B12 were determined. Hepatic levels of SAM and *S*-adenosylhomocysteine (SAH) were measured, as indicator of hypomethylation. ACh-induced EDR and sodium nitroprusside (SNP)-induced endothelium-independent relaxation (EIR), in isolated aorta rings were evaluated.
Results: Hcy concentrations were significantly increased in the folate and B12 deficient groups. SAM and the SAM/SAH ratio were lower in the FD and FB12D than in the control and B12D group. Folate, B12 deficiency, serum Hcy levels and hepatic SAM/SAH ratio did not affect EDR neither EIR.
Conclusions: In adult Wistar rats, chronic folate or folate plus vitamin B12 deficiency generates hypomethylation which is not related to an alteration of endothelial function.

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Introduction

Hyperhomocysteinemia is associated with a higher cardiovascular risk. However, it is not clear if it causes vascular dysfunction directly or is just a marker for other risk factor. Low circulating levels of vitamin B12 and folic acid rise homocysteine levels, and reduce the availability of *S*-adenosylmethionine (SAM), limiting methylation capacity.[1]

Intracellular methylation reactions, which involve methyltransferase activity and SAM as the methyl donor, participate in synthesis and detoxification processes in addition to DNA, RNA, phospholipids and protein methylation . Once a methyl group has been transferred, SAM is converted to *S*-adenosylhomocysteine (SAH) leading to a decreased intracellular SAM/SAH ratio. Under physiological conditions, SAH is hydrolyzed to Hcy and adenosine. This reaction is reversible, with a dynamic equilibrium that strongly favours SAH synthesis rather than hydrolysis. The active form of folate, 5-methyltetrahydrofolate, provides a methyl group that is used to reconvert homocysteine back to methionine through the transmethylation pathway. Thus, folate is important to maintain the availability of SAM.[2–4]

Hyperhomocysteinemia has been associated with impaired endothelium-dependent vasodilatation in the absence of frank atherosclerotic vascular lesions. Nevertheless, this finding is not universal.[5] The association between hyperhomocystinemia and cardiovascular disease may be explained by a low SAM or a high SAH concentration or a low SAM/SAH ratio, or by low concentrations of folate, vitamin B6, or vitamin B12. Moreover, a high SAH is a more sensitive indicator of cardiovascular disease, than an increase in plasma tHcy.[6] Endothelial dysfunction in hyperhomocysteinemic mice, with a heterozygous deficiency of the cystathionine ß-synthase (CBS) gene, was associated with increased tissue levels of SAH in liver and brain.[7] Loehrer et al. found a reduced SAM/SAH ratio, due to elevated SAH levels in plasma and erythrocytes, in hyperhomocysteinemic patients with occlusive vascular disease and in patients with proven cardiovascular disease.[8,9] Other studies in humans, demonstrated a direct association between SAM plasma levels and endothelium-dependent-flow mediated vasodilatation, and an inverse correlation with carotid intima-media thickness in nondiabetic subjects.[10,11] However, the authors recognized some limitations of the studies such as, a considerable prevalence of cardiovascular risk factors in the study population.[10]

The aim of this study was to investigate in an animal model, the effect of hypomethylation secondary to a moderate folate or vitamin B12 deficiency, assessed by hepatic SAM/SAH ratio, on endothelium-dependent vascular relaxation (EDR) and endothelium-independent vascular relaxation (EIR) of isolated aorta rings.

Materials and methods

Forty-eight male Wistar rats (180 ± 12 g) were fed ad libitum with a standard rat chow diet from weanling during 24 days until adult age (180 ± 12 g). At 45 days, they were divided into four groups of 12 rats each: folate and vitamin B12 deficiency (FB12D, 0 mg folic acid/kg, 0 μg/kg vitamin B12), folate deficiency (FD 0 mg folic acid/kg and 50 μg/kg B12) vitamin B12 deficiency (B12D: 8 mg/kg folic acid and 0 μg/kg B12 and control diet (CD)). All rats were fed with Vitamin Mix For AIN-76[a] rodent diet without added folate or cyanocobalamin (Research Diets, INC. 20 Jules Lane New Brunswick, NJ 08901) and were provided with drinking water that was either unsupplemented (FB12D) or supplemented with, 50 μg/kg feed vitamin B12 (FD) or 8 mg/kg feed folic acid (B12D). The control group (CD) was fed with AIN-76A Control Diet.

