The comprehensive effects of hyperlipidemia and hyperhomocysteinemia on pathogenesis of atherosclerosis and DNA hypomethylation in ApoE−/− mice

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Atherosclerosis (AS) is a disease induced by multiple factors, including genetic and environmental elements. The aim of the present study is to investigate the comprehensive effects of high cholesterol, high methionine diet, and apolipoprotein E deficiency (ApoE−/−) on the pathogenesis of AS. ApoE−/− mice were fed with high cholesterol and methionine diet for 15 weeks to induce hyperlipidemia and hyperhomocysteinemia. The methylation levels of genomic DNA (gDNA) and B1 repetitive elements in aortic tissues were measured by both methylation-dependent restriction analysis and nested methylation-specific polymerase chain reaction (PCR). Methylation sequence-bias pattern was assayed by DNA methyl-accepting capacity with restriction endonuclease. The mRNA expression of DNA methyltransferase-1, 3 (DNMT1, 3) was detected by real-time PCR. The concentrations of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) were determined by high-performance liquid chromatography. The results showed hypomethylation of gDNA and B1 repetitive elements. The mRNA expression of DNMT1 was reduced. The levels of SAM, SAH, and SAM/SAH ratio were increased. The atherosclerotic lesion areas strongly correlated with the risk factors. The distribution of DNA demethylation was preferred to non-CpG islands, which may suggest the major impact of hypomethylation on DNA integrity and genomic instability. Overall, our data unequivocally showed that the comprehensive role of high cholesterol, high methionine diet, and ApoE−/− is not uniformly consistent with the role of a single risk factor. The DNA methylation pattern in AS is quite complex and depends on genetic background and many involved risk factors.

Keywords atherosclerosis; hyperhomocysteinemia; hyperlipidemia; ApoE−/− mice; DNA methylation

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Introduction

Atherosclerosis (AS) is a lipid metabolic disturbance and inflammatory process, which leads to local plaque deposition in the vessel wall of arteries, narrowed lumen, and decreased tissue perfusion [1]. Its progressive process involves genetic and environmental elements. Many retrospective and prospective studies in human and animals have shown that risk factors such as hyperlipidemia and hyperhomocysteinemia (HHcy) contribute to atherosclerotic plaque formation and AS development [2,3], but the atherogenic mechanisms related to hyperlipidemia and HHcy remain controversial and obscure so far, especially when mingled with the genetic AS-prone background.

DNA methylation is a mechanism of epigenetic regulation. It 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 the transmethylation reactions [4,5]. Epigenetic alterations of genomic DNA (gDNA) play a critical role in AS. The genomic CpG dinucleotides are grouped in clusters in particular regions called CpG islands, which are methylated resulting in gene silencing and hypomethylated resulting in gene activation in the regulatory areas of transcription. DNA methyl addition is mainly carried out by DNA methyltransferases1, 3 (DNMT1, 3). The changes of DNMT1 and DNMT3 in DNA methylation patterns can lead to global hypomethylation and regional hypermethylation, particularly in the promoter regions of certain genes such as tumor suppressor genes or imprinted genes [6,7]. B1 is the sequence of short interspersed nucleotide elements in mouse, corresponding to the Alu sequence in human [8]. Demethylation of B1 repetitive
elements increases their activity like retrotransposable sequences, which in turn may induce genomic alterations by insertion and/or homologous recombination and deregulate gene transcription [9,10]. It has been shown that B1 repetitive elements account for ~45% in the human genome. They are scattered throughout the genome and its methylation correlates with gDNA methylation. Loss of DNA methylation in B1 repetitive elements might account for the major hypomethylation [11,12]. Increasing evidence shows that gDNA hypomethylation is associated with gene transcriptional activity in the pathogenesis of cardiovascular diseases including AS [13]. However, it remains unclear whether HHcy and hyperlipidemia coordinate to accelerate the development of AS via aberrant B1 repetitive elements. Therefore, we combined high cholesterol, high methionine diet, and apolipoprotein E deficiency (ApoE−/−) together in a single animal model to investigate their comprehensive effects on the pathogenesis of AS.

