Increased plasma S-adenosyl-homocysteine levels induce the proliferation and migration of VSMCs through an oxidative stress-ERK1/2 pathway in apoE−/− mice

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Aims

Although S-adenosyl-homocysteine (SAH) is considered to be a more sensitive predictor of cardiovascular disease than homocysteine, the underlying mechanisms of its effects remain unknown. We investigated the in vivo and in vitro effects of SAH on vascular smooth muscle cells (VSMCs) proliferation and migration related to the development of atherogenesis in apolipoprotein E-deficient (apoE2/2) mice.

Methods and results

A total of 72 apoE2/2 mice were randomly divided into six groups (n = 12 for each group). The control group was fed a conventional diet, the M group was fed a 1% methionine-supplemented diet, the A group was fed a diet that was supplemented with the SAH hydrolase (SAHH) inhibitor adenosine-2, 3-dialdehyde (ADA), the M+A group was fed a diet that was supplemented with methionine plus ADA, and two of the groups were intravenously injected with retrovirus that expressed either SAHH shRNA (SAHH+/−) or scrambled shRNA semi-weekly for 8 weeks. Compared with the controls, the mice in the A, M+A, and SAHH+/− groups had higher plasma SAH levels, larger atheromatous plaques, elevated VSMC proliferation, and higher aortic reactive oxygen species and malondialdehyde levels. In cultured VSMCs, 5 μM ADA or SAHH shRNA caused SAH accumulation, which resulted in increased cell proliferation, migration, oxidative stress, and extracellular-regulated kinase 1/2 (ERK1/2) activation. These effects were significantly attenuated by preincubation with superoxide dismutase (300 U/mL).

Conclusion

Our results suggest that elevated SAH induces VSMC proliferation and migration through an oxidative stress-dependent activation of the ERK1/2 pathway to promote atherogenesis.

Keywords

S-adenosyl-homocysteine • Vascular smooth muscle cells • Oxidative stress • Apolipoprotein E-deficient • Atherosclerosis

1. Introduction

Homocysteine (Hcy) and S-adenosyl-homocysteine (SAH) are critical intermediates of methionine metabolism. In 1969, McCully et al.1 first proposed the hypothesis that a high concentration of plasma Hcy caused vascular disease. Since then, numerous studies have demonstrated that hyperhomocysteinaemia is an independent risk factor for atherosclerotic diseases.2–5 However, large-scale intervention trials using high-dose B vitamins to reduce plasma Hcy levels in cardiovascular patients have not shown an overall clinical benefit.6–8 In addition, Hcy may actually promote the complications of atherosclerosis rather than atherosclerosis itself.9 SAH is the sole metabolic precursor of Hcy in a reversible reaction that is catalysed by SAH hydrolase (SAHH, EC 3.3.1.1). Recently, growing evidence has revealed that SAH may be a better indicator of cardiovascular disease than Hcy.10–13 However, the effects of SAH on atherosclerosis are poorly understood.

Vascular smooth muscle cell (VSMC) proliferation and migration are key events in the development of atherosclerosis. It has been reported that Hcy can induce VSMC proliferation and migration at concentrations that are much higher than those that are observed physiologically,14 but the mechanisms underlying this effect remain

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unclear. Whether SAH, the precursor of Hcy, is involved in VSMC proliferation and migration has not been studied and merits further investigation.

It is widely accepted that oxidative stress, including the elevated production of intracellular reactive oxygen species (ROS), results in VSMC proliferation and migration as well as the development of atherosclerosis.\(^\text{15}\) A large number of studies have suggested that Hcy may promote the development of atherosclerosis through the elevation of oxidative stress.\(^\text{16,17}\) Furthermore, another study indicated that Hcy increases extracellular-regulated kinase 1/2 (ERK1/2) activity, which results in increased oxidative stress.\(^\text{18}\) However, there is no direct evidence that this is an effect of Hcy, and not SAH, on oxidative stress, nor are any precise mechanisms known.

Here, we elevated the SAH concentration by inhibiting SAHH with adenosine-2, 3-dialdehyde (ADA) in mice that were fed a standard diet with or without high-level supplementation of methionine, which is often used to induce hyperhomocysteinaemia, or by knocking down SAHH by intravenous injection of retrovirus expressing SAHH shRNA. The effects of these treatments on atherosclerosis and VSMC proliferation were investigated in apolipoprotein E-deficient (apoE\(^{-/-}\)) mice, which have high levels of cholesterol and spontaneously develop atherosclerotic lesions. Subsequent in vitro analysis directly confirmed that increased SAH resulted in oxidative stress, cellular proliferation, and migration in VSMCs. Furthermore, these effects are mechanistically attributable to SAH-mediated increases in ERK1/2 activity.

