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High-methionine diets accelerate atherosclerosis by HHcy-mediated FABP4 gene demethylation pathway via DNMT1 in ApoE$^{-/-}$ mice

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ABSTRACT

Homocysteine (Hcy) is an independent risk factor for atherosclerosis, but the underlying molecular mechanisms are not known. We investigated the effects of Hcy on fatty acid-binding protein 4 (FABP4), and tested our hypothesis that Hcy-induced atherosclerosis is mediated by increased FABP4 expression and decreased methylation. The FABP4 expression and DNA methylation was assessed in the aorta of ApoE$^{-/-}$ mice fed high-methionine diet for 20 weeks. Over-expression of FABP4 enhanced accumulation of total cholesterol and cholesterol ester in foam cells. The up-regulation of DNA methyltransferase 1 (DNMT1) promoted the methylation process and decreased FABP4 expression. These data suggest that FABP4 plays a key role in Hcy-mediated disturbance of lipid metabolism and that DNMT1 may be a novel therapeutic target in Hcy-related atherosclerosis.

1. Introduction

Epidemiological evidence suggests that hyperhomocysteinemia (HHcy) is an independent risk factor for atherosclerosis [1]. Numerous mechanisms have been suggested to explain the pathogenesis of homocysteine (Hcy)-associated atherosclerosis, including inflammatory injury and dyslipidemia [2–4]. However, the exact mechanism of lipid disturbances with HHcy remains unclear.

Atherosclerosis is a chronic inflammatory disease characterized by deposition of lipids, especially cholesterols, in the arterial endothelium [5,6]. Inflammation and disturbed lipid metabolism are two essential pathogenic processes of atherosclerosis [7]. Hcy is an amino acid involved in inflammation and dyslipidemia during atherosclerosis [8]. Anchoring a target gene that regulates inflammation and lipid metabolism may bring a breakthrough for understanding Hcy-induced atherosclerosis. Fatty acid binding protein 4 (FABP4), a cytoplasmic fatty acid chaperone, is expressed in both adipocytes and macrophages [9]. It plays a vital role in the metabolic dysfunction that underlies the relationship between inflammatory events and lipid metabolism [10]. High FABP4 levels in atherosclerotic lesions are associated with an unstable plaque phenotype and increased risk of cardiovascular events [11]. Low levels of FABP4 in macrophages decrease the accumulation of cholesteryl ester (CE) and secretion of inflammatory cytokines (TNF-a, MCP-1, and IL-6) [12–14]. The expression of FABP4 in macrophages is

Abbreviations: AS, atherosclerosis; Hcy, homocysteine; HHcy, Hyperhomocysteinemia; ApoE$^{-/-}$, apolipoprotein E knockout; FABP4, fatty acid-binding protein 4; DNMT1, DNA methyltransferases 1; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; TC, total Cholesterol; FC, free Cholesterol; CE, Cholesteryl Ester; AZC, azacytidine

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suppressed by cholesterol-lowering statins in vitro. Overexpression of FABP4 facilitates foam cell formation in THP-1 macrophages, and enhances the accumulation of cholesterols and triglycerides [15,16]. Hcy suppresses adipogenesis in 3T3-L1 preadipocytes by decreasing lipid accumulation and downregulating gene expression of adipocyte protein 2 and peroxisome proliferator-activated receptor gamma (PPAR-gamma) [17]. Therefore, we hypothesized that FABP4 may play an important role in Hcy-induced lipid metabolism disorders.

Hcy is an intermediate product of the methionine cycle that affects gene expression by DNA methylation modification [18]. DNA methylation occurs by covalent addition of a methyl group from the methyl donor S-adenosylmethionine (SAM) to the 5′ carbon of the cytosine ring in CpG pairs. SAM is converted to S-adenosylhomocysteine (SAH) during transmethylation. SAH in turn undergoes hydrolysis to form Hcy [19]. Aberrant DNA methylation induces inappropriate gene expression and promotes disease occurrences. Many studies have reported an association between HHcy and aberrant DNA methylation in several diseases, including atherosclerosis, osteoporosis, uremia, and alcoholism [20]. Additionally, we found that DNA methylation, such as LOX-1, ApoE, and MCP-1, is a key molecular target that mediates Hcy-induced atherosclerosis [21,22]. However, there is no study reporting the association between Hcy and FABP4 gene methylation in atherosclerosis.

This study was designed to investigate whether and how HHcy disturbs lipid metabolism. We explored whether HHcy interferes with DNA methylation status of FABP4 gene via DNMT1 in ApoE$^{-/-}$ mice and foam cells.