The major purposes of the present study are to confirm whether the cholesterol could induce rearrangement of DNA methylation pattern besides its pro-hyperlipidemia effect, whether addition of methionine could significantly deteriorate the pro-atherogenic lipids profile under the background of cholesterol diet and whether high cholesterol and high methionine could impact mutually. To clarify the potential mechanisms of hyperlipidemia and HHcy in AS, the methylation levels of B1 repetitive elements that widely distribute in the human genome and are associated with AS were evaluated. Exploiting the features of the aberrant methylation distribution induced by cholesterol and methionine diets may further expand the knowledge about the epigenetic mechanisms in AS.

**Materials and Methods**

**Animals, diet, and treatment protocols**

Male ApoE−/− mice (6 weeks old, 25 g body weight) provided by the Animal Center of Peking University Health Science Center (Beijing, China) were housed individually in cages in a climate-controlled room (24°C). The mice were divided into four groups (12 each) and maintained for 15 weeks on the following diets (KeAoXieLi, Beijing, China): Control group fed with regular mouse diet; Chol group fed with high cholesterol diet (containing 15% lard and 2% cholesterol); MethChol group fed with high cholesterol diet plus 1.7% methionine; and Meth group fed with regular diet plus 1.7% (wt/wt) methionine. The treatment of the laboratory animals and experimental protocol followed the guidelines of General Hospital of Ningxia Medical University and were approved by the Institutional Authority for Laboratory Animal Care. On the morning of the last day of the diet period, the mice in each group were anesthetized by injection with 20% ethylcarbamate enterocelia (2 ml/100 g). Blood was collected by cardiac puncture, and the serum was separated by centrifugation (1000 g for 10 min at 4°C). All the samples were stored at −80°C until further analysis.

**Aorta tissue preparation and atherosclerotic lesion evaluation**

The aorta was excised and placed in plastic cassettes to avoid warping of tissue. The samples were embedded in glycolmethacrylate (Technovit 7100; Kulzer, Wehrheim, Germany) [14]. To quantify plaques by stereological means, ~10 evenly spaced blocks of the aortic segment were sampled. The blocks were exhaustively sectioned at 10 μM and stained with hematoxylin and eosin (H&E). Then, the sections were projected onto a table top at ×40 magnification. An orthogonal grid (2 cm × 2 cm) was superimposed over the projected image, and the number of grid points overlying the intima and media of the aorta were counted for relative atherosclerotic plaque areas [15].

**Determination of serum total Hcy and lipid levels**

The concentrations of Hcy, total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL), and low density lipoprotein (LDL) were measured with ADVIA 2400/Automatic Biochemistry Analyzer (Siemens, Munich, Germany) by conventional enzymatic methodology. Plasma ox-lipid levels (ox-LDL and ox-HDL) were also measured by the enzyme-linked immunosorbert assay (Research & Diagnostics Systems, Minneapolis, USA) to determine whether lesion formation was related to changes of the oxidative lipids.

**Detection of aortic SAM and SAH concentrations**

SAM and SAH concentrations were determined by high-performance liquid chromatography (HPLC) [16]. The aorta (20 mg) was homogenized and mixed thoroughly in 1 ml 20% HClO₄ solution. The homogenated samples were loaded into a C18 column (Shimadzu, Tokyo, Japan) equipped on a Hitachi L2000 HPLC System (Hitachi, Tokyo, Japan). SAM and SAH standards (Sigma, St Louis, USA) were used to identify the elution peaks, and the concentrations of SAM and SAH in the tissues were calculated by using the standard curve.

**Detection of DNMT1, 3 mRNA expression by real-time polymerase chain reaction**

Total RNA was extracted from 80 mg aorta in 1 ml Trizol (Invitrogen, Carlsbad, USA). RNA was reversely transcribed using Revertid™ Premium First Strand cDNA Synthesis Kit (Thermo Scientific, Glen Burnie, USA) according to the manufacturer’s instruction. The primer nucleotide sequences and probe were shown in Table 1. Thermo cycler conditions comprised an initial activation step at 95°C for 10 min, followed by a two-step polymerase
chain reaction (PCR) program of 95°C for 15 s, annealing temperatures (Table 1) for 30 s and 60°C for 30 s for 45 cycles. An amplification curve was obtained for each qPCR run. RNA level of each gene was acquired from the value of the threshold cycle (Ct) related to that of glyceraldehyde phosphate dehydrogenase (GAPDH) through the formula 

\[ \Delta \text{Ct} = \text{Ct}_{\text{GAPDH}} - \text{Ct}_{\text{gene}} \]

