Increased Plasma S-Adenosylhomocysteine–Accelerated Atherosclerosis Is Associated With Epigenetic Regulation of Endoplasmic Reticulum Stress in apoE−/− Mice

Yunjun Xiao, Wei Huang, Jinzhou Zhang, Chaoqiong Peng, Min Xia, Wenhua Ling

Objective—S-Adenosylhomocysteine (SAH) is a better predictor of cardiovascular disease than homocysteine is, and it has been implicated in mediating the pathogenicity of hyperhomocysteinemia in atherosclerosis via an epigenetic mechanism. However, the underlying mechanism remains unclear. Here, we tested the hypothesis whether the effect of SAH on atherosclerosis is involved in epigenetic regulation of endoplasmic reticulum stress.

Approach and Results—A total of 48 apolipoprotein E–deficient mice at 8 weeks were randomly divided into 4 groups (n=12 for each group). The control group was fed a conventional diet, the adenosine dialdehyde group was fed a diet that was supplemented with the SAH hydrolase inhibitor adenosine dialdehyde, and the other 2 groups were intravenously injected with a retrovirus that expressed either SAH hydrolase short hairpin RNA or scrambled short hairpin RNA semiweekly for 16 weeks. Plasma SAH levels and atherosclerotic lesion size were significantly increased in adenosine dialdehyde and SAH hydrolase short hairpin RNA groups when compared with control group. Expression of endoplasmic reticulum stress markers glucose-regulated protein-78 and CEBP-homologous protein was significantly increased in the mice with elevated plasma SAH levels. Moreover, plasma SAH was negatively associated with a decrease in the expression of trimethylated histone H3 lysine 9 and histone methyltransferases. Chromatin immunoprecipitation assays showed a significant decrease in trimethylated histone H3 lysine 9 occupancy at the glucose-regulated protein-78 and CEBP-homologous protein promoters in mice treated with adenosine dialdehyde and SAH hydrolase short hairpin RNA when compared with control mice.

Conclusions—Our results suggest that elevated plasma SAH levels–accelerated atherosclerosis was associated with the activation of endoplasmic reticulum stress via modulation of histone methylation. (Arterioscler Thromb Vasc Biol. 2015;35:60–70. DOI: 10.1161/ATVBAHA.114.303817.)

Key Words: atherosclerosis ■ endoplasmic reticulum stress ■ epigenenomics ■ S-adenosylhomocysteine
SAH Accumulation in apoE−/− Mice

The body weight and food intake among the 4 groups of mice were not significantly different throughout the 16 weeks of the study. The high-density lipoprotein-cholesterol levels were significantly decreased in the ADA and SAHH shRNA groups when compared with that in the C group (22.8±6.1 and 25.5±6.9, respectively, versus 32.9±9.7 mg/dL; P<0.05), whereas the levels of total cholesterol, triglyceride, and non–high-density lipoprotein-cholesterol among the 4 groups were not significantly different (Table I in the online-only Data Supplement). Dietary ADA supplementation or intravenous injection of a recombinant retrovirus expressing SAHH shRNA both resulted in a significant reduction of SAHH protein expression in the aortic tissue (Figure 1A). The SAHH activity of red blood cells was also significantly decreased in the ADA and SAHH shRNA groups when compared with that in the C group (1.5±1.9 and 1.3±1.5, respectively, versus 6.5±1.9 nmol/h per milligram protein; P<0.05; Figure 1B). Furthermore, the relative SAHH mRNA levels were also decreased by 62% and 70% in the ADA and SAHH shRNA groups, respectively, compared with those in the C group (Figure 1C). To investigate how ADA and SAHH shRNA treatment could affect SAHH mRNA levels further, we assessed the effect of ADA and SAHH shRNA on SAHH mRNA stability and total mRNA methylation in vitro. In contrast to the control or scrambled groups, ADA (30 μmol/L) and SAHH shRNA treatment both markedly decreased the half-life of SAHH mRNA (T1/2=6.5 or 6.0 versus 11.2 or 11.1 hours; P<0.05; Figure 1D). Moreover, ADA (30 μmol/L) and SAHH shRNA treatment also markedly inhibited the total mRNA methylation (Figure I in the online-only Data Supplement), which has been shown to be associated with mRNA stability.24,25 As a result of SAHH downregulation, the mice in the ADA and SAHH shRNA groups had notably higher plasma SAH levels when compared with the mice in the C group (75.9±9.8 and 85.5±12.1, respectively, versus 44.5±6.9 nmol/L; P<0.05; Figure 1F). The plasma SAM/SAH ratios in the ADA and SAHH shRNA groups were obviously reduced when compared with those in the C group (0.6±0.18 and 0.59±0.30, respectively, versus 1.23±0.29; P<0.05; Figure 1F). The plasma SAM levels in the ADA and SAHH shRNA groups were slightly lower than in the C group, but there were no significant differences among any of the groups (Figure 1G). Furthermore, the

Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>3meH3K4</td>
<td>trimethylated histone H3 K4</td>
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<tr>
<td>ADA</td>
<td>adenosine deamination</td>
</tr>
<tr>
<td>CHOP</td>
<td>CEBP-homologous protein</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>GRP78</td>
<td>glucose-regulated protein-78</td>
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<td>SAH</td>
<td>S-adenosylhomocysteine</td>
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<td>SAHH</td>
<td>SAH hydrolase</td>
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<td>SAM</td>
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factor 6 also plays a key role in the production of chaperones that facilitate the repair process, including glucose-regulated protein-78 (GRP78). Upregulation of PKR-like ER kinase increases the levels of activating transcription factor 4 and CEBP-homologous protein (CHOP), a potent inducer of apoptosis.15 Numerous studies have shown that homocysteine may promote atherosclerosis through the activation of ER stress,13,14 however, there is no direct evidence that homocysteine and not SAH, is responsible for this effect on ER stress, and no precise mechanisms have been described.