After 8 weeks of feeding with the experimental diets, the animals were killed by a blow to the head.[12] Blood was collected from abdominal aorta for the measurement of serum levels of homocysteine, folate and vitamin B12. The thoracic aorta and liver were removed for the measurement of arterial reactivity and SAM/SAH levels, respectively, as described below.

The experimental protocol was approved by our institution Ethics Committee. Animals were treated in humane conditions.

Laboratory analyses

Serum homocysteine concentration was measured using an Abbott Kit (Abbott IMx homocysteine, Abbott laboratories, Diagnostic division, Abbott Park, I1 60064).

Folic acid and vitamin B12 were measured by the DPC BioMediq Immulite™ 2000 analyser using a chemiluminescent enzyme immunoassay (DPC 4210 Pacific concourse Drive, Los Angeles, CA 90045-6900, USA).

SAM and SAH concentrations in liver homogenates were quantified by HPLC using an Agilent-1100 DAD detector (Hewlett Packard) operating at 260 nm. Frozen liver was weighed, homogenized with $HClO_4$ 0.5 M 1:5 (w/v) in Ultraturrax (Heidolph Diax 900) and centrifuged at 12,500×g for 5 min. Supernatant was filtered through a 0.22 μm Millipore filter. Acid filtrates were directly injected to the HPLC (25 μl). A Hypersil BDS column C18 (53 × 7.0 mm, 3 μm, Alltech Rocket, PA, USA) was used, with a mobile phase that consisted of 40 mM $NH_4H_2PO_4$, 8 mM 1-heptanesulfonic acid, and 18% (v/v) methanol, pH adjusted to 3.0 with HCl. Under these conditions, retention times for SAH and SAM, were 3.3 and 4.4 min, respectively. HPLC analyses were conducted at a flow rate of 3 ml/min at 35 °C. Calibration curves were based on peak area and linear response was obtained between 10 and 1000 pmol for SAM or SAH (Sigma) with a correlation coefficient greater than 0.999 for each curve. The concentrations of SAM and SAH related linearly to the areas under the HPLC chromatogram. Results were expressed as nmol per gram of wet tissue.[13]

Aortic artery reactivity

The thoracic aorta was rapidly removed and carefully cleaned of all fat and connective tissue, taking special care to avoid endothelial damage. Aortic rings (5—8 mm) were mounted immediately on two L-shaped stainless steel hooks in a 30 ml organ bath containing a modified Krebs—Henseleit solution maintained at 37 °C and bubbled with a 95% O_2 and 5% CO_2 gas mixture, as previously described.[12] One of

the hooks was attached through an FT-03 force-displacement transducer to a screw gauge and a model 7 Grass polygraph (Grass Instruments, Quincy, Mass, USA) to record changes in vessel wall tension, while the other was fixed to the bottom of the bath. The resting tensions of the arterial rings were set to 1.5 g by means of the screw gauge. The rings were allowed to equilibrate for 60 min, changing the solution at 15 min intervals to prevent metabolite accumulation. After the stabilization period and before the experiment, a maximal muscle tension was induced by a 70 mM KCl depolarizing solution, as an internal control. The rings were challenged twice until the response reached a plateau, followed by a complete return to the baseline after thoroughly washing to avoid any residual effect of this solution. Following re-equilibration, norepinephrine (NE; 10^{-4} M) was added to the bath and the contractile response was allowed to reach a plateau. Acetylcholine (ACh; 10^{-9}–10^{-6} M) was then added in a cumulative fashion to the bath in ½ $\log_{10}$ increment. The relaxation response was allowed to reach a plateau before adding the next ACh concentration. After washing the rings several times to completely wash out ACh and to attain baseline tension, NE (10^{-4} M) was again added and the relaxation induced by sodium nitroprussiate (NP; 10^{-4} M) was recorded. The maximal relaxation induced with sodium nitroprussiate was achieved with a unique high dose of 100 μM. After the experiment, the wet weight of each ring was recorded. Developed muscular tension was expressed as mg tension/mg wet weight. All changes were expressed as percent of the maximal response achieved by NE in each ring.[12]