Final results were expressed as N-fold differences in target gene expression relative to the GAPDH, termed ‘Ntarget,’ and were determined as follows: 

\[ \text{Ntarget} = 2^{\Delta \text{Ct} \text{(gene)}} - \Delta \text{Ct} \text{(GAPDH)} \]

DNMT activity and gDNA methylation assay
DNMT activity was detected using Epiquik™ DNA Methyltransferase Activity Assay Kit (Epigentek, New York, USA). The nuclear materials were extracted from the aorta samples with Epiquik™ Nuclear Extraction Kit (Epigentek). gDNA methylation assay was performed following the protocol of Methylamp™ Global DNA Methylation Quantification Kit (Epigentek). gDNA was isolated from the aorta using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) and its concentration was adjusted to 6.6 ng/µl with DNA-binding solution.

Nested methylation-specific PCR for B1 repetitive elements methylation assay
Global DNA was isolated from the peripheral blood mononuclear cells of the mice using the Wizard® Genomic DNA Purification Kit. DNA denaturation and bisulfite conversion processes were integrated into one step by EZ DNA Methylation-Gold TM Kit (ZYMO, Los Angeles, USA). Nested methylation-specific (nMS)-PCR consisted of two-step PCR amplification after a standard sodium bisulfite DNA modification. The first step used an outer primer pair set that did not contain any CpG. The second step PCR was carried out with the conventional PCR primers. The primers and product sizes of the nMS-PCR assay are shown in Table 2. To reduce mispriming and to increase efficiency, touchdown (TD) PCR was used in the amplification. Samples were subjected to 30 cycles in a TD program (94°C for 30 s; 67°C for 30 s; and 72°C for 1 min, followed by a 0.5°C decrease of the annealing temperature every cycle). After completion of the TD program, 20 cycles were run (94°C for 45 s, 52°C for 45 s, and 72°C for 45 s), ending with a 5 min extension at 72°C. The PCR products were separated by electrophoresis in 2% agarose gel containing ethidium bromide. DNA bands were visualized by ultraviolet, and calculated by the formula: 

\[ \text{Methylation} = \frac{\text{methylation}}{\text{methylation} + \text{unmethylation}} \times 100\% \]

Table 1 Primer sequences of DNMT 1, 3 for real-time PCR analyses

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank no.</th>
<th>Primer, probe sequence (5’ → 3’)</th>
<th>Length (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT1</td>
<td>NM010066</td>
<td>Forward: GGAGGCCAGCAAGAGAGTA</td>
<td>216</td>
<td>57.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: GGAGACACCCAGACCAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probe: 6-FAM-GACAGAGGGCAGACCAACAAGGCG-TAMRA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNMT3</td>
<td>AF220524.1</td>
<td>Forward: AGAATGCTATGCGGGGTTG</td>
<td>106</td>
<td>51.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: CTCTGGTTTCTGACTTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probe: 6-FAM-TGAACCCAGACGACATTGAA-TAMRA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2 nMS-PCR primers of the B1 repetitive elements

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Primer sequence (5’ → 3’)</th>
<th>Size (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1 repetitive sequence—O</td>
<td>Forward: ATAGAAGTGGATTTATAGTTAGTTATTG</td>
<td>269</td>
<td>64.7</td>
</tr>
<tr>
<td></td>
<td>Reverse: CACTCCAACCTTTTAAACCTTAAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1 repetitive sequence—M</td>
<td>Forward: GTTAGTTATTGGATGGTTATACGG</td>
<td>139</td>
<td>62.1</td>
</tr>
<tr>
<td></td>
<td>Reverse: TACAACAAAAAACAACACTCCGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1 repetitive sequence—U</td>
<td>Forward: GTTAGTTATTGGATGGTTATATGG</td>
<td>139</td>
<td>62.1</td>
</tr>
<tr>
<td></td>
<td>Reverse: TACAACAAAAAACAACACTCCAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