SAH is produced as a product of methylation reactions involving S-adenosylmethionine (SAM) as the methyl donor, including the methylation of DNA, histone, and other proteins.15 The ratio of SAM/SAH has been frequently used as an indicator of the cellular methylation potential.16 Histones can be methylated on either lysine (K) or arginine (R) residues. Lysine residues can be dimethylated, trimethylated, or trimethylated in vivo. It has been reported that trimethylated histone H3 K4 (3meH3K4) is strongly associated with transcriptional activation, whereas 3meH3K9 is predominantly correlated with transcriptional repression.17,18 Production of the latter epigenetic mark is catalyzed by an increasingly large family of SET-domain–containing histone lysine methyltransferases, including G9a, Setdb1, Suvs, and Suvs.19 Several studies have revealed that plasma SAH and homocysteine levels were significantly associated with DNA hypomethylation in atherosclerosis and vascular disease.8,20 Wang et al21 also found that homocysteine inhibited vascular endothelial cell growth and p21ras methylation in the presence of adenine. Therefore, SAH and homocysteine might affect atherosclerosis both directly and indirectly by influencing DNA methylation. Furthermore, SAM and its metabolites methylthioadenosine and SAH could inhibit lipopolysaccharide-induced proinflammatory gene expression via modulation of histone methylation, particularly by decreasing the 3meH3K4 occupancy at the promoters of proinflammatory genes.22 In contrast, elevated levels of SAH in the liver activate the expression of ER stress genes by inhibiting histone methylation and decreasing the 3meH3K9 occupancy at the promoters of ER stress genes.23 However, whether the mechanism underlying the effect of SAH on ER stress is involved in the modulation of histone methylation in atherosclerosis is still unclear.

Here, we elevated the plasma SAH concentration in atheroscleurosis-deficient (apoE−/−) mice by inhibiting SAHH short hairpin RNA (shRNA). The aim of this study was to examine the effects of increased plasma SAH levels on atherosclerosis and the expression of ER stress genes and to determine whether the mechanisms of SAH-induced ER stress might be involved in the modulation of histone methylation.

Results

Dietary ADA Supplementation and Intravenous Injection of SAHH shRNA Induce Plasma SAH Accumulation in apoE−/− Mice

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.
plasma tHcy levels were not significantly different among the 4 groups (Figure 1H).

**Elevated Plasma SAH Levels Promote Atherosclerosis in apoE<sup>−/−</sup> Mice**

To determine the effect of high levels of SAH on the formation of atherosclerotic lesions, sections of the aortic sinus were dissected and stained with Oil red O. After 16 weeks of feeding, advanced atherosclerotic lesions were observed in the aortic sinus in mice from all 4 groups (Figure 2A). The aortic sinus plaque areas were significantly increased in the mice from the ADA and SAHH shRNA groups when compared with those from the control group (721375±97376 and 752300±93125, respectively, versus 433416±80041 μm<sup>2</sup>; P<0.001; Figure 2B). A correlation analysis revealed a positive correlation between the plasma SAH and the aortic sinus lesion area (r=0.690; P<0.001; Figure 2C) and a negative correlation between the plasma SAM/SAH ratio and the aortic sinus lesion area (r=−0.768; P<0.001; Figure 2D). However, there was no significant association between the plasma tHcy or SAM levels and the aortic sinus lesion area. Further correlation analyses within the 4 treatment groups revealed that the associations between the plasma SAH or SAM/SAH ratio and the plaque sizes were significant in each of the 4 groups (Figures II and III in the online-only Data Supplement). Moreover, the associations between SAH and plaque size in the ADA and SAHH shRNA groups were much stronger than those in the control or scrambled groups (r=0.736 and 0.756 versus r=0.601 and 0.583; all P<0.05).

**Increased Plasma SAH Levels Induce ER Stress in apoE<sup>−/−</sup> Mice**

Next, to explore the effects of SAH on ER stress, the expression of ER stress markers, including GRP78 and CHOP, was assessed by immunohistochemical staining in aortic sinus lesions from all 4 groups. Compared with the C or scrambled groups, stronger staining for GRP78 was observed in complex plaque lesions in mice of the ADA and SAHH shRNA groups (Figure 3A). Furthermore, we observed weak CHOP immunostaining in the ADA and SAHH shRNA groups when compared with that in the C group (Figure 3A).
plaque lesions from the C or scrambled groups, but strong staining was observed in lesions in the ADA and SAHH shRNA groups. The expression of CHOP was significantly increased in the ADA and SAHH shRNA groups when compared with the that in the C group (57.13±15.21% and 68.45±17.69% versus 13.74±3.48%; P<0.05; Figure 3B). To assess whether increased plasma SAH levels correlated with increased expression of ER stress markers further, immunoblot and real-time polymerase chain reaction analyses of total aortic tissue lysates were performed. A >4-fold increase in the protein expression of GRP78 (Figure 3C) and CHOP (Figure 3D) was observed in both the ADA and SAHH shRNA groups when compared with those in the C group. The plasma SAH levels correlated positively with the protein levels of GRP78 (r=0.747; P<0.001; Figure 3E) and CHOP (r=0.609; P<0.01; Figure 3F), whereas the plasma SAM/SAH ratio, an indicator of methylation capacity, correlated negatively with the protein levels of GRP78 (r=−0.538; P=0.01; Figure 3G) and CHOP (r=−0.417; P=0.04; Figure 3H). Furthermore, the mRNA levels of GRP78 (Figure 3I) and CHOP (Figure 3J) were also significantly increased in both the ADA and the SAHH shRNA groups when compared with those in the C group. To clarify the role of ER stress in SAH-aggravated atherogenesis and whether the inhibition of ER stress could reverse SAH-aggravated atherogenesis in apoE−/− mice further, we treated apoE−/− mice with adenine deaminase (ADA) or mice that were intravenously injected with a retrovirus that expressed either SAHH short hairpin RNA (shRNA) or scrambled shRNA.

Because 3meH3K9 and 3meH3K4 are correlated with the transcriptional repression and activation, respectively, to investigate the effect of SAH on histone methylation, we assessed the expression of 3meH3K9 and 3meH3K4 among the 4 groups further. Immunofluorescent staining with a specific antibody recognizing 3meH3K9 indicated a greater intensity and more positive nuclear staining in complex lesions of the aortic root in mice from the control or scrambled groups. In contrast, a weaker intensity and fewer 3meH3K9-positive cells were observed in aortic lesions from mice in the ADA and SAHH shRNA groups (Figure 4A, left). Further analyses demonstrated that the relative fluorescence intensity of 3meH3K9 staining was significantly decreased by 79% and 81% in the ADA and SAHH shRNA groups, respectively, when compared with those in the control group (P<0.05; Figure 4A, right). Western blot analyses revealed that the level of 3meH3K9 was also decreased by 78% and 83% in the ADA and SAHH shRNA groups, respectively, when compared with those in the C group (P<0.05; Figure 4B). However, both immunofluorescent staining and Western blot analyses with a specific antibody against 3meH3K4 revealed that there was no significant difference in the level of 3meH3K4 among the 4 groups (Figure V in the online-only Data Supplement). Correlation analyses demonstrated that the plasma SAH concentration was negatively associated with the level of 3meH3K9 (r=−0.748; P<0.001; Figure 4C, left), whereas the plasma SAM/SAH ratio was positively correlated with the level of 3meH3K9 (r=0.591; P=0.015; Figure 4C, right). This result suggested that plasma SAH accumulation induced significant inhibition of 3meH3K9 expression but had no effect on 3meH3K4 expression.

