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Homocysteine induced oxidative stress in human umbilical vein endothelial cells via regulating methylation of SORBS1

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Abstract. – OBJECTIVE: The aim of the present study was to investigate the mechanism of homocysteine (Hcy) induced oxidative stress in the human umbilical vein endothelial cells (HUVECs).

PATIENTS AND METHODS: The HUVECs were isolated from umbilical vein vascular wall of 12 patients and treated with Hcy. The malondialdehyde (MDA) level was measured using the thiobarbituric acid (TBA) method. The expressions of superoxide dismutase 2 (SOD2), endothelial nitric oxide synthase (eNOS), and intercellular adhesion molecule 1 (ICAM-1) were detected by Western blot and RT-PCR. The genome-wide DNA methylation assay was performed using the Infinium Human Methylation 450 BeadChip. The specific DNA methylation was determined using bisulfite sequencing analysis. To evaluate the role of sorbin and SH3 domain-containing protein 1 (SORBS1), the HUVECs were transfected with small interfere RNA (siRNA) targeting SORBS1 (SORBS1-siRNA).

RESULTS: Hcy induced MDA level in HUVECs, and increased ICAM-1 expression both in protein and mRNA levels. The protein and mRNA levels of SOD2 and eNOS were inhibited by Hcy induction. However, the effects of Hcy on MDA level and expressions of SOD2, eNOS, and ICAM-1 were attenuated by folic acid (Fc) and vitamin B12 (B12) treatment. DNA total methylation level in Hcy treated cells was significantly decreased compared to the control group, while the DNA total methylation levels were increased after treatment with Fc and B12. The methylation level of SORBS1 in Hcy treatment group was higher than that of control group. And the methylation level of SORBS1 induced by Hcy was attenuated by Fc and B12 treatment. SORBS1-siRNA transfection induced the MDA levels and reduced the expressions of SOD2 in HUVECs.

CONCLUSIONS: We indicated that Hcy induced oxidative stress in HUVECs via regulating methylation of SORBS1. We also found that Fc and B12 treatment attenuated the oxidative stress and inflammation induced by Hcy in HUVECs. The findings indicated that Fc and B12 might be effective agents for the treatment of Hcy induced AS.

Key Words:
Atherosclerosis (AS), Oxidative stress, Homocysteine (Hcy), DNA methylation, SORBS1.

Introduction

Atherosclerosis (AS) is a kind of chronic inflammatory disease which is characterized by the accumulation of lipids and inflammatory cells in the artery wall[1]. The pathogenesis of AS includes the activation of pro-inflammatory pathways, and increased expression of cytokine or chemokine and oxidative stress. It has been reported that oxidative stress is a critical and final common mechanism in AS[1]. Homocysteine (Hcy) is a non-proteinogenic α-amino acid, and hyperhomocysteinemia (HHcy) leads to inflammation and oxidative stress which may result in AS[2,3]. However, the mechanism of Hcy induced oxidative stress in human umbilical vein endothelial cells has not been fully understood. DNA methylation is a process in which methyl groups are added to the DNA molecule, and then the activity of DNA is altered without changing the sequence[4]. DNA methylation is essential for embryonic development and is associated with a number of normal processes, such as parental gene imprinting, X-chromosome inactivation, and repression of transposable

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retroelement activity[5,6]. However, aberrant DNA methylation is related to many diseases including diabetes[7], various cancers[8] and AS[9]. In addition, it has been found that oxidative stress may induce the alteration of DNA methylation in AS[9]. Thus, targeting DNA methylation might be beneficial for the injury induced by oxidative stress in AS. Hcy is one of the products of the methyltransferase reaction, and aberrant DNA methylation is found to be one of the mechanisms of HHcy induced diseases[10]. Hcy induces the cell proliferation of vascular smooth muscle cell via regulating the methylation status of PTEN, which is involved in cell proliferation[11]. The effect is weakened by the treatment of an antagonistic agent of Hcy[11]. Besides, Ma et al[12] proved that Hcy induced oxidative stress through regulating DNA methylation in endothelial cells. Thus, we suggested that Hcy may affect the DNA methylation of genes involved in the oxidative stress in AS. In the present study, we investigated whether aberrant DNA methylation is involved in Hcy induced oxidative stress in human umbilical vein endothelial cells, and the protective effect of folic acid (Fc) and vitamin B12 (B12) on Hcy induced oxidative stress.

