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ORIGINAL CONTRIBUTION

Betaine supplementation attenuates atherosclerotic lesion in apolipoprotein E-deficient mice

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Abstract
Background Betaine serves as a methyl donor in a reaction converting homocysteine to methionine. It is commonly used for the treatment of hyperhomocysteinemia in humans, which indicates it may be associated with reduced risk of atherosclerosis. However, there have been few data regarding its vascular effect.
Aim of the study To investigate the effect of betaine supplementation on atherosclerotic lesion in apolipoprotein (apo) E-deficient mice.
Methods Four groups of apoE-deficient mice were fed AIN-93G diets supplemented with 0, 1, 2, or 4 g betaine/100 g diet (no, 1, 2, and 4% betaine, respectively). Wild-type C57BL/6 J mice were fed AIN-93G diet (wild-type). Mice were sacrificed after 0, 7, or 14 weeks of the experimental diets. Atherosclerotic lesion area in the aortic sinus, levels of tumor necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1 in aorta and serum, serum lipids, and methylation status of TNF-α promoter in aorta were determined.
Results Linear regression analysis showed that the higher dose of betaine was related to smaller atherosclerotic lesion area ($\beta = -11.834$, $P < 0.001$). Compared with no-betaine mice after 14 weeks, mice receiving 1%, 2%, or 4% betaine had 10.8, 41, and 37% smaller lesion area, respectively. Betaine supplementation also reduced aortic expression of TNF-α in a dose-dependent way in four groups of apoE-deficient mice, and Pearson correlation revealed that atherosclerotic lesion area was positively associated with aortic TNF-α level ($r = 0.777$, $P < 0.001$). Although serum TNF-α levels were lower in betaine-supplemented mice than in no-betaine mice after fourteen weeks of treatment ($P < 0.001$), we did not observe a significant dosage effect ($P = 0.11$). However, methylation level of TNF-α promoter did not differ among groups at any time. In this study, apoE-deficient mice receiving betaine supplementation for 14 weeks had higher concentrations of serum total cholesterol ($P < 0.01$), LDL cholesterol ($P < 0.05$), and lower body weight ($P < 0.05$) than no-betaine mice.
Conclusions These data suggest that despite exacerbating hyperlipidemia in apoE-deficient mice, betaine may exert its anti-atherogenic effect by inhibiting aortic inflammatory response mediated by TNF-α.

Keywords Betaine · Atherosclerosis · Inflammation · Lipids · DNA methylation

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Introduction

Atherosclerosis is a pathological condition that underlies several important adverse vascular events including coronary artery disease and stroke. There are few effective pharmacological approaches to treatment of this disease because of its complex pathogenesis. The view that atherosclerosis is a chronic inflammatory disease [24] is now

widely accepted. A large number of inflammatory factors are involved in atherogenesis, for example interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1) [26]. Recent evidence indicates that atherosclerosis is also related to aberrant DNA methylation [3, 13, 15, 16, 22]. DNA hypermethylation and hypomethylation both occurred before the appearance of any histologically detectable aortic lesions in four-week-old apoE-deficient mice [16]. These findings indicate that approaches which could affect inflammatory response and DNA methylation may be effective in treating atherosclerosis.

Betaine (trimethylglycine) is formed in vivo as the oxidation product of choline or can be obtained externally from various foods [30]. It has three active methyl groups and is an alternative methyl donor for homocysteine remethylation. Betaine supplementation (1.5–6 g/d) reduces fasting and post-methionine loading plasma homocysteine concentrations in human subjects [19, 25]. It also can ameliorate alcoholic and non-alcoholic fatty liver disease by attenuating steatosis, oxidative stress, and fibrosis [1, 12]. Considering that both hyperhomocysteinemia and inflammation are main mechanisms of atherosclerosis, these data imply that betaine is a promising agent to counteract atherosclerosis. However, the effect of betaine supplementation on atherogenesis has not yet been well studied.

The apoE-deficient mouse is an animal model that is widely used for determining the efficacy of dietary intervention in retardation of atherosclerosis. They develop severe hypercholesterolemia and spontaneous atherosclerotic lesions that mimic, in distribution and appearance, those observed in humans [33]. The purposes of this study were to determine whether betaine supplementation could attenuate early atherosclerotic lesion in apoE-deficient mice and, further, to explore the possible mechanisms by which betaine affects the development and progression of atherosclerosis.