O, outer primer; M, methylation primer; U, unmethylation primer.
Purified gDNA (5 µg) was digested overnight with 10-fold excess HpaII or BssHII according to the manufacturer’s protocols (New England Biolabs, Beverly, USA), and were purified with E.Z.N.A Cycle-pure Kit (Omega BIO-TEK, Norcross, USA). Undigested gDNA served as controls. Then, 0.5 µg purified gDNA was co-incubated with bacterial SssI methylase (2 U) and [methyl-3H]SAM (3 mCi per sample) in tubes for 2 h at 37°C in a buffer of 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 10 mM ethylenediaminetetraacetate ion (final volume 25 µl). The reaction was stopped by placing tubes on the ice. Then 25 µl aliquots from each reaction mixture were applied onto GF/C filter discs, and the GF/C filter discs were counted by scintillation counting. The results were expressed as [methyl-3H] incorporation/0.5 µg DNA.

Statistical analysis
Results are expressed as mean ± SEM. The data were analyzed using one-way ANOVA and the additional analysis using the Student–Newman–Keuls test for multiple comparisons within treatment groups or t-test for two groups. P < 0.05 was considered significant.

Results
Serum total Hcy and lipids levels in ApoE−/− mice
After 15 weeks experimental diet, the serum total Hcy (tHcy) and lipid levels were measured (Fig. 1 and Table 3).

The MethChol group fed with high cholesterol and methionine diet had the highest tHcy and lipid levels. The Chol group and the Meth group also showed increased levels of tHcy and lipids. This result may indicate some mutual detrimental impacts of high cholesterol and methionine diet on each other.

Atherosclerotic lesions examination and its correlation with Hcy level
The atherosclerotic lesion was examined and analyzed by cross-sectional stereological method for the aortic root [Fig. 2(A,B)]. The areas of atherosclerotic lesions were significantly increased in Chol group, MethChol group, and Meth group, respectively, up to 1.44-, 2.40-, and 1.45-fold compared with that of the control group, and the size of atherosclerotic lesion area positively correlated with tHcy levels [R = 0.6862, P < 0.0001, Fig. 2(C)]. The atherosclerotic lesion in MethChol group was most severe and widespread, which may suggest the coordinative effects of Hcy and cholesterol on accelerating the development of AS.

Hcy-induced gDNA and B1 repetitive elements hypomethylation in ApoE−/− mice
DNA methylation pattern has been considered as a useful molecular marker for AS [18]. Our results showed that the MethChol group displayed the most obvious hypomethylation change and its gDNA methylation level decreased to 11.72% and 9.15% compared with that of Chol and Meth groups. The methylation levels of gDNA in the Chol group and Meth group decreased to 11.49% and 13.99% compared with that of the control group [Fig. 3(A)]. The gDNA methylation level negatively correlated with the tHcy levels [R = −0.6618, P < 0.0001, Fig. 3(B)]. The results may mean that Hcy-induced gDNA hypomethylation could be an important pathogenic route for AS development. The methylation of B1 repetitive elements showed very similar result with that of the gDNA methylation (Fig. 4), which further validated the demethylation effects of HHcy and hyperlipidemia on mouse genome.

![Figure 1](https://example.com/figure1.png)

Figure 1 Levels of serum total Hcy. Values are expressed as mean ± SEM. n = 12. *P < 0.05, **P < 0.01.

<table>
<thead>
<tr>
<th>Group</th>
<th>TC (mM)</th>
<th>TG (mM)</th>
<th>HDL (mM)</th>
<th>LDL (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.06 ± 0.11</td>
<td>0.43 ± 0.14</td>
<td>1.28 ± 0.05</td>
<td>0.41 ± 0.01</td>
</tr>
<tr>
<td>Chol</td>
<td>15.83 ± 1.06*</td>
<td>0.92 ± 0.16*</td>
<td>0.63 ± 0.05*</td>
<td>0.79 ± 0.07*</td>
</tr>
<tr>
<td>MethChol</td>
<td>24.02 ± 2.20**</td>
<td>1.58 ± 0.22**</td>
<td>0.48 ± 0.03**</td>
<td>1.88 ± 0.42**</td>
</tr>
<tr>
<td>Meth</td>
<td>11.32 ± 0.83*</td>
<td>0.77 ± 0.19*</td>
<td>0.36 ± 0.02*</td>
<td>0.62 ± 0.05*</td>
</tr>
</tbody>
</table>