Increased Plasma SAH Levels Inhibit Histone Methylation

Because 3meH3K9 and 3meH3K4 are correlated with transcriptional repression and activation,17,18 respectively, to investigate the effect of SAH on histone methylation, we assessed the expression of 3meH3K9 and 3meH3K4 among the 4 groups further. Immunofluorescent staining with a specific antibody recognizing 3meH3K9 indicated a greater intensity and more positive nuclear staining in complex lesions of the aortic root in mice from the control or scrambled groups. In contrast, a weaker intensity and fewer 3meH3K9-positive cells were observed in aortic lesions from mice in the ADA and SAHH shRNA groups (Figure 4A, left). Further analyses demonstrated that the relative fluorescence intensity of 3meH3K9 staining was significantly decreased by 79% and 81% in the ADA and SAHH shRNA groups, respectively, when compared with those in the control group (P<0.05; Figure 4A, right). Western blot analyses revealed that the level of 3meH3K9 was also decreased by 78% and 83% in the ADA and SAHH shRNA groups, respectively, when compared with those in the C group (P<0.05; Figure 4B). However, both immunofluorescent staining and Western blot analyses with a specific antibody against 3meH3K4 revealed that there was no significant difference in the level of 3meH3K4 among the 4 groups (Figure V in the online-only Data Supplement). Correlation analyses demonstrated that the plasma SAH concentration was negatively associated with the level of 3meH3K9 (r=−0.748; P<0.001; Figure 4C, left), whereas the plasma SAM/SAH ratio was positively correlated with the level of 3meH3K9 (r=0.591; P=0.015; Figure 4C, right). This result suggested that plasma SAH accumulation induced significant inhibition of 3meH3K9 expression but had no effect on 3meH3K4 expression.

SAH Accumulation-Induced ER Stress Is Involved in the Inhibition of Histone Methylation

To examine whether the SAH accumulation-induced ER stress is involved in the inhibition of histone methylation, we quantitatively assessed the levels of 3meH3K9 occupancy at the promoters of ER stress genes further. We used the aortic specimens from the mice with the most severe atherosclerotic
lesions and the highest plasma SAH levels in each group for these analyses. We conducted chromatin immunoprecipitation assays and semiquantitative polymerase chain reaction analysis and observed that the occupancy of 3meH3K9 at the GRP78 and CHOP promoters in mice from the ADA and SAHH shRNA groups was significantly decreased when compared with the control mice (decreased by 44% and 59%, respectively, for GRP78 and by 54% and 63%, respectively, for CHOP; Figure 5A). Correlation analyses showed that the 3meH3K9 occupancy at the GRP78 and CHOP promoters correlated negatively with the plasma SAH levels ($r = -0.793; P < 0.01$ and $r = -0.667; P < 0.01$, respectively; Figure 5B and 5C) and positively with the plasma SAM/SAH ratio ($r = 0.507; P < 0.05$ and $r = 0.641; P < 0.01$, respectively, Figure 5D and 5E).

The results suggested that reduced 3meH3K9 occupancy at the ER stress gene promoters because of SAH accumulation resulted in the activation of ER stress.

SAH Accumulation Inhibits Histone Methylation via the Inhibition of Histone Methyltransferases

Given that SAH is a potent inhibitor of the activity of several methyltransferases and that it also regulates the expression of methyltransferases at the transcriptional level,\textsuperscript{15,27,28} we hypothesized that SAH accumulation-inhibited histone

Figure 3. Effect of elevated plasma S-adenosylhomocysteine (SAH) levels on the expression of endoplasmic reticulum stress markers. A, Representative micrographs of glucose-regulated protein-78 (GRP78) immunostaining in aortic sinuses of apoE$^{-/-}$ mice from the 4 groups (left) and quantification of the staining (right). B, Representative micrographs (left) and quantification (right) of CEBP-homologous protein (CHOP) immunostaining in aortic sinuses of apoE$^{-/-}$ mice from the 4 groups. C and D, Immunoblot analysis of GRP78 and CHOP protein expressions in aortic tissue lysates from apoE$^{-/-}$ mice. Representative Western blots are shown above each bar graph with the image intensities in each group, expressed as ratios with $\beta$-actin$\pm$SD (n=6–8, *$P < 0.05$ vs control or scrambled groups). E–H, Correlation analyses of the relationship between the plasma SAH or the SAM/SAH ratio and the GRP78 or CHOP protein expression. I and J, Quantitative real-time reverse-transcriptase polymerase chain reaction analysis of GRP78 and CHOP mRNA expression in aortic extracts from apoE$^{-/-}$ mice. The mRNA levels were normalized to GAPDH and presented as the fold change compared with the control mice (n=6–8, *$P < 0.05$ vs control or scrambled groups).
methyltransferases. As expected, the total H3K9 methyltransferase activity was significantly decreased by >50% in the ADA and SAHH shRNA groups when compared with those in the control group (Figure 6A). Moreover, the total H3K9 methyltransferase activity was negatively associated with the SAH levels (r = −0.591; P = 0.002; Figure 6B) and positively associated with the SAM/SAH ratio (r = 0.519; P = 0.009; Figure 6C). Furthermore, we examined the effect of SAH accumulation on the expression of different histone H3K9 methyltransferases, including Suv39h1, Suv39h2, and Setdb1, among which Suv39h1 mediates the trimethylation of H3K9 to 3meH3K9, and we observed that the levels of aortic Suv39h1 transcripts were significantly downregulated in the ADA and SAHH shRNA groups when compared with those in the control mice (Figure 6D). Furthermore, the mRNA expression of G9a, which mediates the dimethylation of H3K9 to 2meH3K9, was also significantly decreased in the ADA and SAHH shRNA groups when compared with those in the control group (Figure 6E), whereas the mRNA expression of the other 2 methyltransferases was similar among the 4 groups (Figure 6F and 6G). Correlation analyses revealed that the plasma SAH levels were negatively correlated with the expressions of Suv39h1 and G9a (r = −0.525; P < 0.01 and r = −0.473; P = 0.02, respectively; Figure 6H and 6I), whereas the plasma SAM/SAH ratios were positively correlated with the expression of Suv39h1 and G9a (r = 0.679; P < 0.001 and r = 0.405; P = 0.049; respectively; Figure 6J and 6K), which is consistent with the hypothesis that the expression of these enzymes plays a regulatory role in histone methylation.