Materials and methods

Animals and diets

Male wild-type C57BL/6 J mice ($n = 18$) and apoE-deficient mice on a C57BL/6 J background ($n = 54$) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mice were acclimated on a standard AIN-93G diet [23] for one week. At seven weeks of age, six wild-type and six apoE-deficient mice were sacrificed for baseline analysis. The remaining wild-type mice continued to consume AIN-93G diet alone (wild-type group, $n = 12$) while the rest of the apoE-deficient mice were randomly divided into four groups and fed AIN-93G diets supplemented with 0, 1, 2, or 4 g betaine/100 g diet (no, 1, 2, and 4% betaine groups, $n = 12$, respectively). Betaine was purchased from Cultor, Finnsugar Bioproducts (Finland). Mice were housed three per cage with free access to food and water. Body weight and consumption of diet were recorded weekly. After seven and fourteen weeks of experimental diets, six mice from each group were sacrificed by cervical dislocation under anesthesia. This study was approved by the Institutional Animal Care and Use Committee at Sun Yat-Sen University.

Assessment of atherosclerotic lesion area

Fatty streaks were quantified by evaluation of oil red O-stained lesions in the aortic sinus as previously described [21] with a few modifications. Upon sacrifice, the heart was removed and fixed in 10% buffered formalin for more than 48 h. It was then cut just below the beginning of the aortic sinus and the upper portion was embedded in OCT compound (Sigma, USA). Six 8-μm sections (spaced 80 μm apart) per mouse obtained throughout the aortic sinus (400 μm) were stained with oil red O and counterstained with hematoxylin. Lesion areas were measured by a technician who was unaware of the study using Image-Pro Plus software (Media Cybernetics, USA). The mean percentage of lesion to lumen area of six sections was used as the final value for each mouse.

Analysis of aortic TNF-α and MCP-1

Aortic TNF-α and MCP-1 protein levels were analyzed by western blot. After animals were sacrificed, dissected aortas were homogenized in ice-cold buffer containing 20 mM Tris–HCl (pH 7.0), 1% Triton X-100 and protease inhibitors. Protein concentrations were determined using BCA protein assay kit. Total proteins (60–120 μg) were subjected to electrophoresis on 12% SDS-polyacrylamide gels (Bio-Rad, USA), followed by electrophoretic transfer to PVDF membranes (Amersham, USA) overnight at 30 V. Membranes were blocked with 5% skim milk in Tris-buffered saline/Tween (TBST) for 2 h at room temperature, and then incubated with polyclonal anti-TNF-α (1:100; Boster, People's Republic of China), polyclonal anti-MCP-1 (1:100; Boster), and monoclonal anti-β-actin (1:10,000; Kangcheng, People's Republic of China) antibody for 2 h at room temperature. After three washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000) for 1 h at room temperature. After three washes with TBST, bands were stained with BeyoECL Plus substrate and the density of each band was analyzed with Quantity version 5.0 software from Bio-Rad. β-Actin was used as internal control and the

levels of TNF-α and MCP-1 proteins were expressed as the ratio of TNF-α/β-actin and MCP-1/β-actin, respectively.

Serum inflammatory factors and lipids

Mice were anesthetized and fasting blood was drawn by eyeball removal. Animals were sacrificed after the terminal blood samples. Serum was separated and stored at −40 °C until further analysis. Serum TNF-α and MCP-1 concentrations were measured with mouse ELISA kits (Bender Medsystems, Austria). Serum concentrations of total cholesterol (TC), triglyceride (TG), HDL cholesterol (HDL-C), and LDL cholesterol (LDL-C) were determined using commercially available kits (Biosino, People's Republic of China) on a automatic biochemistry analyzer (A25 Biosystem, Spain).