Table 3 The levels of lipids in ApoE−/− mice

Values are expressed as mean ± SEM. *P < 0.05, **P < 0.01 vs. Control. n = 12.
SAM, SAH concentrations, and DNMT1, 3 mRNA expressions in ApoE<sup>2/2</sup> mice

SAM plays an important role in transmethylation reaction, while SAH is a potent inhibitor of cellular transmethylation [19,20], so SAM, SAH can exert remarkable regulatory effects on DNA methylation. After 15 weeks experimental diets, compared with the control group, the concentrations of aortic SAM, SAH, and the ratio of SAM/SAH increased to 1.70-, 2.40-, 1.80-fold, 3.17-, 6.21-, 5.36-fold, and 1.35-, 1.86-, 1.46-fold in Chol, MethChol, and Meth groups (Table 4), respectively, which displayed a similar pattern to that of Hcy. These data may indicate that the atherogenic diets can cause a parallel increase of SAM, SAH, and SAM/SAH ratio. They may co-act together on the pathogenesis of aberrant DNA methylation in AS, but their increase did not show a preferential profile for hypomethylation, which should be low SAM, high SAH, and low ratio of SAM/SAH.

Another key factor for DNA methylation regulation is the DNMTs. The mRNA expression of DNMT1, 3 was displayed in Fig. 5. The expression of DNMT1 mRNA had a very similar tendency with the changes of gDNA methylation. The lowest mRNA level occurred in MethChol group, which also had the lowest methylation level. Since the DNMT1 is the essential maintenance methyltransferase, its decrease can play a major role in the pathogenesis of hypomethylation in AS. The expression of DNMT3 mRNA showed an opposite tendency to the DNMT1 expression and the changes of gDNA methylation although there was not significant difference between groups, which suggests that the DNMT3 alteration is not implicated in the pathogenesis of hypomethylation but may be involved in hypermethylation. To corroborate the change of mRNA expression of DNMTs, the DNA methyltransferase activity was detected. The result showed a highly coincident correlation of methyltransferase activity with the DNMT1 mRNA expression, but not with the DNMT3 (Fig. 6).
DNA methylation-dependent restriction analysis and the sequence bias of demethylation

Figure 7 showed the methyl-accepting capacity of gDNA highly coincided with the gDNA methylation levels. The MethChol group had the lowest methylation level and the highest methyl-accepting capacity. HpaII, specific for C↓CGG sequences, significantly decreased the methyl-accepting capacity again. The most remarkable impact is in the MethChol group where the Methylation-accepting capacity reduced to 48.30% compared with that in control group. Although a mild decrease of methyl-accepting capacity was observed, the four groups did not show any differences, so the BssHII did not exert an important impact on the methyl-accepting capacity. These data show that the hyperlipidemia and HHcy can cause the DNA demethylation and coordination of the two atherosclerotic risk factors will significantly strengthen the demethylation effect. The sequence bias of demethylation had a preference of C↓CGG over the CpG islands. The most CpG islands located in the promoter area of genes while the C↓CGG sequences widely distributed, and the sequence bias to non-CpG islands area just coincided with the hypomethylation bias of repetitive elements.

Table 4 SAM and SAH concentrations, and SAM/SAH ratio in aortic tissue

<table>
<thead>
<tr>
<th>Group</th>
<th>SAH (nM)</th>
<th>SAM (nM)</th>
<th>SAM/SAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.45 ± 0.34</td>
<td>45.82 ± 1.35</td>
<td>8.28 ± 0.25</td>
</tr>
<tr>
<td>Chol</td>
<td>12.66 ± 0.78*</td>
<td>141.54 ± 4.78*</td>
<td>11.18 ± 0.98*</td>
</tr>
<tr>
<td>MethChol</td>
<td>17.00 ± 0.98*</td>
<td>284.19 ± 6.87*</td>
<td>15.38 ± 1.22*</td>
</tr>
<tr>
<td>Meth</td>
<td>13.38 ± 0.26*</td>
<td>245.69 ± 8.41*</td>
<td>12.08 ± 1.05*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. *P < 0.05 vs. Control. n = 12.