To explore how SAH regulates Suv39h1 and G9a gene expression, we identified the putative Suv39h1 and G9a promoter region using the Ensembl genome browser (http://www.ensembl.org) and cloned ≈2 kb of the sequence upstream of the transcriptional start site into the pGL3-Luc vector and performed a luciferase reporter assay. After the reporter plasmid was transfected into the mouse macrophage line (RAW264.7), we treated the cells with ADA (30 μmol/L) or SAHH shRNA for 24 hours. The Suv39h1 and G9a promoter activities were increased 8- and 18-fold, respectively. However, these increases were significantly inhibited by ADA or SAHH shRNA treatment (Figure VI in the online-only Data Supplement). This result consistently indicates that intracellular SAH accumulation as a result of ADA and SAHH shRNA treatment directly regulates the promoter activity of the Suv39h1 and G9a genes.

**Discussion**

In the present study, we first reported that elevation of plasma SAH levels in apoE−/− mice with normal plasma homocysteine levels by treatment with a SAHH inhibitor or SAHH shRNA was significantly associated with atherosclerotic lesion progression. Moreover, elevated plasma SAH levels were correlated with the increased expression of ER stress markers (GRP78 and CHOP) and the inhibition of 3meH3K9 and histone methyltransferases. A chromatin immunoprecipitation assay demonstrated that there was a decrease in the levels of...
3meH3K9 at the promoter regions of ER stress genes in mice with elevated plasma SAH levels. Our results suggest that atherosclerosis promoted by elevated plasma SAH levels might be associated with the induction of ER stress via modulation of histone methylation.

In our study, we promoted SAH accumulation in vivo and in vitro by targeting SAHH using the inhibitor ADA and by SAHH shRNA interference. Our findings suggested that ADA and SAHH shRNA treatment not only decreased SAHH activity but also inhibited SAHH expression at the transcriptional level, which is consistent with a recent study.29 Furthermore, ADA-induced suppression of SAHH mRNA may be the result of a feedback mechanism in which excess SAH suppresses SAHH expression at the transcriptional level. Because SAH is an inhibitor of mRNA-methyltransferases, excess SAH can inhibit mRNA methylation, which is associated with mRNA stability.25 Consistently, in our study, we found that ADA and SAHH shRNA treatment significantly decreased SAHH mRNA stability and total mRNA methylation in vitro. Actually, the intracellular SAH level has a stronger effect on the epigenetic changes than the plasma SAH; the plasma SAH is derived from tissues, such as the aorta, and is simply a reflection of the intracellular pools. However, the mechanisms by which SAH is transported into and out of cells are still unclear. One possibility is that SAH might indirectly enter or exit cells by hydrolyzing to homocysteine and adenosine. Second, studies conducted with isolated rat hepatocytes have shown that SAH is not taken up by cells, but rather that it binds to acceptors on the cell surface, which may lead to the transport of SAH into the cells.30 Third, phospholipid methylation on the plasma membrane also uses SAM as methyl donor, which is converted to SAH, indirectly leading to the transport of SAH into or out of the cells. Finally, some studies in which cells were treated directly with SAH indicate that SAH might be able to enter cells directly.31

Although hyperhomocysteinemia is a risk factor for atherosclerosis, the inconsistent results of cohort studies and the negative results of clinical intervention trials suggest that the causal relationship between homocysteine and cardiovascular disease is still controversial.32,33 However, previous studies have reported that severe hyperhomocysteinemia (>100 μmol/L) accelerated the atherosclerotic progression in animal models, and administration of a superphysiological dose of homocysteine (∼1 mmol/L) increased the pathogenesis of atherosclerosis in cultured cells.34–36 However, these results do not reflect the actual effects of moderately elevated homocysteine levels on atherosclerosis progression in clinical patients. Moreover, several studies have reported that moderately elevated plasma tHcy (∼15 μmol/L), which is similar to the physiological level in humans, had no effect on atherosclerotic lesions.37,38 Therefore, hyperhomocysteinemia may cause complications associated with atherosclerosis but cause this condition on its own.

In addition to homocysteine, numerous studies have shown that SAH might be a primary cause of atherosclerosis in hyperhomocysteinemia,39 and it may be a more sensitive indicator of cardiovascular disease and atherosclerosis than homocysteine.5 Recently, several studies have reported that
renal vasodilation may not be impaired by homocysteine itself but by another factor related to methionine; furthermore, aortic endothelium-dependent relaxation was also significantly impaired in hyperhomocysteinemia mice with increased tissue levels of SAH.40,41 It was hypothesized that SAH might also directly impair endothelium function and promote atherosclerosis in hyperhomocysteinemia patients or animal models. However, there is no direct evidence on the relationship between SAH and atherosclerosis, and the effect of homocysteine cannot be excluded. In the current study, we first used the SAHH inhibitor ADA or SAHH shRNA to induce an animal model of chronically elevated plasma SAH levels. We found that the elevated SAH levels significantly promoted atherosclerotic progression in apoE−/− mice with normal homocysteine levels. This result indicates that the effect of SAH on atherosclerosis is independent of homocysteine, which is in consistent with our previous finding that the plasma SAH level was significantly associated with cardiovascular risk independent of plasma homocysteine levels.7

Furthermore, in our previous study, although the plasma tHcy levels in apoE−/− mice fed on a high-methionine diet increased more than 3-fold relative to the control mice with normal tHcy levels (~5 μmol/L), no acceleration of atherosclerotic lesion formation was observed in mice with mild hyperhomocysteinemia (~15 μmol/L).35 In contrast, in an animal model, a just 2-fold increase in the plasma SAH levels (~100 μmol/L) was required to cause severe hyperhomocysteinemia and accelerated the atherosclerotic progression.35 This result suggested