\section*{2. Methods}

\subsection*{2.1 Materials}

ApoE\(^{-/-}\) mice on a C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in our animal facility. VSMCs that were derived from C57BL/6J mouse aorta and 293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The reagents and antibodies that were used are listed in Supplementary material online.

\subsection*{2.2 Retrovirus preparation}

To construct RNA interference expression vectors that targeted mouse SAHH (NM_016661), four SAHH shRNAs (shown in Supplementary material online, Table S1) were obtained and recombined into the pSilencer 5.1 Retroviral Expression Vector (ABI, USA). The resultant vectors were named pSilencer 5.1-U6 Retro-anti-SAHH (1–4). The fidelity of the recombinants and a negative control pSilencer 5.1-U6 Retro scrambled vector were confirmed by polymerase chain reaction and DNA sequencing. To generate high-titer viral stocks, 2 \times 108 293T packaging cells were co-transfected with pSilencer 5.1-U6 Retro-anti-SAHH, the helper vector pCLAmpho and pRRL-CMV-GFP using the reagent FuGENE 6 (Roche) according to the manufacturer’s instructions. The viruses were collected and concentrated 100-fold by centrifugation. The concentrated virus stocks were titered using 293T cells based on GFP expression.\(^\text{19}\)

\subsection*{2.3 Cell culture and retrovirus infection}

VSMCs were cultured routinely in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% foetal bovine serum (FBS), penicillin (50 U/mL), and streptomycin (50 \mu g/mL) at 37 °C in a humidified atmosphere of 5% CO\(_2\) and 95% air. 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 \mu g/mL polybrene and were selected with puromycin (0.5 \mu g/mL) to isolate stable clones.

\subsection*{2.4 Western blotting}

The aorta was perfused with saline, and the adventitia and intima were removed using microsurgical instruments under microscopy as previously described.\(^\text{20}\) The tissue homogenates and cell lysates were collected for western blotting as previously described, and detailed information is available in Supplementary material online.\(^\text{21}\)

\subsection*{2.5 Animal experiments}

The animal experiments conformed to the Guide for the Care and Use of Laboratory Animals that was published by the US National Institute of Health (NIH Publication No. 8523, revised 1985). \(72,8\)-week-old apoE\(^{-/-}\) mice were systematically assigned to 6 groups with similar mean body weights and treated for 8 weeks as follows: 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); the M group \((n = 12)\) received a methionine-enriched diet in which the control diet was supplemented with 10 g methionine/kg food; the A group \((n = 12)\) was maintained on a control diet that contained 0.04 g ADA/kg food; the M+A group \((n = 12)\) was maintained on a diet that contained 10 g methionine/kg food and 0.04 g ADA/kg food; the SAHH\(^{-/-}\) group was maintained on the control diet and received 200 \mu L intravasal injections of retrovirus solution with a minimum titer of 2 \times 10^9 gfu/mL semi-weekly; the scrambled negative control group received retrovirus that expressed a scrambled shRNA. 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 pricking 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 h light/dark cycle with free access to food and autoclaved water. We weighed the mice every week and calculated the consumption of water and food for each group. The study and all of the procedures were approved by the Sun Yat-Sen University Animal Experiment Committee.

\subsection*{2.6 Metabolites and plasma cholesterol measurement}

Plasma and cellular Hcy, SAH, and S-adenosyl-methionine (SAM) concentrations were measured as previously described,\(^\text{22,23}\) and there is detailed information available in Supplementary material online. The plasma total cholesterol and triglycerides were measured by commercial kits according to the manufacturer’s instructions.

\subsection*{2.7 Quantification of atherosclerotic lesions and immunohistochemistry}

The hearts were harvested, weighed, and stored in 10% formalin buffer solution at 4 °C. Half of the hearts were cut transversely and embedded in OCT compound and frozen for quantification of plaque areas, and half of them were imbedded by paraffin for immunohistochemical analysis of \(\alpha\)-smooth muscle actin (SMA), anti-proliferating cell nuclear antigen (PCNA), and Ki67 as previously described.\(^\text{24}\) The detailed information is available in Supplementary material online.