Methylation assay for TNF-α promoter

Genomic DNA was extracted from the aorta with a kit (TaKaRa, Japan) following the manufacturer's instructions. The methylation status of the CpG islands in the promoter region of the mouse TNF-α gene was determined by bisulfite modification and subsequent methylation-specific PCR (MSP), as previously described [10], with some modifications. Briefly, 2 μg genomic DNA was used for bisulfite modification. MSP was performed using methyl-specific primers (M forward, 5′-CGATTTAGAGATTAC GGTTCGAGC-3′; M reverse, 5′-AATAAACGCCGT CCTATAACAACG-3′) and unmethyl-specific primers (U forward, 5′-TTGATTTAGAGATTATGGTTTGAGT GT-3′; U reverse, 5′-ATAAACACCATCCTATAACAACA CC-3′). The length of both expected methyl and unmethyl products was 168 bp. Methyl amplification conditions for TNF-α promoter were: initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 30 s, annealing at 63 °C for 30 s, and extension at 70 °C for 30 s for 30 cycles, followed by a stabilization at 70 °C for 5 min. Unmethyl amplification followed the same procedure with the exception of annealing temperature, which was 65 °C. Final PCR products were separated on 2% agarose gels and visualized under UV illumination.

Statistical analysis

Results were expressed as mean ± SD. Student's *t*-test and one-way ANOVA were used to examine differences between or among groups, respectively. Multiple comparison was done with the least significant difference (LSD) test. Linear regression was conducted to assess the relationship between the dose of betaine and atherosclerotic lesion area, and aortic and serum expression levels of TNF-α. Pearson correlation was used to examine the association between aortic TNF-α and atherosclerotic lesion area. A two-tailed $P < 0.05$ was considered significant. Analyses were performed with SPSS 13.0 software.

Results

Body weight and food intake

There was a progressive increase in body weight over 14 weeks in all groups ($P < 0.001$). The initial body weight of five groups did not differ from one another. After 14 weeks, the no-betaine group had lower body weight than the wild-type group ($P = 0.017$) and betaine supplementation further reduced the body weight compared with the no-betaine group ($P < 0.05$) (Fig. 1). Betaine was well tolerated and no adverse effects were observed. Food intake did not differ among groups at any time (data not shown).

Atherosclerotic lesion area

After 14 weeks of treatment, oil red O staining of aortic sinus showed that there was no visible fatty streak in wild-type mice. However, lesions of different degrees were observed in four groups of apoE-deficient mice (Fig. 2a). Supplementation with 1, 2, and 4% betaine led to 10.8% ($P = 0.40$), 41% ($P = 0.019$), and 37% ($P = 0.020$), respectively, reduction in atherosclerotic lesion area compared with the no-betaine group (Fig. 2b). Linear regression showed higher dose of betaine was related to smaller atherosclerotic lesion area ($\beta = -11.834$, $P < 0.001$) (Fig. 3).

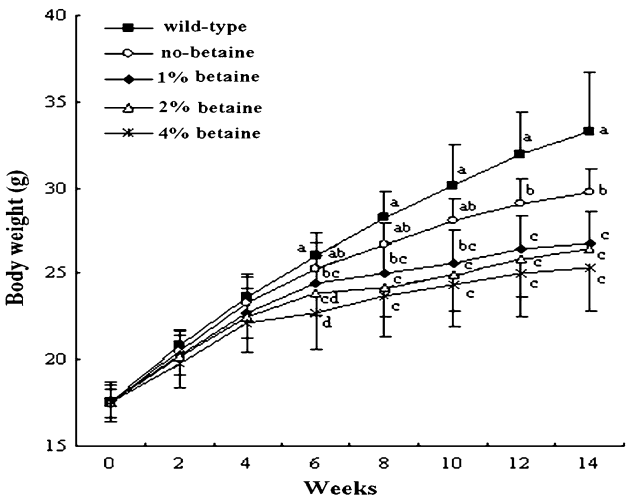

Fig. 1 Body weights of wild-type mice fed AIN-93G diet (wild-type) and apoE-deficient mice fed AIN-93G diet supplemented with 0, 1, 2, or 4 g betaine/100 g diet. Values are means ± SD, $n = 6$–12. Within a week, means without a common letter differ, $P < 0.05$