DNA methylation-dependent restriction analysis and the sequence bias of demethylation

The effect of HHcy on lipid oxidation

One of the deleterious effects of HHcy is its ability to generate reactive oxygen species thereby producing oxidative stress. The consequent oxidized lipids, especially the

Figure 4 The methylation status of B1 repetitive elements detected by nMS-PCR  (A) A representative electrophoresis picture of B1 repetitive elements. M, methylated PCR product; U, unmethylated PCR product. (B) The methylation levels of B1 repetitive elements were detected by nMS-PCR. Values are expressed as mean ± SEM. n = 12. *P < 0.05. (C) B1 repetitive elements methylation had a negative correlation with Hcy levels. R = -0.7605, P < 0.0001.

Figure 5 mRNA expression of DNMT1 and DNMT3 analyzed by real-time PCR Each gene RNA level was acquired from the value of the threshold cycle (Ct) as related to that of GAPDH through the formula ΔCt = CtGAPDH - Ctgene. Final results were expressed as N-fold differences in target gene expression relative to the GAPDH, termed ‘Ntarget,’ and were determined as follows: Ntarget = 2^(-ΔCt(gene)-ΔCt(GAPDH)), where ΔCt values of the GAPDH and sample were determined by subtracting the Ct value of the target gene from the Ct value.
ox-LDL, have been acknowledged as an initiating and accelerating factor in AS [21]. The results of serum ox-LDL and ox-HDL concentrations showed a correlation between the progression of AS and the DNA methylation levels. The highest Ox-LDL concentration and ox-HDL/HDL ratio occurred in the MethChol group (Fig. 8). The atherosclerotic lesion of the MethChol group was most severe and the methylation level was the lowest. The gDNA methylation level negatively correlated with the ox-LDL levels \[ R = -0.5841, \ P < 0.0001, \ \text{Fig. 8(B)} \]. The \( R \) value \[ R = 0.6081, \ P < 0.0001, \ \text{Fig. 8(C)} \] for the correlation of ox-LDL and atherosclerotic lesion area showed positive.

Figure 6 DNMT activity of the aorta DNMT activity was detected using EpiQuik™ DNA Methyltransferase Activity Assay Kit. Values are expressed as mean ± SEM. \( n = 12 \). *\( P < 0.05 \).

Figure 7 The methylation-accepting capacity of gDNA Blank control: without any endonucleases digestion; HpaII: HpaII digestion; BssHII: BssHII digestion. a, Control; b, Chol; c, MethChol; d, Meth. Each value represents the number of methyl groups incorporated into 0.5 \( \mu \)g DNA. Values are expressed as mean ± SEM. \( n = 12 \).

Figure 8 Levels of oxidative lipids and the correlation between ox-LDL and gDNA methylation, ox-LDL, and atherosclerotic lesion area (A) The oxidative lipids levels and ox-HDL/HDL ratio in ApoE\(^{-/-}\) mice. Values are expressed as mean ± SEM. \( n = 12 \). (B) ox-LDL showed a negative correlation with gDNA methylation. \( R = -0.5841, \ P < 0.0001 \). (C) ox-LDL exhibited a positive and significant correlation with atherosclerotic lesion area. \( R = 0.6081, \ P < 0.0001 \).
These data are in agreement with the current knowledge about the lipids oxidation, and also expand our认识 the atherogenic diets on the genetic AS-prone background can lead to AS through the demethylation and oxidative stress routes.

Discussion

We have previously reported that Hcy can cause cell-type-specific hypomethylation in endothelial cells, vascular smooth muscle cells (VSMCs), and foam cells [22,23]. It suggests that Hcy metabolism and methylation status may be differentially regulated, resulting in tissue-specific pathology in HHcy. The present study was to establish a high cholesterol, high methionine diet, and ApoE-deficient genetic background with the purpose to investigate the comprehensive effects on the AS pathogenesis. This combination is clinically relevant, and can imitate the physiological and pathophysiological situations in vivo. Since the gene background may profoundly influence the responses of body to environmental factors and the combination of different factors may influence each other significantly, the result may differ from that in single factor involved research.

First, our results showed that the ApoE^{-/-} mice displayed evident HHcy and hyperlipidemia. The high cholesterol diet not only induced a significant increase of plasma lipids, but also a moderate increase of serum Hcy level. The methionine added into the cholesterol diet, remarkably raised both Hcy and lipids levels, which indicated a clear coordinative strengthening impact of them on each other. The methionine added into the cholesterol diet, remarkably raised both Hcy and lipids levels, which indicated a clear coordinative strengthening impact of them on each other. The methionine added into the cholesterol diet, remarkably raised both Hcy and lipids levels, which indicated a clear coordinative strengthening impact of them on each other.