\subsection*{2.8 Proliferation assays}

The establishment of growth curves and the cell proliferation reagent WST-1 (Roche Molecular Biochemicals, Germany) were used to measure cell proliferation.\(^\text{25,26}\) Briefly, 4 \times 10^4 cells were plated in triplicate, harvested, and counted at 24, 48, 72, and 96 h. The number of viable cells was determined using a Z2 Particle Count and Size Analyzer (Beckman-Coulter, Miami, FL, USA). For WST-1 assay, cells were plated in quadruplicate into 96-well microtitre plates at 5 \times 10^3 cells/well and cultivated for 24 h and treated at 37 °C in a humidified 5% CO\(_2\) incubator.
After incubation for 24 h, the cells were incubated for additional 4 h in the presence of a WST-1 labelling mixture (10 μL per well). The absorbance of the samples, against a background control (medium alone) as a blank, was measured at 450 nm with using a microliter plate (ELISA) reader (Molecular Devices, Sunnyvale, CA, USA).

2.9 Wound healing assay
The cells were scraped with the end of a 1 mL pipette tips (time 0). Plates were washed twice with phosphate-buffered saline (PBS) to remove detached cells, and incubated with the complete growth medium. Cell migration into the wound empty space was monitored by microscopy at various times followed. Quantitation was done by measuring the distance of the wound edge of the migrating cells from the start point to the migrated point from three independent experiments.

2.10 Transwell assay
The migration assay was performed in 24-well transwell plates of 6.5 mm diameter with polycarbonate membrane filters containing 8 μm pores.

Figure 1 Metabolite concentrations in apoE<sup>−/−</sup> mice. (A) The expression of SAHH in the aortic medium. Dietary ADA supplementation and intravenous injection of SAHH shRNA-expressing retrovirus significantly reduced SAHH expression. The expression of SAHH was quantified using NIH Image software (right panels). The plasma levels of Hcy (B), SAH (C), SAM (D), and the SAM/SAH ratio (E) are indicated for each treatment group of apoE<sup>−/−</sup> mice. After quantitative reductive cleavage of all disulfide bonds, plasma Hcy was subsequently measured by high-performance liquid chromatography and ammonium 7-fluorobenzo-2-oxa-1, 3-diazone-4-sulfonate (SBDF) fluorescence detection. Plasma SAH and SAM were measured by stable-isotope dilution liquid chromatography–electrospray injection tandem mass spectrometry (n = 10–12). *P < 0.05, **P < 0.01 vs. C group mice.
Aliquots of 2 × 10^4 cells in 300 μL medium without FBS were seeded into the upper wells of the cell inserts. The lower transwell compartment contained 600 μL of DMEM, 0.4% FBS, and 0.2% bovine serum albumin, with or without 5 μM ADA. After incubating for 5 h at 37°C, the non-migrating cells were removed with cotton swabs from the upper surface of the membrane and washed with PBS. The cells were then fixed with methanol for 10 min at 4°C followed by haematoxylin and eosin staining. The number of cells that migrated to the lower surface of the filter was determined by counting the cells in 10 representative fields. All experiments were performed in triplicate and were repeated at least three times.

### 2.11 Statistical analysis

The data are expressed as the mean ± SE. The significance was determined by a one-way ANOVA using SPSS 13.0 software (SPSS, Chicago, IL, USA). The correlations between selected pairs of variables were evaluated with the Pearson correlation coefficient and a linear regression analysis. A value of P < 0.05 was considered to be statistically significant.

### 3. Results

#### 3.1 Dietary ADA and intravenous injection of SAHH shRNA induce SAH accumulation in vivo

Body weight and food intake among the six groups of mice were not significantly different throughout the 8 weeks of study (data not shown). To obtain the most effective retrovirus against SAHH, we performed a preliminary examination of four different shRNAs named shRNA1–4 (shown in Supplementary material online, Table S1) that targeted SAHH mRNA at different positions and were cloned into the pSilencer 5.1 Retroviral Expression Vector. As indicated in Supplementary material online, Figure S1 and Supplementary material online, Table S2, the most effective sequence was shRNA1, which resulted in an approximately 85% decrease in SAHH protein and a concomitant greater than six-fold increase in SAH concentration. Dietary ADA (groups A and M+A) or intravenous injection of recombinant retrovirus expressing SAHH shRNA1 (group

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**Figure 2** Atherosclerotic lesions and VSMC proliferation were increased in apoE−/− mice with high levels of plasma SAH. (A) The aortic sinuses were sectioned and stained for lipids using Oil red O (left panel; 100 ×; scale bar: 400 μm). The relative atherosclerotic plaque area is expressed as the percentage of the total cross-sectional vessel wall area (right panel). Immunohistochemical staining of aortic sinus sections was performed with the rabbit monoclonal antibodies anti-SMA (B), anti-PCNA (C), or anti-Ki67 (D) and their expression was quantified (n = 10–12). *P < 0.05, **P < 0.01 vs. group C.