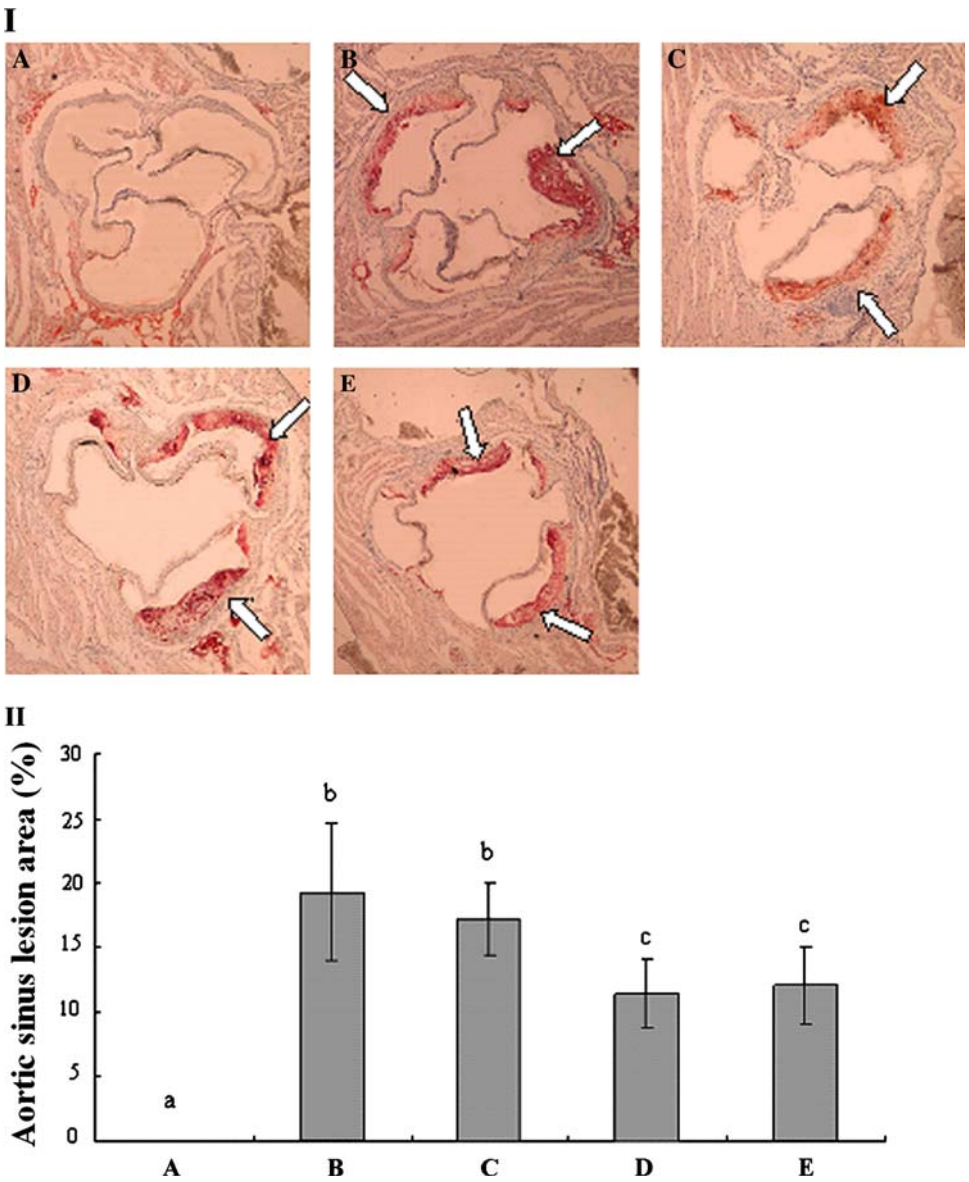

Fig. 2 Cryostat sections (**a**) and atherosclerotic lesion area (**b**) of aortic sinus in wild-type mice fed AIN-93G diet (wild-type, *A*) and apoE-deficient mice fed AIN-93G diets supplemented with 0 (no, *B*), 1 (1% betaine, *C*), 2 (2% betaine, *D*), or 4 (4% betaine, *E*) g betaine/100 g diet for 14 weeks. In **a**, the fatty streaks were stained red (*arrows*; original magnification, 40×). In **b**, values are mean ± SD, $n = 6$. Bars without a common letter differ, $P < 0.05$

Aortic TNF-α and MCP-1

After 14 weeks, protein levels of TNF-α were higher in the no-betaine group than in the wild-type group ($P < 0.001$), and betaine supplementation reduced TNF-α expression in apoE-deficient mice in a dose-dependent way ($P < 0.001$) (Fig. 4). Correlation analysis revealed a significant positive association between aortic TNF-α and atherosclerotic lesion area ($r = 0.777$, $P < 0.001$) (Fig. 5). However, there was no significant difference in MCP-1 protein levels among apoE-deficient mice (data not shown).