Second, Cash et al. [26] first proposed that DNA hypomethylation is involved in the development of cardiovascular disease. The plasma Hcy is associated with the inhibition of methyltransferases that cause gDNA hypomethylation in VSMCs, peripheral white blood cells, and atherosclerotic lesions [27–29]. More solid evidence from animal studies also suggested that gDNA hypomethylation is associated with atherosclerotic lesions [30]. The aberrant methylation pattern, including hypo- and hypermethylation, is an early marker of AS in ApoE^{-/-} mice fed with normal rodent diet, which preceded any histological sign of AS [31,32]. In our ApoE^{-/-} mice fed with high cholesterol and methionine diet, the significant features are the evident HHcy, gDNA, and B1 repetitive elements hypomethylation in genome, which differed significantly from that in the simple ApoE^{-/-} mice without environmental factors. The atherosclerotic lesion areas were strongly correlated with the coordination of the risk factors. It is reasonable to conjecture that B1 repetitive elements in mouse may have the similar functions with Alu elements in human. The hypomethylation of B1 repetitive elements in our experimental model indicated that it was involved in the AS pathogenesis. Methylation-dependent restriction endonucleases that recognize unmethylated cytosine could cut certain specific CG sequences but not the methylated mCG. The HpaI specific for C\downarrow CGG sequences and BssHII for CpG islands (GC\downarrow GCGC) were used in this study for investigating the Hcy-induced alteration in DNA methylation and its potential sequence bias. Digestion with HpaI or BssHII restriction endonucleases resulted in a destruction of corresponding CG loci and loss of the potential methylation-accepting sites, which meant a reduced methyl-accepting capacity [33]. The distribution of DNA demethylation was preferred to non-CpG islands, which may suggest the major impacts of hypomethylation on DNA integrity and genomic instability and the regulation of some special genes function.

Furthermore, in our previous research [34], Hcy was found to induce gDNA hypomethylation, elevation of SAH, and decrease of SAM and SAM/SAH ratio in primary culture of human vascular smooth muscle cells (HVSMCs). Since SAM is the major methyl group donor, while the SAH is a potent inhibitor of cellular transmethylation reaction, a reasonable deduction is that the hypomethylation is a passive process due to the decrease of SAM and SAM/SAH ratio and the elevation of SAH. In the present study, however, it was found that the SAH, SAM, and SAM/SAH ratio all increased along with the hypomethylation, which was different from the data in HVSMCs and did not support the causal role of the changes of SAH, SAM, and SAM/SAH ratio in initiation of hypomethylation in ApoE^{-/-} mice fed with high cholesterol and methionine diet. These data emphasize the current knowledge that different genetic background combined with different risk factors may cause various responses through corresponding routes.

Additionally, the investigation about the reasons of DNA hypomethylation unveiled the dominant role of DNMT1, which displayed obvious downregulation of mRNA expression and a reduced activity. The genesis of hypomethylation in our animal model may be mainly due to the reduced DNMT1. Hiltunen et al. [35] have shown that only a few rounds of replication of contractile medial VSMCs are required to develop a significant hypomethylation in the VSMCs genome, and that the DNMT1 is upregulated. But it is hard to explain the co-existence of hypomethylation and upregulation of DNMT1, and it is conflicted with our result of the reduced DNMT1. In our experimental model, the reduced activity of the maintenance methyltransferase DNMT1 was consistent with the hypomethylation. The reduced DNMT1 cannot methylate the hemimethylated CpGs during replication. Therefore, by copying DNA
methylation patterns from parental to daughter strands, the hypomethylation will soon ensue.

In conclusion, the pathogenesis of AS by coordinating genetic and environmental risk factors may differ from the role of single factor. The high cholesterol, high methionine diet, and ApoE deficiency can induce significant hypomethylation of gDNA and B1 repetitive elements. The hypomethylation is mainly due to the reduced DNMT1 but not the alteration of SAM, SAH, and SAM/SAH ratio.

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References