(Corning, NY, USA).
(Figure 1A). As a result, the mice in groups A, M+A, and SAHH+/− had notably higher plasma SAH levels compared with the mice in group C (Figure 1C; 53.2 ± 2.9, 81.6 ± 5.0, or 59.4 ± 4.2, respectively, vs. 32.1 ± 2.1 nmol/L; P < 0.05). Among the six groups of mice, only the mice in group M had significantly increased plasma Hcy and SAM levels compared with the control mice (Figure 1B and D; P < 0.05). The plasma SAM/SAH ratios in groups A, M+A, and SAHH+/− were significantly reduced compared with that of control mice (Figure 1E; 0.87 ± 0.08, 0.48 ± 0.05, or 0.98 ± 0.09, respectively, vs. 1.31 ± 0.21; P < 0.05). The levels of total cholesterol and triglycerides in the A, M+A, and SAHH+/− group mice were slightly lower than those in mice that were fed the control diet, but there was no significant difference among any of the groups (Supplementary material online, Table S3).

### 3.2 Elevated SAH promotes atherogenesis and VSMC proliferation in vivo

To determine the effect of accumulated SAH on the formation of atherosclerotic lesions, sections of the aortic sinus were taken and stained with Oil red O. We found that there were significant increases in the plaque areas of the A, M+A, and SAHH+/− group mice compared with the control mice (Figure 2A; 38.3, 78.5, and 45.0 increases, respectively). The correlation analysis among all of the groups showed that there was a positive correlation between plasma SAH and aortic sinus lesion areas (r = 0.592; P < 0.01). Furthermore, we examined VSMC proliferation in the aortic sinus, which were identified by the presence of SMA, PCNA, and Ki67 staining. As expected, a notable number of SMA-positive cells...
were observed in the intima and media of mice in the A, M+A, and SAHH+/− groups but fewer were observed in group M (Figure 2B and Supplementary material online, Figure S2). Further immunohistological analysis also showed profound expression of PCNA and Ki67 in the aortic medium from these three groups compared with that of the control mice (Figure 2C and D; $P<0.01$).

Among the six groups, the mice in group M+A had the largest lesions and the most significant increase in SMA, PCNA, and Ki67 expression.

### 3.3 Increased plasma SAH levels induce oxidative stress in apoE−/− mice

It is well established that oxidative stress, especially the production of ROS, is implicated in VSMC proliferation and migration and the development of atherosclerosis. To examine the effects of SAH accumulation on oxidative stress in vivo, we assessed the levels of ROS in the sections of the aortic sinus and the levels of MDA in the aortic medium. Relative analysis demonstrated that DHE fluorescence was increased significantly by 20, 56, and 37%, respectively, in the A, M+A, and SAHH+/− groups compared with the C group mice (Figure 3A). No differences were observed in the group M mice. Furthermore, the mice from group M+A had higher levels of MDA in the aortic medium than group A or group SAHH+/− (Figure 3B).

To specifically identify the effect of elevated SAH-induced oxidative stress on ERK1/2 activation in VSMCs, homogenate supernatants only from the aortic medium were assayed by western blotting. As shown in Figure 3C, the mice from groups A, M+A, and SAHH+/− had increased phosphorylated ERK1/2 expression compared with group C mice. There was also a significant increase in phosphorylated ERK1/2 expression in the M group.
3.4 SAH accumulation induces VSMC proliferation and migration in vitro

To explore the underlying mechanisms of the effects of SAH on VSMC proliferation and migration in vitro, we assessed SAH accumulation following the addition of 5μM ADA or infection with retroviral SAHH shRNA1. The intracellular levels of Hcy, SAH, SAM, and SAM/SAH are indicated in Supplementary material online, Table S3. As expected, both ADA and shRNA knockdown induced a significant accumulation of SAH with a concomitant decrease in Hcy and SAM concentrations compared with WT VSMCs (13.75 ± 0.49 or 15.32 ± 0.33, respectively, vs. 2.46 ± 0.33 × 10^{-2} nM/10^7 cells; P < 0.01). The stable transduction of scrambled shRNA did not affect SAH levels.