Material and Methods

Cell Isolation and Identification

Cell Isolation and Culture

The present study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Sun Yat-Sen University. The human umbilical vein endothelial cells (HUVECs) were isolated from umbilical vein vascular wall of 12 patients as described previously[13]. Briefly, HUVECs were isolated by a collagenase treatment, then cultured in a human endothelium SFM medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 50 mg/l Endothelial Cell Growth Supplement (ECGS, BD Biosciences, Franklin Lakes, NJ, USA), 100 U/ml penicillin (HyClone, South Logan, UT, USA) and 100 μg/ml streptomycin (HyClone, South Logan, UT, USA) in a 37°C -5% CO2 incubator.

Immunofluorescence Assay

The HUVECs (1×105/well) were seeded into 6-well plate and then fixed by 4% paraformaldehyde for 30 min. After blocking with 5% fetal bovine serum (FBS), the rabbit anti-human VIII factor (vWF) antibody (dilution 1: 100, BOSTER Biological Technology, Wuhan, China) was added and incubated at 37°C for 2 h. Next, the Alex Fluor 488 labeled goat anti-rabbit secondary antibody (dilution 1:100, Cell Signaling Technology, CST, Danvers, MA, USA) was added and incubated at 37°C for 1 h in the dark. After that, the cells were stained by 5 μg/ml of DAPI at room temperature for 3 min and then sealed. Finally, the cells were examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Flow Cytometry

The HUVECs were collected and resuspended in the stain buffer. The cells were incubated with 5 μl LFITC labeled CD31 antibody or isotype control antibody (BioLegend, San Diego, CA, USA) at 37°C for 30 min. After washing for three times, the cells were resuspended in 200 μl stain buffer and analyzed using a flow cytometer.

Cell Treatment

The isolated HUVECs were separated to four groups: a) Blank; b) Hcy group, cells treated with Hcy (1 mM, Sigma-Aldrich, St. Louis, MO, USA) for 5 days; c) Hcy + Fc (Sigma-Aldrich, St. Louis, Mo, USA) group, cells treated with Hcy (1 mM) and Fc (100 mM) for 5 days; d) Hcy + Fc + B12 (Sigma-Aldrich, St. Louis, MO, USA) group, cells treated with Hcy (1 mM), Fc (100 mM), and B12 (100 mM) for 5 days. After incubation under different conditions for 5 days, the HUVECs were collected for further experiments.

Cell Transfection

The isolated HUVECs were separated to three groups: a) Blank group, cells without transfection; b) Control group, cells transfected with NC-siRNA (Forevergen Biosciences, Guangzhou, China); c) Sorbin and SH3 domain containing 1 gene (SORBS1)-siRNA group, cells transfected with SORBS1-siRNA (Forevergen Biosciences, Guangzhou, China). The transfection was performed using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA) and incubated for 72 h.

Western Blot

The proteins of HUVECs were extracted by RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). The protein concentrations were determined using the BCA Protein Assay Kit (Beyotime, Shanghai, China). The proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred

onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). After blocking with 5% non-fat milk at room temperature for 1 h, the membranes were incubated with primary antibodies against superoxide dismutase 2 (SOD2) (dilution 1: 500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), endothelial nitric oxide synthase (eNOS) (dilution 1: 800, Santa Cruz Biotechnology, Santa Cruz, CA, USA), intercellular adhesion molecule 1 (ICAM-1) (dilution 1:600, Santa Cruz Biotechnology, Santa Cruz, CA, USA), sorbin and SH3 domain-containing protein 1 (SORBS1) (dilution 1:500, Novus Biologicals, Littleton, CO, USA), and GAPDH (dilution 1: 400, Cell Signal Technology, Danvers, MA, USA) at 4°C overnight. Next, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (dilution 1:3000, Boster) at 37°C for 1 h. The blots were visualized using an enhanced chemiluminescence detection kit (BD Biosciences, Franklin Lakes, NJ, USA). The intensity of the blots was detected using Quantity One software (Bio-Rad Hercules, CXA, USA).