Serum TNF-α and MCP-1

The initial concentrations of the two inflammatory factors were higher in apoE-deficient mice than that in wild-type mice ($P < 0.05$ for both factors). After seven weeks of treatment, the 1% betaine group had lower concentrations of serum TNF-α ($P = 0.001$) and MCP-1 ($P = 0.011$) than the no-betaine group. Betaine supplementation for 14 weeks reduced serum TNF-α ($P < 0.001$) but not MCP-1 compared with the no-betaine group (Table 1). Linear regression showed there was no significant negative relationship between the dose of betaine and serum TNF-α ($P = 0.11$) (data not shown).

Serum lipids

ApoE-deficient mice had significant elevation in concentrations of serum lipids except TG compared with wild-type mice at the beginning of the study (all $P < 0.001$). After 14 weeks, the 2 and 4% betaine groups still had

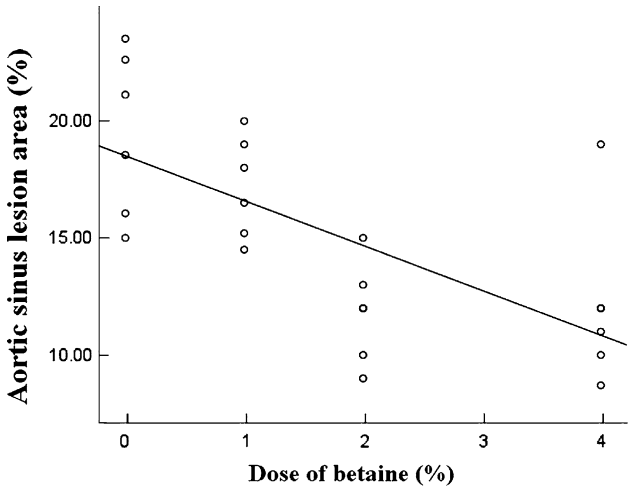

Fig. 3 Linear regression analysis of atherosclerotic lesion area vs dose of betaine in apoE-deficient mice fed AIN-93G diets supplemented with 0, 1, 2 or 4 g betaine/100 g diet

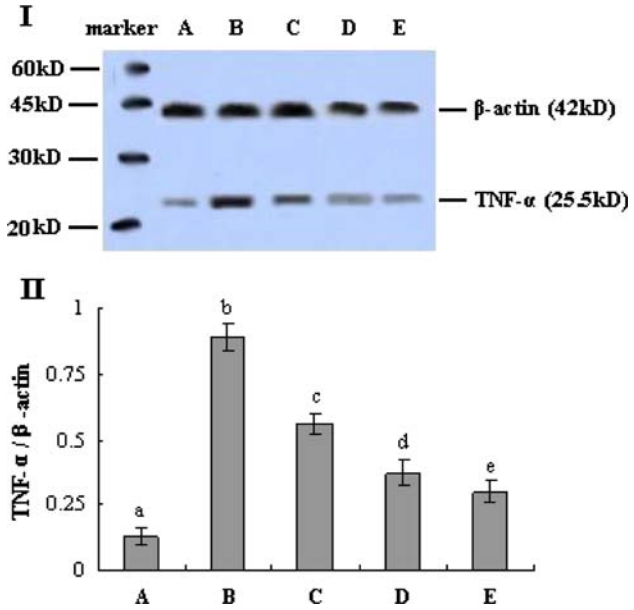

Fig. 4 **a** Western blot analysis of TNF-α protein expression in aorta in wild-type mice fed AIN-93G diet (wild-type, *A*) and apoE-deficient mice fed AIN-93G diets supplemented with 0 (no, *B*), 1 (1% betaine, *C*), 2 (2% betaine, *D*), or 4 (4% betaine, *E*) g betaine/100 g diet for 14 weeks. **b** TNF-α protein levels were expressed as the ratio of TNF-α/β-actin. Values are mean ± SD, $n = 6$. Bars without a common letter differ, $P < 0.05$

higher concentrations of TC ($P < 0.01$) and LDL-C ($P < 0.05$) than the no-betaine group. However, after seven weeks of betaine supplementation, serum TC ($P < 0.002$) and LDL-C concentrations ($P < 0.02$) increased even more compared with no-betaine mice. Serum HDL-C concentration was higher in betaine-supplemented groups after seven weeks ($P < 0.003$) and in the 4% betaine group after 14 weeks ($P = 0.046$) compared with the no-betaine group