Statistical analysis

Statistical analyses were performed using STATISTICA for Windows version 4.5 (StatSoft Inc Tulsa OK, USA 1993). Descriptive data are expressed as mean ± standard deviation. Comparisons between groups were done using ANOVA for repeated measures. Post-hoc comparisons between groups when ANOVA was significant, were done using the Schaffé test. Correlation between variables were analyzed by Pearson's and multiple regression models.

Results

At the end of the eight weeks feeding the four diets, the animals of each group exhibited a similar increase in body weight (Fig. 1). Serum levels of folate, vitamin B12, Hcy, hepatic SAM and SAH concentrations, and SAM/SAH ratio are shown in Table 1. Serum folate levels were lower in FB12D and FD group than in B12D and CD group ($p < 0.0001$). Animals fed the control diet had the highest folate serum concentration. Vitamin B12 levels were lower in the FB12D and B12D group than in the FD and CD group ($p < 0.0001$). CD and FD group had similar vitamin B12 serum concentrations. The highest Hcy concentration was obtained in the FB12D and FD groups and the lowest, in the CD group ($p < 0.007$).

Liver SAM concentrations and SAM/SAH ratios were lower in the FB12D and FD groups ($p < 0.001$ and $p = 0.006$, respectively). Liver SAH concentrations were similar in all groups. SAM correlated inversely with serum Hcy levels

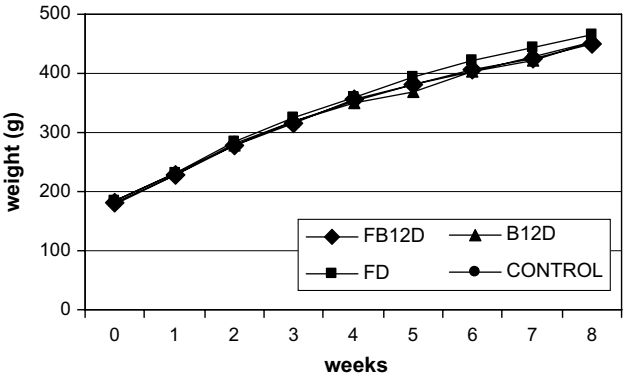

Figure 1 The effect of the four dietary regimens, folate and vitamin B12 deficiency (FB12D), folate deficiency (FD), vitamin B12 deficiency (B12D) and control, on weight gain in rats, during 8 weeks.

($r = -0.42$, $p = 0.003$), and with folate levels ($r = 0.60$, $p < 0.0001$), but not with vitamin B12 levels. In a multiple regression model where the dependent variable was SAM and the independent variables were Hcy, folate and vitamin B12, only folate was a significant predictor of SAM with a beta of 0.65 ($p = 0.001$).

Endothelium-dependent and independent vasorelaxation were similar in all groups as shown in Figs. 2 and 3. Fig. 2 shows the relaxation curves for ACh concentrations ranging from 10^{-9} to 10^{-6} M. Higher concentrations were not tested since no differences were observed in control experiments. There was no association between vascular reactivity and serum levels of Hcy, folate, vitamin B12, SAM, SAH or SAM/SAH ratio.