RT-PCR

Total RNA of HUVECs was extracted using TRIzol reagent (Life Technologies, Grand Island, NY, USA). Reverse transcription was performed to generate cDNA using the FSQ-101 ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) following the manufacturer's instructions. Then, the RT-PCR reactions were performed using SYBR® Premix Ex TaqTM II (TaKaRa, Otsu, Shiga, Japan) on ABI 3730 automated sequencer (Applied Biosystem, Foster City, CA, USA). The primers of SORBS1 were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The other primers used in the study were synthesized by Invitrogen (Carlsbad, CA, USA): SOD2, 5'-ATGT TGAG CCGG GCAG TGTG C -3', 5'-CTGA AGAG CTGT CTGG GCTG T -3'; eNOS, 5'-CCAG CTAG CCAA AGTC ACCA T-3', 5'–GTCT CGGA GCCA TACA GGAT T-3'; ICAM-1, 5'-TCAA AAGT CATC CTGC CCCG -3', 5'-TGCT CAGT TCAT ACAC CTTC CG -3'; GAPDH, 5'-ACCA CAGT CCAT GCCA TCAC-3', 5'-TCCA CCAC CCTG TTGC TGTA -3'. The mRNA expressions were quantified using the 2-ΔΔCT method.

Detection of Malondialdehyde (MDA) Level

The MDA levels of HUVECs with different treatments were measured by thiobarbituric acid (TBA) method using a commercial kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. The absorbance at 532 nm was detected using microplate reader.

Genome-Wide DNA Methylation Assay

The genomic DNA was extracted from the HUVECs with different treatments using QLAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Then, the genome-wide DNA methylation was assessed using the Infinium Human Methylation 450 BeadChip kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. Briefly, the DNA samples were prepared using EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA). Then, the whole-genome amplification reaction was performed, and then an enzymatic end-point fragmentation, precipitation and resuspension in hybridization buffer were also carried out. Subsequently, hybridization to chips, washing, extension, and imaging were performed according to the protocol. The signals were then converted to beta (β)-value. The β-values of each CpG site ranging from 0 to 1 reflected the percentage methylation levels from 0-100%, respectively.

DNA Methylation Analysis

For methylation analyses, genomic DNA was isolated from the HUVECs with different treatments using QLAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Bisulfite sequencing analysis was performed as previously described[14]. The primers used for the bisulfite sequencing analysis were as follows: 5'-TGGG TAAA TTGA GGTT TAGG AGTT A-3', 5'-TCTA AAAC TCAA CRCT AACT TCAC C-3'.

Statistical Analysis

The data were expressed as means ± standard deviation (SD). Statistical analysis was performed using SPSS software for Windows (version 11.0, SPSS Inc., Chicago, IL, USA). Differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. A $p<0.05$ was considered as a statistically significant difference.

Results

The HUVECs Were Isolated Successfully

As shown in Figure 1A (left), the vWF positive cells exhibited green fluorescence in the cyto-

Table I. Differentially methylated genes from preliminary screening.

Genes	Hcy (AVG β)	Hcy+Fc (AVG β)	Hcy+Fc+B12 (AVG β)	Blank (AVG β)
SORBS1	0.89	0.36	0.34	0.33
AIPL1	0.98	0.09	0.08	0.06
GABRG1	0.97	0.12	0.15	0.14
SNW1	0.98	0.87	0.09	0.08
UNG	0.67	0.70	0.13	0.01
CX	0.91	0.17	0.92	0.26

Notes: "AVG β" means the average of β-value.

plasm, and can be confirmed to the HUVECs. As shown in Figure 1A (right), the nuclei exhibited blue fluorescence after DAPI staining. The results proved that purity of the isolated cells was almost 100%. Overexpression of CD31 is a character of vein endothelial cells[16]; therefore, flow cytometry was applied for the confirmation of HUVECs. The percentage of CD31-positive cells in negative control, isotype control and CD31 antibody treatment groups were 0.1%, 1.2%, and 91.7%, respectively, indicating the purity of the HUVECs was 91.7%.