We next determined the proliferation status of the cells. Both SAHH^{+/−} VSMCs and ADA-treated VSMCs showed higher proliferation at each time point than WT VSMCs (Figure 4A). Similarly, we found significantly higher WST-1 signals from ADA-treated and SAHH^{+/−} VSMCs compared with WT VSMCs (Figure 4B). We further assessed cell migration using a wound-healing assay and a trans-well assay. These assays demonstrated that SAH accumulation induced a dramatic increase in the cellular migration of SAHH^{+/−} VSMCs and ADA-treated VSMCs (Figure 4C and D). However, the proliferation and migration of the scrambled VSMCs were comparable with WT VSMCs.

3.5 Oxidative stress-induced ERK1/2 activation is essential for SAH-induced cell proliferation and migration

Next, we investigated the effects of SAH accumulation on oxidative stress in vitro. As shown in Figure 5A and B, the oxidation of DHE was markedly increased in SAHH^{+/−} VSMCs and ADA-treated VSMCs, but not in scrambled shRNA VSMCs. The MDA concentration in SAHH^{+/−} VSMCs and ADA-treated VSMCs was consistently significantly increased compared with that in WT VSMCs (Figure 5C; 0.31 ± 0.05 and 0.38 ± 0.06, respectively, vs. 0.24 ± 0.03 μM/mg protein; P < 0.05). Interestingly, pre-treatment with superoxide dismutase (SOD) (300 U/mL) for 30 min suppressed VSMC proliferation in both SAHH^{+/−} VSMCs and ADA-treated VSMCs although thrombin (10 U/mL), which was used as a positive control, significantly promoted WT VSMC proliferation (Figure 6A). The combination of SOD (300 U/mL) and catalase (200 U/mL) completely blocked the proliferation of SAHH^{+/−} VSMCs (Supplementary material online, Figure S3). Similarly, SOD also attenuated VSMC migration in both SAHH^{+/−} VSMCs and ADA-treated VSMCs (Figure 6B).

We also examined whether SAH accumulation induces the activation of ERK1/2 signalling, which is crucial in regulating cell proliferation. Compared with WT VSMCs, treatment with 10% FBS increased the levels of phospho-ERK1/2 in SAHH^{+/−} VSMCs and, to a lesser extent, increased phospho-ERK1/2 in ADA-treated VSMCs.
VSMCs. However, total ERK1/2 remained unchanged (Figure 6C). The phosphorylation of ERK1/2 was almost completely blocked in the presence of PD98059. Furthermore, pre-treatment with SOD (300 U/mL) attenuated the phosphorylation of ERK1/2 in both SAHH+/- VSMCs and ADA-treated VSMCs (Figure 6D).

4. Discussion

These results have clearly demonstrated that the inhibition of SAHH by pharmacological inhibition (ADA) or genetic down-regulation (retroviral SAHH shRNA) resulted in SAH increase, enlarged atheromatous plaques, elevated VSMC proliferation, and oxidative stress compared with control mice. Furthermore, in vitro studies have confirmed that intracellular SAH accumulation induced VSMC proliferation and migration through an oxidative stress-dependent activation of ERK1/2.

Several recent studies have indicated that SAH is a more sensitive indicator of vascular disease than an elevated concentration of plasma Hcy.10,13 However, the precise mechanisms of the effects of SAH remain unknown. SAHH inhibition using excess adenosine and Hcy, either added alone or in combination, has been used to increase SAH levels in biological models.28,29 In the present study, in addition to the inhibitor ADA, SAHH shRNA retrovirus was successfully generated and used to specifically upregulate SAH levels both in vivo and in vitro. We intravenously injected apoE-/- mice with recombinant retrovirus expressing SAHH shRNA1. The western blotting analysis showed that the SAHH expression in the aorta of SAHH+/- mice was reduced to 45.0% of the level in group C, resulting in a significant increase in plasma SAH levels (Figure 1).