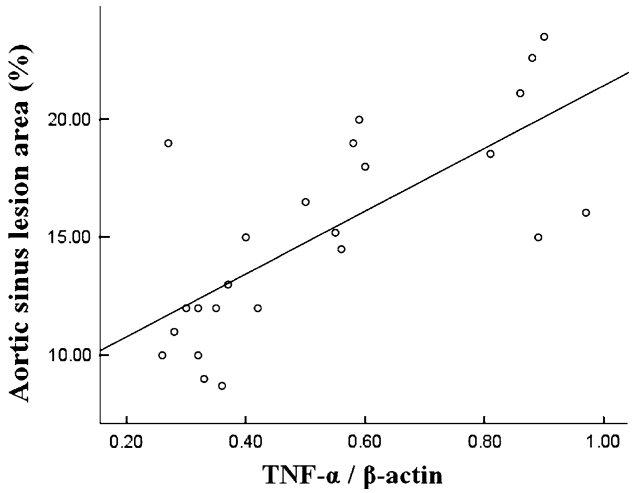

Fig. 5 Pearson correlation analysis of atherosclerotic lesion area vs aortic TNF-α protein level in apoE-deficient mice fed AIN-93G diets supplemented with 0, 1, 2 or 4 g betaine/100 g diet

Table 1 Serum TNF-α and MCP-1 concentrations in wild-type mice fed AIN-93G diet (wild-type) and apoE-deficient mice fed AIN-93G diets supplemented with 0, 1, 2 or 4 g betaine/100 g diet (no, 1%, 2%, and 4% betaine, respectively)

Time (week)	Group	TNF-α (pg/ml)	MCP-1 (pg/ml)
0	Wild-type	47.25 ± 3.14^{a}	44.58 ± 3.92^{a}
	ApoE-deficient	51.42 ± 3.21^{b}	51.29 ± 4.44^{b}
7	Wild-type	47.25 ± 3.14^{a}	49.66 ± 2.46^{a}
	No-betaine	65.11 ± 4.10^{b}	60.81 ± 5.62^{b}
	1% betaine	56.11 ± 5.49^{c}	54.46 ± 4.33^{c}
	2% betaine	60.65 ± 5.00^{bc}	56.77 ± 2.44^{bc}
	4% betaine	62.07 ± 3.42^{bd}	58.80 ± 4.35^{bc}
14	Wild-type	48.52 ± 4.45^{a}	51.90 ± 2.06^{a}
	No-betaine	79.40 ± 4.68^{b}	67.05 ± 6.82^{b}
	1% betaine	56.33 ± 3.86^{c}	65.29 ± 6.01^{b}
	2% betaine	63.04 ± 4.67^{d}	63.00 ± 5.81^{b}
	4% betaine	65.52 ± 3.97^{d}	69.90 ± 7.31^{b}

Values are mean ± SD, $n = 6$. Means in a column without a common subscript letter differ, $P < 0.05$

at the corresponding time. In apoE-deficient mice, serum TG concentration did not differ with or without betaine supplementation at seven or 14 weeks (Table 2).

Methylation of TNF-α promoter

The results from electrophoresis of MSP products showed that most of the CpG islands of the TNF-α promoter were partially methylated, i.e., both methylated and unmethylated bands were detected (Fig. 6). For TNF-α promoter, there were no significant differences in methylation frequency among groups at any time (data not shown).