Treatment With Fc and B12 Attenuated the Oxidative Stress Induced by Hcy

The results in the Figure 2A showed that Hcy increased the MDA level in the HUVECs, and

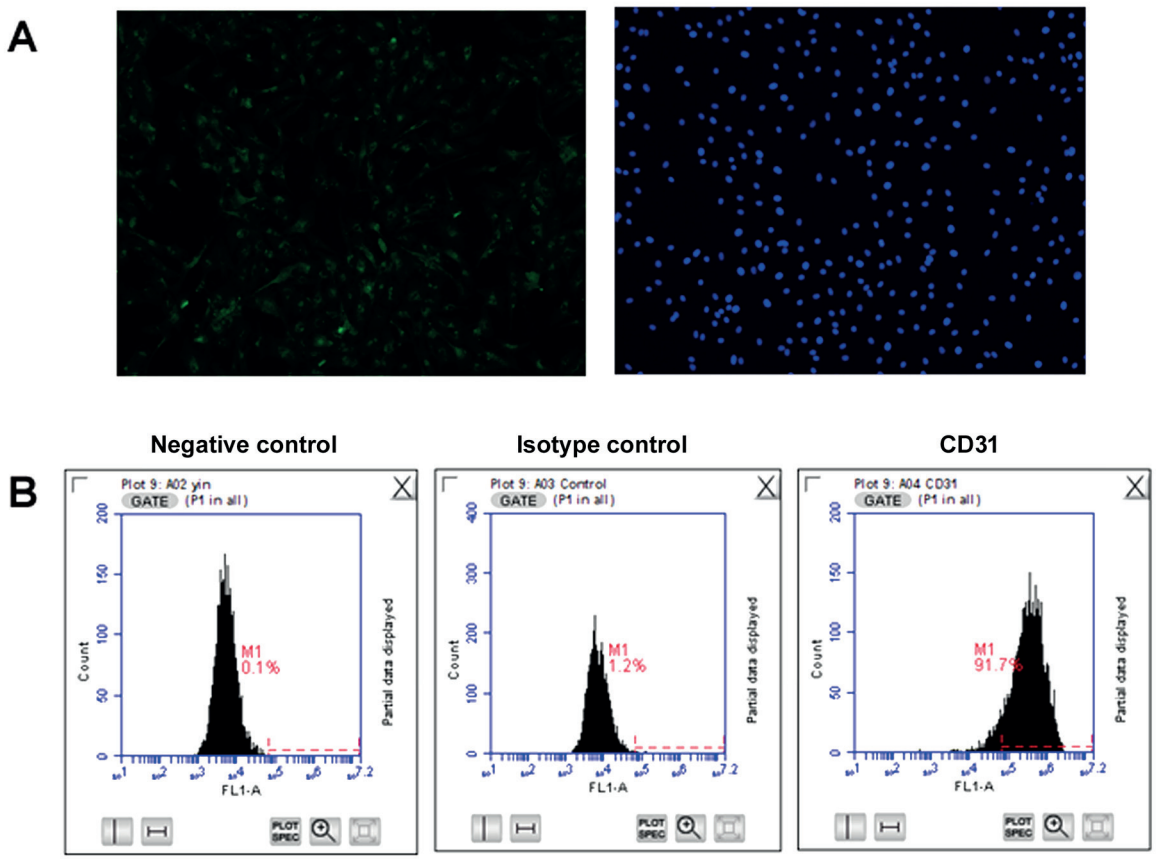

Figure 1. The HUVECs were isolated successfully. (A) The isolated cells were detected by immunofluorescence assay. The vWF positive cells exhibited green fluorescence in the cytoplasm (left). The nuclei exhibited blue fluorescence after DAPI staining (right). (B) The flow cytometry was applied for the confirmation of HUVECs.

treatment with Fc or Fc + B12 significantly attenuated the induction ($p<0.05$). As shown in Figures 2B and 2C, the protein and mRNA levels of SOD2 in Hcy induced cells were decreased compared with control group ($p<0.05$). However, protein and mRNA levels of SOD2 were increased in the cells treated with Fc or Fc + B12, compared to the cells induced by Hcy ($p<0.05$). Combination of Fc and B12 exhibited a stronger effect on the protein and mRNA levels of SOD2 compared with Fc treatment ($p<0.05$). Besides, Hcy also inhibited the protein and mRNA levels of eNOS, and the inhibition was attenuated by Fc or Fc + B12 treatment (Figures 2D and 2E, $p<0.05$).