Figure 6. Effects of plasma S-adenosylhomocysteine (SAH) accumulation on total histone H3 K9 (H3K9) methyltransferase activity and the mRNA levels of histone H3K9 methyltransferase genes. A, Total H3K9 methyltransferases activity in aortas from apoE−/− mice in the 4 groups (n=6–8; *P<0.05 vs control or scrambled groups). B and C, Correlation analyses of the relationship between plasma SAH or the S-adenosylmethionine (SAM)/SAH ratio and the total H3K9 methyltransferase activity. D–G, Quantitative real-time reverse-transcriptase polymerase chain reaction analysis of Suv39h1, G9a, Suv39h2, and Setdb1 mRNA levels in apoE−/− mice. The mRNA levels were normalized to GAPDH and are presented as the fold change compared with control mice (n=6–8; *P<0.05 vs control or scrambled groups). H–K, Correlation analysis of the relationship between plasma SAH or the SAM/SAH ratio and the Suv39h1 and G9a mRNA levels.
that a mild elevation of the plasma SAH level is more likely to promote the development of atherosclerosis than a mild elevation of the homocysteine level, which is also consistent with the finding that plasma SAH is a more sensitive marker of cardiovascular disease than plasma homocysteine. In support of this view, Valli et al. found that elevated serum SAH levels, but not elevated homocysteine levels, were associated with cardiovascular risk in patients with chronic kidney disease. Furthermore, Sipkens et al. also reported that SAH induces phosphatidylserine exposure and apoptosis in endothelial cells independently of homocysteine. Taken together, our results suggest that plasma SAH causes pathological vascular effects independently of homocysteine, and the hyperhomocysteinemia-induced vascular pathology may be mainly because of SAH accumulation.

Another interesting finding in our study is that the accumulated plasma SAH-accelerated atherosclerotic lesions were linked with the activation of ER stress. It has been demonstrated that homocysteine was shown to induce ER stress and apoptotic cell death in cultured endothelial cells and apoE−/− mice. Several studies have demonstrated that both SAH and homocysteine could promote apoptosis of endothelial cells and induce the proliferation and migration of smooth muscle cells, which may both be involved in the induction of oxidative stress. Therefore, there might be some similar or overlapping mechanisms underlying the effects of SAH and homocysteine on atherosclerosis. Furthermore, it is well known that elevations in plasma tHcy are highly correlated with parallel increases in plasma SAH, and a high concentration of homocysteine can also indirectly increase the intracellular SAH levels through a reversible reaction catalyzed by SAHH to promote SAH synthesis. Although the activation of ER stress was associated with the development of atherosclerotic lesions in hyperhomocysteinemia apoE−/− mice, we cannot exclude the possible effect of elevated SAH levels because the intracellular and plasma SAH levels were unknown in previous studies. Moreover, a previous study indicated that elevated liver SAH was associated with the activation of ER stress in mice with alcoholic liver injury. This result suggests that SAH may be able to induce ER stress similar to homocysteine. However, the relationship between SAH and ER stress in atherosclerosis has not been directly reported to date. In this study, we first reported that the markers of ER stress (GRP78 and CHOP) were significantly increased in the advanced lesions of mice with elevated plasma SAH when compared with the control mice. Further analyses revealed that plasma SAH was positively correlated with the expression of ER stress markers (Figure 3). This result suggested that ER stress could also be activated in mice with elevated SAH levels and normal homocysteine levels. Hence, we speculated that the activation of ER stress and aggravated atherosclerosis in hyperhomocysteinemia may partly be ascribed to the effect of simultaneously elevated SAH levels, but the mechanism underlying the SAH-induced ER stress is unclear.

Recently, it has been shown that epigenetic regulation of gene expression plays an important role in the development of vascular disease and attracts considerable attention. For example, dysregulation of histone methylation resulted in changes in the expression of proinflammatory genes and endothelial or inducible nitric oxide synthase genes in diabetes mellitus or vascular disease. Given that SAH is involved in all methylation reactions in the body, including DNA and histone methylation, our previous studies and those of others all demonstrated that chronic elevation of homocysteine levels results in parallel increases in intracellular or plasma SAH, which is associated with DNA hypomethylation in vascular diseases or atherosclerosis related to hyperhomocysteinemia. Hence, these findings suggested that the epigenetic mechanism of the pathogenicity of hyperhomocysteinemia in atherosclerosis might be partly mediated by its precursor, SAH. However, the contribution of SAH-mediated histone methylation to atherosclerotic lesions remains poorly understood. In the current study, to investigate whether the effects of elevated SAH levels on ER stress and atherosclerosis are related to the mediation of histone methylation, we performed immunofluorescent staining and chromatin immunoprecipitation assays to explore the effects of SAH on histone methylation and the methylated histone occupancy at the promoters of genes related to ER stress. Our findings demonstrated that increased plasma SAH was associated with a reduction in the trimethylation of H3K9 and a decrease in the occupancy of repressive 3meH3K9 at the promoter regions of ER stress-related genes (Figures 4 and 5). Because 3meH3K9 is mainly associated with the transcriptional repression of genes, the SAH accumulation-mediated decrease in 3meH3K9 occupancy at the promoters of ER stress genes resulted in the activation transcription of ER stress-related genes. Hence, SAH accumulation-induced ER stress may be mediated by the inhibition of 3meH3K9. In support of this concept, Esfandiari et al. showed that elevated hepatic SAH levels also decreased the 3meH3K9 occupancy at the promoters of ER stress-related genes in mice with alcoholic liver injury. However, Ara et al. reported that SAM and its metabolites or SAH could reduce 3meH3K4 occupancy at the promoters of tumor necrosis factor α or inducible nitric oxide synthase in response to lipopolysaccharide. However, in our study, we observed no effect of SAH accumulation on 3meH3K4 expression (Figure V in the online-only Data Supplement). This discrepancy may be explained by differences in the experimental conditions, such as the use of in vitro or in vivo systems or the inflammation status. According to the evidence, we inferred that accumulated disruption of the dynamic equilibrium of activating or repressive histone methylation by SAH might induce the changes in the expression of genes related to diseases.

Moreover, SAH is a potent inhibitor of the activity of several methyltransferases and also regulates the expression of methyltransferases at the transcriptional level. For example, the elevation of SAH levels decreased the levels of O6-methylguanine DNA methyltransferase mRNA in different cell lines. DZNep, an inhibitor of SAHH, indirectly regulated not only the expression of the 3meH3K27 histone methyltransferase EZH2 but also the H3K9 histone methyltransferase SETDB1 at the transcriptional level. The genes are mediated by SAH accumulation in human lung cancer cells. To examine whether the inhibition of histone methylation by SAH accumulation is mediated by the inhibition
of histone methyltransferases further, we assessed the total H3K9 methyltransferase activity and the mRNA expression of 4 H3K9 methyltransferases and analyzed the relationship between SAH and these enzymes. The results showed that the plasma SAH levels were negatively associated with the total H3K9 methyltransferase activity and the levels of mRNA for the H3K9 methyltransferases Suv39h1 and G9a (Figure 6). Luciferase reporter assays demonstrated that SAH regulated Suv39h1 and G9a mRNA expression by inhibiting their promoter activity. Furthermore, because SAH/Naïve local nuclear localization coincides with high rates of mRNA synthesis, a portion of the SAHH colocalizes with RNA polymerase II, and inhibitors of SAH reduce both the methylation and the synthesis of poly(A)+RNA.