Discussion

In this study, we did not find an effect of hypomethylation, secondary to a moderate folate or vitamin B12 deficiency, on endothelium-dependent relaxation of the aorta, induced by acetylcholine. Similar results were published by Devlin using mice with a genetic defect in the homocysteine remethylation pathway (*Mthfr*$^{+/-}$) which sensitizes animals to hyperhomocysteinemia caused by dietary folate deficiency.[14] This lack of effect on endothelial function, contrasts with other findings in cystathionine beta-synthase-deficient mice fed a folate replete, methionine-enriched diet.[7,15] One explanation is that we studied wild animals with diet modifications, unlike other investigators that used genetic hyperhomocysteinemic models, that could have different mechanisms of vascular damage. The exposure period to high homocysteine levels could be also a confounding variable, since Dayal found endothelial dysfunction in CBS$^{+/-}$ hyperhomocysteinemic mice after 15 weeks but not after 7 weeks of hyperhomocysteinemia, even in the absence of folate deficiency.[7]

The experimental model was adequate, demonstrated by the differences in serum folate, vitamin B12 and Hcy levels with the diet in each study group. Serum levels of these vitamins and Hcy were similar to those reported by other authors, using the same experimental model.[16,17] The amount of vitamins of each experimental diet was

Table 1 Weight, serum vitamin levels and liver SAM and SAH concentration in rats fed without folate and vitamin B12 (FB12D), folate (FD), vitamin B12 (B12D) and controls

	FB12D	FD	B12D	Control
Initial weight g	180.58 ± 10.69	184.42 ± 11.87	182.92 ± 11.59	178.75 ± 10.91
Final weight g	451.42 ± 34.64	465.00 ± 43.62	453.25 ± 50.15	452.58 ± 40.09
Serum Hcy umol/L	24.26 ± 5.09^{b}	22.92 ± 3.04^{b}	17.34 ± 2.65^{c}	10.00 ± 3.50^{a}
Serum folate nmol/L	31.81 ± 16.00^{b}	29.87 ± 22.73^{b}	74.96 ± 35.67^{c}	186.52 ± 55.54^{a}
Serum B_{12} pmol/l	200.25 ± 53.34^{b}	874.84 ± 315.71^{a}	178.48 ± 59.10^{b}	797.86 ± 91.41^{a}
Liver SAM mol/g wet tissue	55.49 ± 9.40^{b}	48.83 ± 8.19^{b}	63.64 ± 17.23^{c}	75.48 ± 7.54^{a}
Liver SAH mol/g wet tissue	34.42 ± 9.66	33.98 ± 9.41	33.57 ± 8.53	30.47 ± 8.28
SAM/SAH	1.72 ± 0.49^{b}	1.55 ± 0.54^{b}	2.02 ± 0.67	2.61 ± 0.60^{a}

ANOVA $p < 0.007$.
SAM = *S*-adenosylmethionine, SAH = *S*-adenosylhomocysteine.
[a] and [b] = significantly different, [c] and [b, a] significantly different by Scheffé post-hoc analysis.

calculated according to the requirements for the species (NRC, 1995). Vitamin B12 and folate concentrations of the control diet (commercial diet) were higher than the NRC recommendations. Serum levels of vitamin B12 were similar in FD and C groups. Vitamin B6 was not measured, but we can assume that it was within normal values, since the vitamin mix used has adequate levels of this vitamin. Conversely serum folate levels were related to the amount of folate in each diet. This disparity is because body homeostasis of these vitamins is different. Vitamin B12 is absorbed by a saturable mechanism and folate is absorbed both by a saturable (active transportation) and a non-saturable mechanism (passive diffusion).[18]

The present study also demonstrates that a moderate depletion of folate but not of vitamin B12 induces a reduction in SAM and the SAM/SAH ratio in the liver. Hypomethylation has been associated with age, atherosclerosis and cancer.[19] Global DNA hypomethylation was demonstrated in humans, rabbits, and ApoE knock-out mice with advanced atherosclerosis, both in vascular tissue and peripheral blood cells.[20–22] In addition, circulating homocysteine levels correlated with the extent of DNA methyl-group loss in advanced atherosclerosis[22] and in patients with end stage renal disease.[23] Therefore, most of the studies that associate hypomethylation with cardiovascular disease use models with pre-existent cardiovascular risk or genetic alterations. In this study we have chosen a pure animal model to measure only the effect of global hypomethylation due to dietary folate or Vitamin B12 deficiencies on vascular reactivity, to avoid the well known effect of aging, lipids alterations, oxidative stress or inflammation on vascular function. We did not look for histological damage of the endothelium, because functional changes in arteries precede histological alterations in humans and animals. Moreover, rats are resistant to develop atherosclerotic plaques secondary to hypercholesterolemia or hyperhomocysteinemia,[24] and