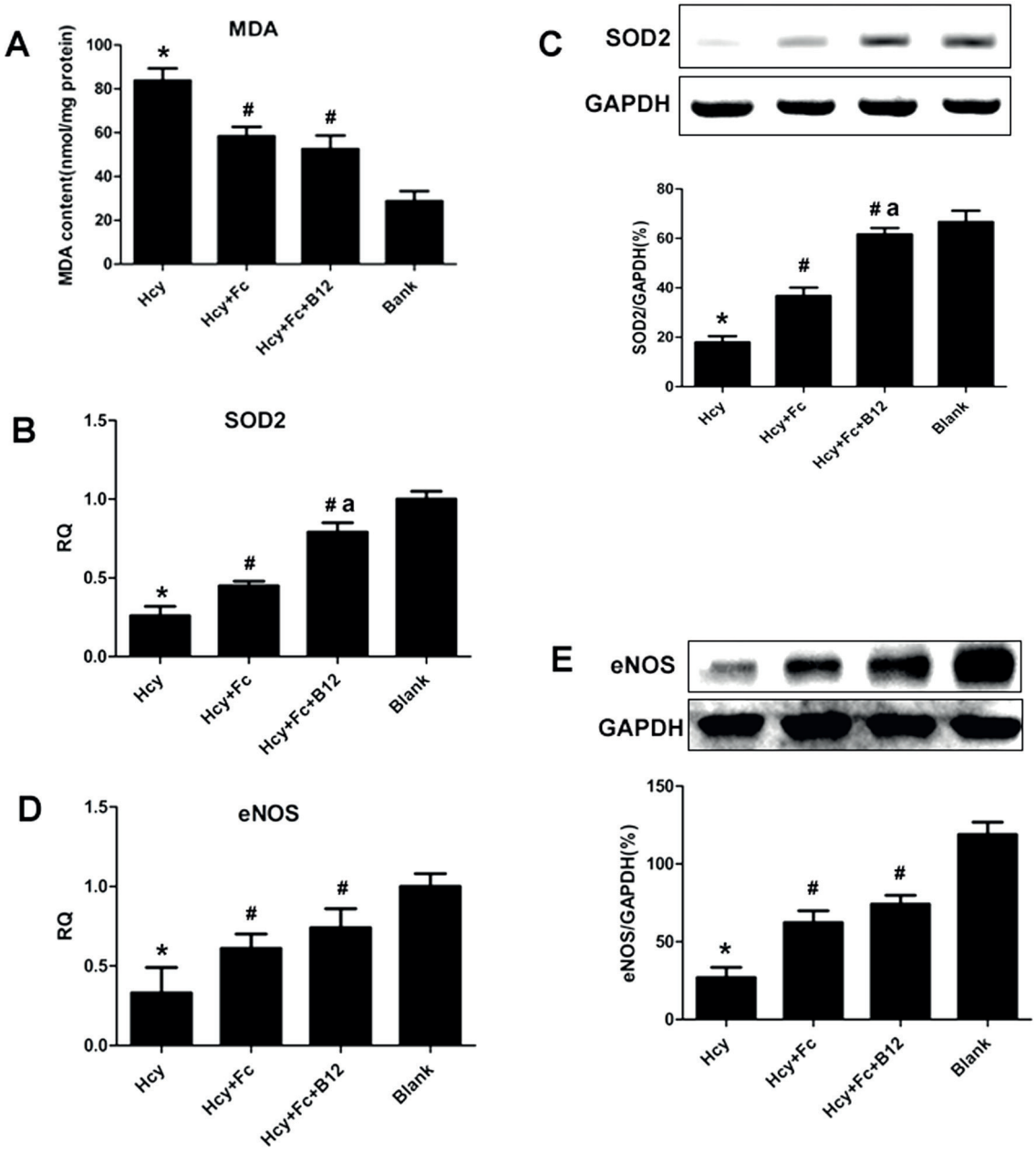

Figure 2. Treatment with Fc and B12 attenuated the oxidative stress induced by Hcy. ***(A)*** The MDA level was measured using thiobarbituric acid (TBA) method. ***(B)*** The mRNA levels of SOD2 were detected by RT-PCR. ***(C)*** The protein levels of SOD2 were detected by Western blot. ***(D)*** The mRNA levels of eNOS were detected by RT-PCR. ***(E)*** The protein levels of eNOS were detected by Western blot. *$p<0.05$ vs. blank control; #$p<0.05$ vs. Hcy treatment group; $^a p<0.05$ vs. Hcy + Fc treatment group.