Furthermore, it is speculated that the inhibition of histone methyltransferase mRNA by SAH accumulation may also be mediated by the inhibition of mRNA methylation.

In conclusion, plasma SAH accumulation accelerated the progression of atherosclerosis in apoE−/− mice with normal SAH accumulation may also be mediated by the inhibition of mRNA methylation. The results showed that the plasma SAH levels were negatively associated with the total H3K9 methyltransferase activity and the levels of mRNA for the H3K9 methyltransferases Suv39h1 and G9a (Figure 6). Luciferase reporter assays demonstrated that SAH regulated Suv39h1 and G9a mRNA expression by inhibiting their promoter activity. Furthermore, because SAH/Naïve local nuclear localization coincides with high rates of mRNA synthesis, a portion of the SAHH colocalizes with RNA polymerase II, and inhibitors of SAH reduce both the methylation and the synthesis of poly(A)+RNA. Therefore, it is speculated that the inhibition of histone methyltransferase mRNA by SAH accumulation may also be mediated by the inhibition of mRNA methylation.

Acknowledgments

We thank Dr Luo (Department of Nutrition, School of Public Health, Xi’an Jiaotong University, Xi’an, China) for constructing the SAH hydrolase short hairpin RNA interference expression vectors.

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Disclosures

None.

References


Significance

S-adenosylhomocysteine has been implicated in mediating the pathogenicity of hyperhomocysteinemia in atherosclerosis via an epigenetic mechanism, but the underlying mechanisms remain unclear. In the present study, we demonstrated that an elevation of plasma S-adenosylhomocysteine levels is positively associated with the development of atherosclerotic lesions in ApoE-deficient mice with normal plasma homocysteine levels. Furthermore, we found that elevated plasma S-adenosylhomocysteine was associated with a proatherogenic role of S-adenosylhomocysteine in cystathionine beta-synthase-deficient mice.
Increased Plasma S-Adenosylhomocysteine–Accelerated Atherosclerosis Is Associated With Epigenetic Regulation of Endoplasmic Reticulum Stress in apoE−/− Mice
Yunjun Xiao, Wei Huang, Jinzhou Zhang, Chaoqiong Peng, Min Xia and Wenhua Ling

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## Supplemental Materials

**Supplemental Table I.** Body weight, plasma lipid, and cholesterol levels of the four groups of ApoE<sup>-/-</sup> mice.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=12)</th>
<th>Scrambled (n=12)</th>
<th>ADA (n=12)</th>
<th>SAHH shRNA (n=12)</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>27.5±2.2</td>
<td>28.8±1.9</td>
<td>28.2±2.1</td>
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<tr>
<td>Cholesterol (mg/dL)</td>
<td>358.3±56.2</td>
<td>354.6±70.8</td>
<td>361.4±71.7</td>
<td>375.5±65.4</td>
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<tr>
<td>Triglyceride (mg/dL)</td>
<td>81.7±18.4</td>
<td>84.5±17.8</td>
<td>79.8±19.3</td>
<td>83.3±15.9</td>
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<tr>
<td>HDL-C (mg/dL)</td>
<td>32.9±9.7</td>
<td>36.1±11.3</td>
<td>22.8±6.1*</td>
<td>25.5±6.9*</td>
</tr>
<tr>
<td>Non-HDL-C (mg/dL)†</td>
<td>325.3±56.8</td>
<td>317.3±73.4</td>
<td>337.8±74.2</td>
<td>350.1±67.7</td>
</tr>
</tbody>
</table>

* P<0.05 vs. ApoE<sup>-/-</sup> mice fed the control diet by one-way ANOVA. † Non-HDL cholesterol concentration was calculated by using total cholesterol subtracted HDL cholesterol.
Supplemental Figure legends

Supplemental Figure I. Effects of intracellular SAH accumulation by ADA and SAHH shRNA treatment on mRNA methylation in mouse macrophage line (RAW264.7). Cells were incubated with or without ADA (30 μmol/L) or SAHH shRNA for 24 h and then labeled with L-[methyl-\(^3\)H]-methionine and \(^{14}\)C-uridine as described under Materials and methods. The polyadenylated mRNA was isolated and the amount of \(^3\)H and \(^{14}\)C incorporated in mRNA was determined by liquid scintillation counting. The results represent the mean values of three independent experiments. Data was expressed as mean ± SD. * \(P<0.05\) vs. control or scrambled groups.

Supplemental Figure II. Correlation analyses of the relationship between plasma SAH levels and total atherosclerotic plaque areas within the four groups of control, Scrambled, ADA, and SAHH shRNA-treated mice.

Supplemental Figure III. Correlation analyses of the relationship between plasma SAM/SAH ratio and total atherosclerotic plaque areas within the four groups of control, Scrambled, ADA, and SAHH shRNA-treated mice.

Supplemental Figure IV. Effects of ER stress inhibition by 4-phenyl butyric acid (PBA) on SAH-aggravated atherogenesis in apoE\(^{-/-}\) mice. Representative micrographs of Oil red O-stained cryostat sections of aortic sinuses from the apoE\(^{-/-}\) mice of the six groups (left panels) and quantification of total atherosclerotic plaque areas (right panels). \(n=10\) each group, * \(P<0.05\) vs. ADA or SAHH shRNA groups.

Supplemental Figure V. Effect of elevated plasma SAH levels on the expression of histone methylation marker 3meH3K4. (A) Representative aortic sinuses sections of apoE\(^{-/-}\) mice were immunofluorescence stained with specific antibody to 3meH3K4 and a secondary antibody labeled with FITC, then viewed with fluorescent microscopy (left panels). Digital scans of the immunofluorescence stained aortic sinus were quantified using Image Pro Plus software (right panels, \(n=6\) to \(8\)). (B) Western blot analysis of 3meH3K4 protein expression in aortic tissue among the four groups. Representative western blot are shown on left panels and quantification was expressed as ratios with histone H3 ± SD (right panels).