2. Materials and methods

2.1. Animals

Six-week-old male wild-type mice ($n = 12$) and ApoE$^{-/-}$ mice ($n = 36$) with a C57BL/6J genetic background (Animal Center of Peking University Health Science Center, Beijing, China) were housed in a temperature-controlled (24 °C) facility with a 12-h light/dark cycle and free access to food and water. The mice were equally divided into four groups and maintained for 20 weeks on the following diets (KeAoXieLi, Beijing, China): (1) normal control (N-control) group, where C57BL/6 J mice were fed with regular diet; (2) ApoE$^{-/-}$ mice control (A-control) group, where ApoE$^{-/-}$ mice were fed with the same diet as N-control group; (3) Meth group, where ApoE$^{-/-}$ mice were fed with regular diet plus 1.7% methionine; and (4) Meth-F group, where ApoE$^{-/-}$ mice were fed with regular diet plus 1.7% methionine (wt/wt), 0.006% folate, and 0.0004% vitamin B_{12}. Mice were anesthetized by intraperitoneal (i.p.) pentobarbital (50 mg/kg body weight) and a bolus dose of 100 mg/kg, if the animal struggled in pain during monitoring. The adequacy of anesthesia was assessed by monitoring toe-pinch reflex and regular respiration. Aortic tissues were frozen in liquid nitrogen and stored at −80 °C until analysis. The research protocol of this study was approved by the Committee on the Ethics of Animal Experiments of the Health Science Center of Ningxia Medical University. This study also conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health.

2.2. Cell culture

Human monocytic leukemia THP-1 cells were cultured in RPMI 1640 (GIBICO, USA) medium containing 10% heat-inactivated fetal bovine serum (HI-FCS; 56 °C, 45 min), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C in a 5% CO_2 incubator. THP-1 monocytes were cultured with 100 nmol/L PMA (Sigma–Aldrich, St Louis, USA) for 24 h to facilitate differentiation into macrophages. The adherent macrophages were washed thrice with phosphate-buffered saline (PBS). The macrophages were transformed into foam cells by incubation with 50 mg/L ox-LDL (Sigma–Aldrich) in the medium together with different concentrations of Hcy (50, 100, 200, and 500 μmol/L), DNA methylation inhibitor-azacytidine (10 μmol/L AZC), and its antagonists (30 μmol/L vitamin B_{12} and 30 μmol/L folate containing 100 μmol/L Hcy) for 48 h. Only the cell preparations with a 95% or greater viability were used. For each experiment, a minimum of three independent experiments were performed.

2.3. Tissue preparation and evaluation of atherosclerotic lesions in ApoE$^{-/-}$ mice

After blood sampling, mice were sacrificed and their hearts were flushed with saline. The aortas were embedded in OCT and snap-frozen in liquid N_2. The inferior vena cava was cut to allow the perfusate to exit. Frozen sections of 10 mm thickness were taken in the region of the proximal aorta, starting from the end of the aortic sinus and 300 mm distally. This was done according to the technique of Paigen et al. [23]. Sections were stained with Oil Red O and counterstained with hematoxylin. Quantitative analysis of lipid-stained lesions was performed on sections starting just beyond the end of the aortic sinus. The lipid-stained lesions were measured by digitizing morphometry and reported in mm^2 per lesion.

2.4. Detection of serum Hcy and lipid concentrations in ApoE$^{-/-}$ mice

Blood samples were collected from the mice at the start of the experiment. Serum Hcy concentrations and lipid profiles, including total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL) and low density lipoprotein (LDL) were measured using an automatic biochemistry analyzer (SIEMENS, Germany).

2.5. qRT-PCR for FABP4, DNMT1, DNMT3a and DNMT3b mRNA

Total RNA from ApoE$^{-/-}$ mice and cultured cells were isolated by using Trizol reagent (Invitrogen, Grand Island, USA) and reversely transcribed via the Revert Aid first strand cDNA synthesis kit (MBI, Vilnius, Lithuania). qRT-PCR was carried out on the FTC3000 qRT-PCR detection system under the following conditions: 45 cycles at 95 °C for 45 s, the annealing temperature for 45 s and extension 60 °C for 60 s. The oligonucleotide sequence of primers and probes is listed in Table 1. The mRNA level of each gene was calculated from the value of the threshold cycle (Ct) of the qRT-PCR, as related to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH): $Ct = Ct_{(GAPDH)} - Ct_{(sample)}$. The final results were expressed as a N-fold difference in the target gene expression. The relationship to the calibrator, termed as "Ntarget," was determined as follows: $N_{target} = 2^{Ct(sample)-Ct(calibrator)}$. The Ct values of the calibrator and the sample were determined by subtracting the Ct value of the target gene from the Ct value.

2.6. Western blot for FABP4, DNMT1, DNMT3a, and DNMT3b protein

The protein was resolved electrophoretically by SDS–PAGE (10% polyacrylamide) and transferred to the polyvinylidene difluoride membranes on a platform shaker. It was washed thrice for 5 min each in Tris-buffered saline plus Tween-20 (TBST). The membrane was incubated with a polyclonal anti-FABP4 antibody (ab66682, 1:1000; Abcam), monoclonal anti-DNMT1 antibody (ab13537, 1:1000; Abcam), polyclonal anti-DNMT3a antibody (ab23565, 1:1000; Abcam), or polyclonal anti-DNMT3b antibody (ab2851,