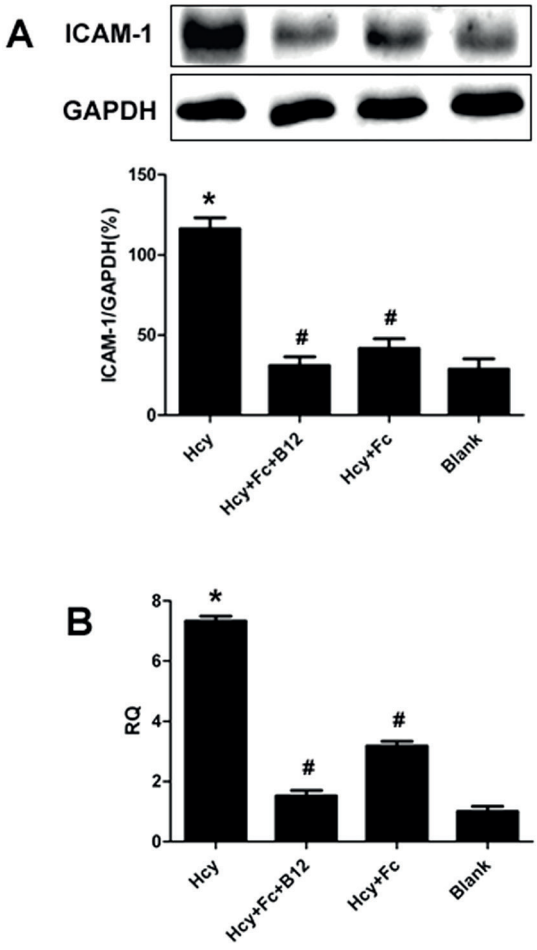

Figure 3. Treatment with Fc and B12 attenuated the inflammation induced by Hcy. ***(A)*** The protein levels of ICAM-1 were detected by Western blot. ***(B)*** The mRNA levels of ICAM-1 were detected by RT-PCR. $^{*}p<0.05$ vs. blank control; $^{\#}p<0.05$ vs. Hcy treatment group; $^{a}p<0.05$ vs. Hcy + Fc treatment group.

Treatment with Fc and B12 Attenuated the Inflammation Induced by Hcy

ICAM-1 is an important factor in the inflammation response, and the results of Western blot proved that Hcy induced ICAM-1 expression, and the induction was reversed by Fc or Fc + B12 treatment (Figure 3A, $p<0.05$). Besides, the results of RT-PCR showed that the mRNA level of ICAM-1 was also induced by Hcy; however, the effect of Hcy was attenuated by Fc or Fc + B12 treatment (Figure 3B, $p<0.05$).

Treatment With Fc and B12 Attenuated the Methylation of SORBS1 Induced by Hcy

Since DNA methylation plays an important role in AS, the genome-wide DNA methylation was assessed. We found that the genomic DNA methylation level in Hcy treated cells was significantly decreased compared to the control group, while the genomic DNA methylation levels were increased after treatment with Fc or Fc + B12 (Figure 4A, $p<0.05$). Differentially methylated genes were selected as follows: the absolute value of β values difference between Hcy treated group and blank group was more than 0.5; the methylation occurred in 5'UTR and TSS region; the differentially methylated genes were observed in all of the four groups. Finally, six genes were selected including SORBS1, AIPL1, GABRG1, SNW1, UNG, and CX (Table I). According to the published papers, only SORBS1 has been reported to be related with cardiovascular disease[12,13]. Therefore, SORBS1 was selected for the further investigation. Bisulfite sequencing analysis was performed to further assess the methylation of SORBS1. As shown in Table II, methylation level of SORBS1 in Hcy treatment group was higher than that of control group. And the methylation level of SORBS1 in Fc or Fc + B12 treatment group was lower than Hcy treatment group. The data provided similar results with the chip assay. Furthermore, we also examined the protein and mRNA levels of SORBS1. The protein and mRNA levels of SORBS1 in Hcy treated cells were decreased compared with control group (Figures 4B and 4C, $p<0.05$). However, protein and mRNA levels of SORBS1 were increased in the cells treated with Fc or Fc + B12, compared to the cells induced by Hcy (Figures 4B and 4C, $p<0.05$).