Supplemental Figure VI. Effects of intracellular SAH accumulation by ADA and SAHH shRNA treatment on G9a and Suv39h1 promoter activity in RAW264.7. Putative promoter regions of (A) G9a and (B) Suv39h1 genes were cloned into pGL3-Luc vector. The plasmids were transfected into RAW264.7 for 24 h, and then were treated by ADA (30 μmol/L) or SAHH shRNA for a further 24 h. Luminescence was monitored using a Glomax 96 microplate luminometer. The results represent the mean values of three independent experiments. Data was expressed as mean ± SD. * \(P<0.05\) vs. control or scrambled groups.
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Materials and Methods

Animal procedures
Male ApoE–/– mice on the C57BL/6 background were obtained from the Jackson Laboratory and a breeding colony was maintained at our facility. At eight weeks of age, mice were systematically assigned to four groups of similar mean body weights and fed for 16 weeks with control diet and experimental interventions: the C group (n=12) was maintained on a standard rodent maintenance diet as recommended by the American Institute of Nutrition–93 purified diet (AIN-93G) (Harlan Teklad); the ADA group (n=12) was maintained on a control diet that contained 0.04 g ADA /kg food (Sigma; according to a daily oral dose of 10 mg/kg body weight of ADA per animal); the SAHH shRNA group (n=12) was maintained on the control diet and received 200 μL intravenous injections of retrovirus solution with a minimum titer of $2 \times 10^9$ gfu/mL semi-weekly, the construction of SAHH shRNA interference expression vectors and retrovirus preparation was described previously1; the scrambled negative control group (n=12) received retrovirus that expressed a scrambled shRNA.

In order to examine the role of endoplasmic reticulum (ER) stress in SAH-induced atherosclerosis in ApoE–/– mice, we treated ApoE–/– mice with ADA or SAHH shRNA with 4-phenyl butyric acid (PBA), a chemical chaperone that can alleviate ER stress2. Briefly, sixty male ApoE–/– mice at four months of age were randomly divided into six groups (n=10 for each group). Four groups including C, Scrambled, ADA, and SAHH shRNA were treated as the above procedures for 8 weeks. The other two groups were intraperitoneal injected before ADA and SAHH shRNA with the chemical chaperone PBA (P21005, Sigma-Aldrich, St. Louis, MO, USA) (100 mg/kg/time) twice a week for 8 weeks, at the same time mice also received ADA and SAHH shRNA treatment for 8 weeks.

Before the injections, the mice were anaesthetized by inhalation of isoflurane for 3-5 min using an automatic delivery system (Isoflurane Vaporizer, Vaporizer Sales and Services, Rockmart, GA, USA) that provides a steady concentration of 1.5% isoflurane. Loss of reflex was detected by picking the animal’s feet and legs with forceps. The mice were kept in accordance with standard animal care requirements and housed in a temperature-controlled (24°C) room with a 12-hour light/dark cycle with free access to food and autoclaved water. We weighed the mice every week and calculated the consumption of water and feed for each group. The study and all procedures were approved by the Shenzhen Center for Disease Control and Prevention Animal Experiment Committee.

Cell Culture
Mouse macrophage lines (RAW264.7) were cultured in 6-well plates with RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in 5% CO₂. The cells were seeded into six-well plates 24 h prior to infection at approximately 80% confluence. The cells were then infected with retrovirus that was generated using the most effective shRNA in the presence of 8 μg/mL polybrene and were selected with puromycin (0.5μg/mL) to isolate stable clones.

Plasma methionine metabolites measurement
At the end of study, the mice were anesthetized and exsanguinated by withdrawing the maximum amount of blood from the orbital vein. Fasting blood samples were obtained in chilled EDTA-containing microtubes and centrifuged immediately, and the plasma was stored
at -80°C until further study. Plasma total homocysteine (tHcy) was measured by high-performance liquid chromatography (HPLC) and fluorescence detection. Plasma SAH and SAM were measured by stable-isotope dilution liquid chromatography-electrospray injection tandem mass spectrometry (HPLC-MS/MS). The interassay CVs for SAH and SAM were 5.4% and 4.7%, and the intraassay CVs were 6.5% and 3.8%, respectively. Mean recoveries were 95.3% for SAH and 94.5% for SAM. The lower limits of detection were 1.0 nmol/l and 2.0 nmol/l for SAH and SAM, respectively.

**Plasma lipid analysis**
Plasma total cholesterol, HDL cholesterol, and triglyceride were determined by enzymatic colorimetric assays using commercial Cholesterol Reagent and Triglycerides GPO reagent kits according to the manufacturer’s instructions. Non-HDL cholesterol concentration was calculated by using total cholesterol subtracted HDL cholesterol.

**Assay of SAHH activity**
Assay of red blood cell haemolysates was carried out by a method described previously. The assay mixture used for the determination of the synthetic reaction of SAH contained 200 μM [8-14C]adenosine and 3mM DL-homocysteine in 80mM potassium phosphate buffer, pH 7.0, containing 80mM KCl, 0.2 % bovine serum albumin, and 10mM 2-mercaptoethanol. The temperature was 37°C. At the end of reaction the mixture was centrifuged and measured by HPLC-MS/MS for SAH concentration. The results were expressed as nmol/h per mg protein.

**Quantification of atherosclerotic lesions**
The hearts were harvested, weighed and stored in 10% formalin buffer solution at 4°C and then cut transversely and embedded in OCT compound and frozen. 10-μm cryostat sections of the aortic root were taken from where the 3 aortic valves first appeared up to where the aortic valves disappeared, and then stained with Oil red O and counterstained with hematoxylin. The lesion area was measured by means of direct image capture from an RGB camera (JVCKy-F30B) and quantified using Image Pro Plus software by an investigator blinded to the treatment of the animals. The mean atherosclerotic lesion areas from 5 sections were calculated for each mouse.

**Immunohistochemistry and Immunofluorescence analyses**
After the deparaffinization, the endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 10 minutes. Antigen retrieval was performed for 30 minutes at 95°C in citrate buffer, pH 6.0 and a 10-minute incubation in 0.1% Triton X. After blocking with 5% normal goat or rabbit serum, sections were incubated with primary antibody to GRP78 (1:50)(Santa Cruz), CHOP (1:50)(Santa Cruz), 3meH3K4 (1:100) (Abcam), and 3meH3K9 (1:100) (Abcam) overnight at 4°C, followed by goat anti-rabbit or rabbit anti-goat horseradish peroxidase-conjugated or fluorescein isothiocyanate (FITC) labeled secondary antibodies for 30 minutes. Sections were developed in Diaminobenzidine (DAB) substrate and counterstained with hematoxylin for nuclear staining. Eight aortic sinus in each group were examined and at least 2-3 sister sections of each aortic sinus were analyzed. Images were digitized and analyzed with Image Pro Plus software by an investigator blinded to the treatment of the animals.