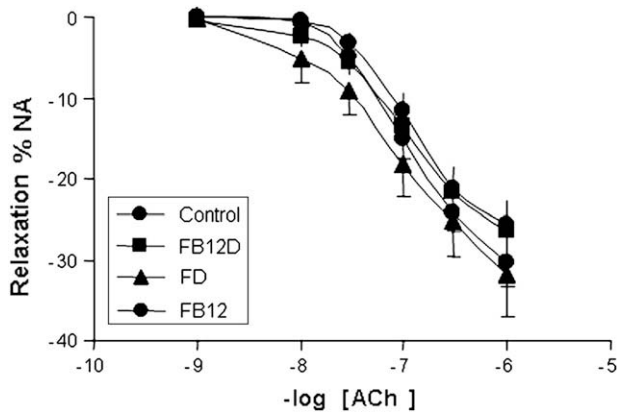

Figure 2 Concentration-response curves for acetylcholine (10^{-9}–10^{-6} M) (−log ACh)-induced relaxation determined in thoracic aortic rings of rats fed without folate and vitamin B12 (FB12D), folate (FD), vitamin B12 (B12D) and control. The developed tension of each cumulative dose is expressed as percentage of maximal contractile response achieved by norepinephrine (10^{-4} M) (NA). Points represent the mean ± SD of 7 independent determinations.

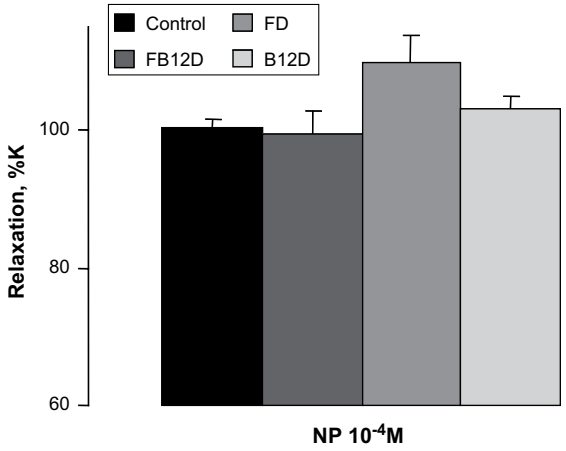

Figure 3 Sodium nitroprusside (10^{-4} M) (NP) evoked vasorelaxation in thoracic aortic rings of rats fed without folate and vitamin B12 (FB12D), folate (FD), vitamin B12 (B12D) and controls. The developed tension by NP is expressed as percentage of the maximal contractile response achieved by 70 mM KCL (K) depolarizing solution in each ring. Points represent the mean ± SD of 7 independent determinations.

scattered lipid staining in aortic sections can only be observed in older (10 month old), homozygous *Mthfr* knockout mice.[19]

Other explanations for the lack of positive results, could be that we observed a decrease in SAM/SAH ratio in the liver due to a decrease in SAM concentration and not to an elevation of SAH, that is more indicative of inhibition of methylation reactions than SAM/SAH ratio.[25] It is also possible that the degree of hypomethylation varies among organs[26] and what we found in the liver is not reflecting accurately what is happening in the aorta. Unfortunately we and other authors[7] are not able to reliably measure SAM or SAH in vascular tissue.

In conclusion, folate deficiency during eight weeks generates hypomethylation, which is not related to a disruption of the endothelial function in adult Wistar rats.

Conflict of interest

The authors declare no conflict interest.

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