Table 2 Serum lipid concentrations in wild-type mice fed AIN-93G diet (Wild-type) and apoE-deficient mice fed AIN-93G diets supplemented 0, 1, 2 or 4 g betaine/100 g diet (no, 1%, 2%, and 4% betaine, respectively)

Time (week)	Group	TC (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	TG (mmol/L)
0	Wild-type	2.43 ± 0.18^{a}	0.45 ± 0.06^{a}	0.22 ± 0.10^{a}	1.83 ± 0.35^{a}
	ApoE-deficient	9.51 ± 0.65^{b}	1.39 ± 0.23^{b}	0.97 ± 0.29^{b}	1.45 ± 0.31^{a}
7	Wild-type	2.70 ± 0.26^{a}	0.53 ± 0.04^{a}	0.27 ± 0.08^{a}	1.53 ± 0.53^{a}
	No-betaine	10.68 ± 0.74^{b}	1.51 ± 0.14^{b}	0.88 ± 0.21^{b}	1.83 ± 0.41^{a}
	1% betaine	14.24 ± 2.01^{c}	2.20 ± 0.53^{c}	1.95 ± 0.56^{c}	1.90 ± 0.69^{a}
	2% betaine	14.83 ± 2.23^{c}	2.19 ± 0.31^{c}	1.95 ± 0.86^{c}	1.83 ± 0.36^{a}
	4% betaine	16.88 ± 2.07^{d}	2.54 ± 0.46^{c}	2.30 ± 1.08^{c}	1.80 ± 0.46^{a}
14	Wild-type	3.53 ± 0.63^{a}	0.53 ± 0.04^{a}	0.19 ± 0.04^{a}	1.64 ± 0.26^{a}
	No-betaine	12.77 ± 0.90^{b}	2.14 ± 0.16^{b}	1.37 ± 0.17^{b}	1.97 ± 0.33^{a}
	1% betaine	13.99 ± 0.92^{b}	2.16 ± 0.19^{bc}	1.42 ± 0.26^{b}	1.76 ± 0.21^{a}
	2% betaine	16.50 ± 3.48^{c}	2.40 ± 0.49^{bc}	2.22 ± 0.86^{c}	1.95 ± 0.12^{a}
	4% betaine	17.60 ± 2.20^{c}	2.50 ± 0.35^{c}	2.46 ± 0.87^{c}	1.96 ± 0.17^{a}

Values are mean ± SD, $n = 6$. Means in a column without a common subscript letter differ, $P < 0.05$

TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglyceride

Discussion

As far as we are aware, this study describes for the first time the effect of betaine supplementation on the formation of atherosclerotic fatty streak in apoE-deficient mice. The results showed that betaine could significantly inhibit atherogenesis in the aortic sinus of apoE-deficient mice.

ApoE is a major component of several classes of lipoproteins and is critical for maintaining overall plasma cholesterol homeostasis [7]. In this study, we confirmed a previous report that apoE-deficient mice have severe hypercholesterolemia compared with wild-type counterparts [33]. However, this study also demonstrated that betaine supplementation further exacerbated hyperlipidemia, which was consistent with several research results for humans [1, 20, 25]. The exact mechanism of this adverse effect is not clear and remains to be elucidated. At present, the most likely explanation derives from an obligate requirement for phosphatidylcholine (PC) to secrete very-low-density lipoprotein (VLDL) [28]. Biosynthesis of PC is mainly through two pathways: combination of cytidine diphosphatecholine with diacylglycerol and methylation of phosphatidylethanolamine (PE). Endogenous betaine is formed from choline, and perhaps betaine supplementation spares this use of choline so that more is available for PC biosynthesis in the liver [31]. Betaine could provide adequate methyl groups to facilitate the methylation of PE to form PC. The two functions work synergistically to enhance synthesis and secretion of VLDL, which subsequently results in increased transportation of lipids from hepatocytes to the peripheral circulation, eventually leading to elevation in circulating TC and LDL-C concentrations. Despite exacerbating dyslipidemia in apoE-deficient mice, no increase in atherosclerotic lesion area was observed, suggesting that betaine inhibits the development of atherosclerosis independently of lipid-lowering effects.

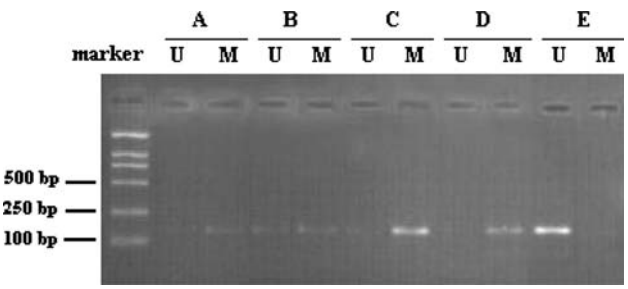

Fig. 6 Representative examples of MSP analysis of TNF-α promoter in aorta in wild-type mice fed AIN-93G diet (wild-type, *A*) and apoE-deficient mice fed AIN-93G diets supplemented with 0 (no, *B*), 1 (1% betaine, *C*), 2 (2% betaine, *D*), or 4 (4% betaine, *E*) g betaine/100 g diet for 14 weeks. *M* and *U* correspond to methylated and unmethylated PCR products, respectively

