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ORIGINAL ARTICLE

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

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KEYWORDS
Methylation folate;
Homocysteine;
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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