**RNA extraction and quantitative real-time PCR (RTqPCR)**
Total RNA was isolated by TRIzol reagent (Invitrogen) following the manufacturer’s instructions. RTqPCR primers were designed using Primer Express software 3.0. PCR assays
were performed using the ABI 7500 machine and conducted in triplicate wells for each sample. Baseline values of amplification plots were set automatically and threshold values were kept constant to obtain normalized cycle times and linear regression data. The following reaction mixture per well was used: 10 μL Syber Green mix, 0.8 μL of primers at the final concentration of 10 μM, 1 μL ROX reference dye, 1 μL cDNA, and 7.2 μL RNAse free water. For all experiment the following PCR conditions were used: denaturation at 95°C for 10 min, following by 40 cycles at 95°C for 15 seconds then at 60°C for 60 seconds. Quantitative normalization of cDNA in each sample was performed using GAPDH as internal control. Relative quantification was performed using the 2^(-ΔΔCt) method.

**Isolation of mRNA and methylation assay**
Polyadenylated mRNA was isolated using Qligotex mRNA mini kit (Qiagen). RNA methylation was determined by the incorporation of radioactivity from L-[methyl-3H]-methionine into RNA and RNA synthesis by incorporation of [14C]-uridine. After the cells were treated with ADA (30 μmol/L) and SAHH shRNA for 24 h, 50 μCi L-[methyl-3H]-methionine/mL and 0.2 μCi [14C]-uridine/mL were added into the cells and incubated for additional 24 h. Radioactivity incorporated in mRNA was determined by liquid scintillation counting. The result of mRNA methylation was expressed by changes in the ratio 3H/14C in mRNA.

**Histone extraction**
Histone proteins were extracted from the nuclear extract as previously described. Briefly, an equal volume of cold 0.2 M H2SO4 was added to nuclei, and the supernatant was then collected by centrifugation at 12,000 rpm for 15 min at 4°C, and was added to 100% trichloroacetic acid (TCA). Histones were precipitated, and were washed with 0.05 M HCl. After centrifugation, the histone pellet was dried at room temperature, and was used for western blot analysis.

**Western blot analysis**
Total tissue protein lysates or histone proteins from the aortas were solubilized in SDS-PAGE sample buffer, separated on 10% SDS-polyacrylamide gel, and transferred electrophoretically onto polyvinyl denedifluoride (PVDF) membranes. After incubation for 1 hour at room temperature with 5% BSA in Tris-buffer saline (TBS)-0.1% Tween-20, membranes were incubated overnight at 4°C with the primary antibodies to SAHH (1: 500) (Proteintech Group), GRP78 (1:200) (Santa Cruz), CHOP (1:100) (Santa Cruz), 3meH3K4 (1:100) (Abcam), 3meH3K9 (1:100) (Abcam), and histone H3 (1:500) (Santa Cruz) followed by horseradish peroxidase-conjugated secondary antibodies, the membranes were developed with the chemiluminescent substrate (Pierce). Band intensities were quantified using Quantity One software and control for equivalent protein loading was assessed with an anti-β-actin antibody (Sigma).

**Chromatin immunoprecipitation (ChIP) assay**
ChIP was carried out using the EZ ChIP kit (Millipore). Briefly, aortas tissues were minced and cross-linked in 1% formaldehyde for 10 minutes at room temperature. Cross-linking was stopped by addition of glycine to a final concentration of 125 mM for 5 minutes. Nuclear extracts were prepared and resuspended in sonication buffer (0.75%SDS, 2 mM EDTA, and 50 mM Tris-HCl [pH 8.0]). The chromatin was sheared to 200-1000 bp by sonication and precleared with protein G agarose beads. Ten percent of original precleared chromatin was
removed for use as a control for total input DNA. Each ChIP assay was performed using 500ng of chromatin and 2μL of rabbit polyclonal 3meH3K9 primary antibody (Abcam) or normal rabbit IgG (Santa Cruz) incubation overnight at 4°C with rotation. Protein G agarose was used to recover the immune complexes at 4°C 2 hours. After washes and elutions, DNA was reverse cross-linked overnight at 65°C, RNase treated for 30 minutes at 37°C, proteinase K treated for 2 hours at 45°C, and purified using a spin column to a final volume of 50μL. PCR was performed with the primer sequences of promoter regions of GRP78, F, GGTGTCAAGAAGGAAAGGGAAA, R, GTTACAAATTGGCCAGGCAAT; and CHOP, F, GACACCGGTGCCAACATT, R, CCGCTTTCTGATTGGTAGGCT; to determine the extent of 3meH3K9 binding to the promoter region of GRP78 and CHOP. PCR products were separated by electrophoresis through 2% agarose gels, visualized using ethidium bromide, and quantitated with ImageQuant Software. Data were normalized with input control.

Total H3K9 methyltransferases activity assay
Mouse aortas were prepared from which nuclear extracts were made using a Nuclear Protein Extraction kit (Beyotime Biotechnology). The protein concentrations were determined using the Biocinchoninic Acid assay kit (Beyotime Biotechnology). Total H3K9 methyltransferases activity levels were quantified using an innovative colorimetric assay with the EpiQuik™ In Situ Histone H3K9 Methyltransferases Activity Assay Kit (Epigentex Group), according to the kit’s instruction. The results were expressed as absorbance units 450 nm.h⁻¹.mg protein⁻¹.

Luciferase assay
Approximately 2 kb promoter regions of Suv39h1 and G9a genomic sequence were identified from Ensembl Genome Brower (http://www.ensembl.org) and were cloned into the pGL3-Luc vector. The promoter construct was transfected into RAW264.7 on a 60 mm plate, using 5 μL Lipofectamine 2000 (Life Technologies). The cells were also co-transfected with the β-gal plasmid to normalize for transfection efficiency. Total cell lysates were prepared from cells 24 h post-transfection using 1× Passive Lysis Buffer and were then assessed for firefly luciferase activity. Luminescence was monitored using a Glomax 96 microplate luminometer (Promega).

Statistical analyses
Results are expressed as means ± SD. Differences among groups were assessed by one-way ANOVA followed by posthoc tests. Correlations between selected pairs of variables were evaluated with the Pearson correlation. A probability value <0.05 was taken as statistically significant.

References