Atherosclerosis is a chronic disease of the arterial wall which is much more than endothelial dysfunction, proliferation, and migration of smooth muscle cells. Recent studies suggest that inflammation plays a central role in the development and progression of atherosclerosis, including the formation of early fatty streak [9, 24, 26]. TNF-α and MCP-1 are two pro-inflammatory cytokines which have potent atherogenic effects. TNF-α could upregulate the expression of adhesion molecules, chemokines, and scavenger receptors, which in turn facilitate the recruitment of monocytes/macrophages to the lesions and increase macrophage uptake of oxidized LDL [18]. MCP-1 is an important chemokine and directly associated with the migration and infiltration of monocytes/macrophages into

the arterial wall [4]. Thus, we investigated whether betaine could affect these two factors. After 14 weeks of treatment, betaine supplementation decreased aortic and serum protein levels of TNF-α but not MCP-1 in apoE-deficient mice. Moreover, betaine lowered aortic TNF-α expression in a dose-dependent way, indicating that betaine may attenuate atherosclerotic lesion by inhibiting the aortic inflammatory response mediated by TNF-α.

DNA methylation, i.e., addition of methyl groups to cytosine within CpG islands, is a form of epigenetic regulation that has the potential to silence gene expression [2]. Recent evidence indicates that DNA methylation is an important pathogenesis in diseases such as cancer and atherosclerosis [5, 6, 29]. In contrast to cancer research, the study of DNA methylation aberration in atherosclerosis is just beginning and only a very limited number of individual genes have been screened [13, 15, 22]. Dietary status of methyl donors, for example folate and choline, can affect DNA methylation [11, 14, 17, 27]. Betaine has three active methyl groups and is crucial for synthesis of *S*-adenosylmethionine, which serves as the direct methyl donor for DNA methylation. Here we examined for the first time whether TNF-α-specific DNA methylation was altered in atherogenesis and if it was affected by betaine supplementation. The results of MSP assay showed that TNF-α promoter did not have aberrant CpG island methylation and betaine had no significant effect on its methylation level in atherogenesis. In this study, electrophoresis bands specific for methyl and unmethyl primers were observed by the naked eye and promoter methylation status was expressed as either methylation or unmethylation. This method could not tell the difference in degree of methylation and may not be sensitive enough to detect small differences. It may be necessary to use other methods to quantify methylation level. It is also possible that the methylation status of the specific CpG islands selected by our assay is not indicative of the overall methylation status of the TNF-α promoter which has three CpG islands. This issue requires further investigation.

Although betaine supplementation had no effect on methylation status of the TNF-α promoter in apoE-deficient mice, it significantly reduced aortic and serum TNF-α protein levels after 14 weeks of treatment. This indicates that promoter methylation is not the sole determinant of gene expression, which has been confirmed by studies of other genes [8, 31].

Betaine has a close relationship with metabolism of body fat. It could significantly accelerate fat combustion by up-regulating the activity of hormone-sensitive lipase in abdominal adipose tissue [32]. Betaine also increases the free carnitine content of liver [32]; carnitine is able to transport activated long-chain fatty acids from the cytosol to the mitochondrial matrix, where they are broken down via β-oxidation. Thus, our finding of decreased body weight in apoE-deficient mice receiving betaine for 14 weeks may be explained by an increase in the activity of enzymes related to lipid catabolism and facilitating the transportation of fatty acids. However, our data are inconsistent with a study on human which demonstrated that betaine did not affect body weight after 12 weeks of intervention [25]. So the precise role of betaine in body fat metabolism remains to be elucidated.

In brief, our study shows that by exerting its anti-inflammatory effect of reducing aortic TNF-α level in a dose-dependent way, betaine attenuates atherosclerotic lesion despite exacerbating hyperlipidemia in apoE-deficient mice. Although betaine is a methyl donor with three active methyl groups, in this study we did not observe that it could affect DNA methylation of the TNF-α promoter.

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