Vascular cells, including macrophages, lymphocytes, endothelial cells, and VSMCs, are involved in the formation of atherosclerotic lesions.30 In particular, the proliferation and migration of VSMCs are critical events in atherogenesis.31 Our data demonstrated that SAH accumulation induced the proliferation and migration of VSMC both in vivo and in vitro. In vivo, SMA, PCNA, and Ki67 staining indicated that there is a positive correlation between SAH levels and VSMC proliferation and migration into the intima (Figure 2). In vitro, SAHH shRNA was more effective than ADA supplementation at inducing cell proliferation and migration. Interestingly, SAHH shRNA-mediated knockdown resulted in a higher level of intracellular SAH, indicating that the observed effects on cell proliferation and migration corresponded to the degree of SAH elevation (Figure 4). Previous studies have suggested that Hcy can induce VSMC proliferation and migration.32 – 34 In this study, we established a cell model in which SAHH expression was stably knocked down using an shRNA vector. This approach lessens the confounding effects of Hcy changes on the proliferation and migration of VSMCs, thus allowing us to make conclusions more directly attributable to SAH elevation alone.
Taken together, these results suggest that SAH-induced VSMC proliferation and migration may be a potential mechanism by which SAH promotes atherosclerosis.

It is well established that high levels of dietary methionine induce hyperhomocysteinaemia in both humans (as detected using an oral methionine load test) and experimental animals. A previous study indicated that hyperhomocysteinaemia was associated with a significant increase in plaque size. However, a methionine load (group M) in the present study did not result in an increase in the lesion area, even though Hcy levels were significantly elevated compared with control mice (Figure 2). There are two possible reasons for the observed differences between our study and the previous report. First, the use of different amounts of methionine or different experimental periods may be responsible for the observed inconsistency. Secondly, the increased level of SAM in the M group may also account for the discrepancy. A recent study suggested that SAM exerts protective effects against restenosis after balloon injury in a rat model of type 2 diabetes by reducing the proliferation and inducing the apoptosis of VSMCs, modifying the inflammatory processes, and reducing oxidative stress. Conversely, mice in groups A and M+A had significantly higher SAH levels, which were positively correlated with plaque areas. In contrast, there was no association between Hcy concentration and the plaque area. Therefore, our findings supported the hypothesis that SAH is a more sensitive predictor of atherosclerosis than Hcy.

Oxidative stress has been implicated in the initiation and development of atherosclerosis. Although SAH induces cardiomyocyte apoptosis in a concentration-dependent manner through a mechanism that requires nuclear ROS production, there is limited knowledge regarding whether oxidative stress contributes to the harmful effects of SAH on vascular disease. It has been previously demonstrated that Hcy alone caused relatively low oxidative stress, but a marked increase in intracellular H$_2$O$_2$ was observed when Hcy was combined with SAH. These effects were attenuated by the addition of SOD, suggesting that the effect of SAH is directly related to increased oxidative stress. We report here that increased oxidative stress was apparent in the A, M+A, and SAHH/−/− group mice in response to high levels of SAH. In the in vitro experiments, we also found that both SAHH/−/− VSMCs and ADA-treated VSMCs have consistently higher levels of ROS (Figure 5). Pre-treatment with SOD significantly attenuated the SAH-induced cell proliferation and migration (Figure 6), clearly indicating that oxidative stress is necessary for SAH-induced VSMC proliferation and migration.

It is well established that the ERK1/2 signalling pathway regulates VSMC proliferation and migration. Guyton et al. demonstrated that the ERK1/2 protein is phosphorylated by exposure to exogenous H$_2$O$_2$ and that this phenomenon can be inhibited by pre-treatment with classical antioxidants. Although the activation of ERK1/2 by ROS has been well studied, little is known about the signalling mechanisms by which SAH stimulates proliferation and migration in VSMC. In the present study, we found that SAH accumulation increased the phosphorylation of ERK1/2, which was blocked by the inhibitor PD98059 (Figure 6). Furthermore, pre-treatment with SOD attenuated ERK1/2 phosphorylation both in SAHH/−/− VSMCs and ADA-treated VSMCs. These results were consistent with the in vivo findings that dietary ADA supplementation, the combination of methionine plus ADA and SAHH shRNA, increased ERK1/2 phosphorylation in the aortic medium. One interesting finding is that the ERK1/2 phosphorylation in the group M mice was comparable to the group A mice, which is not coincident with the changes in plaque areas and the proliferation markers. This discrepancy may be attributed to a higher SAH/SAH ratio (Figure 1E), which has been associated with alleviated ERK1/2 inhibition in betaine-supplemented alcoholic fatty liver disease. Taken together, these data suggested that the effects of SAH on the proliferation and migration of VSMCs may be associated with the oxidative stress-mediated activation of the ERK1/2 pathway.

In conclusion, we demonstrate that increased SAH levels induce VSMC proliferation and migration, which may represent an important mechanism by which SAH promotes atherosclerosis. These effects of SAH are mediated through an oxidative stress-dependent activation of the ERK1/2 pathway.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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