Knockdown of SORBS1 Induced Oxidative Stress and Reduced Anti-Oxidative Activity

To investigate the role of SORBS1 in oxidative stress, the SORBS1 was knocked down by transfection with SORBS1-siRNA. Observation of green fluorescence was considered as success transfection. The fluorescent counting was performed 48 and 72 h after transfection. As shown in Figure 5A, the GFP expression was higher on 72 h after transfection, and the transfection efficiency was more than 80%. The protein and mRNA levels of SORBS1 were also detected using Western blot and RT-PCR, respectively. The results of Figures 5B and 5C showed that protein and mRNA levels of SORBS1 were significantly decreased in the cells transfected with SORBS1-siRNA ($p<0.05$). The results indicated that the SORBS1 was knocked down successfully. After SORBS1-siRNA transfection, the MDA levels and SOD2 expressions were measured. As shown in Figure 6A, the MDA levels were signi-

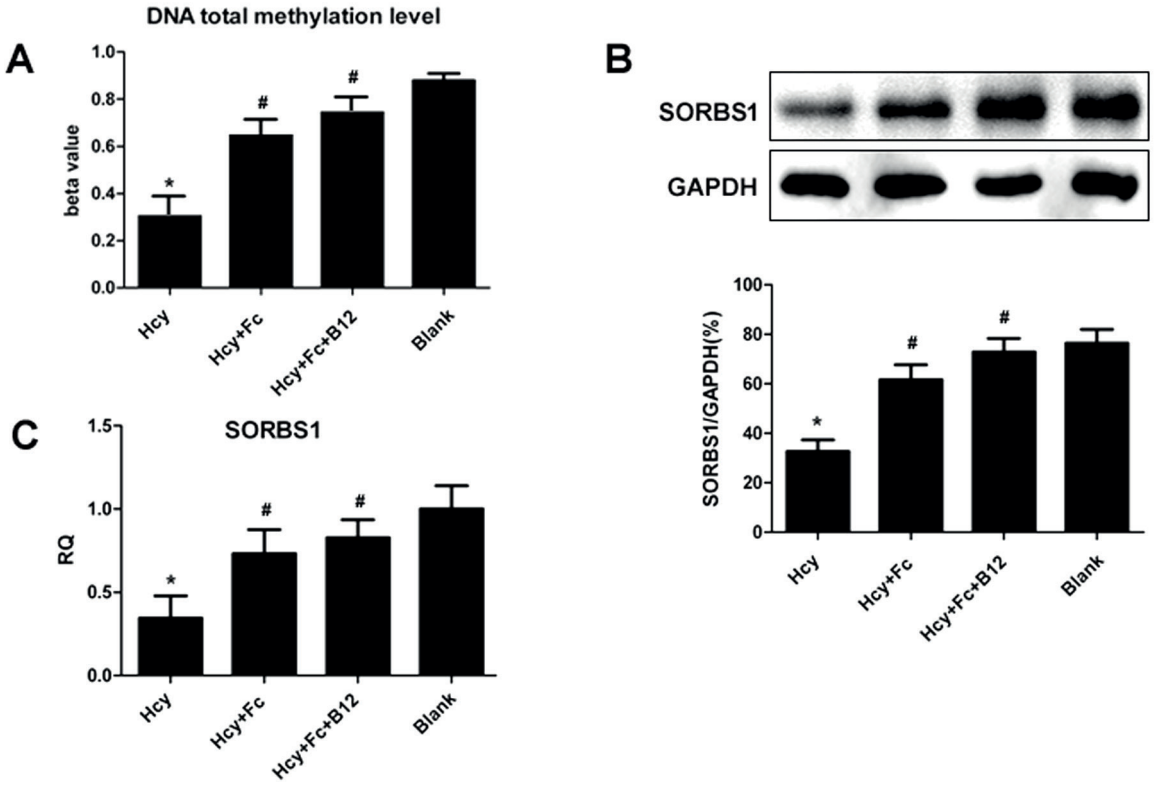

Figure 4. Treatment with Fc and B12 attenuated the methylation of SORBS1 induced by Hcy. ***(A)*** The genome-wide DNA methylation assay was performed using the Infinium Human Methylation 450 BeadChip. ***(B)*** The protein levels of SORBS1 were detected by Western blot. ***(C)*** The mRNA levels of SORBS1 were detected by RT-PCR. *p<0.05 vs. blank control; #p<0.05 vs. Hcy treatment group; ap<0.05 vs. Hcy + Fc treatment group.

ficantly increased after SORBS1-siRNA transfection compared to the NC-siRNA transfection (p<0.05), indicating that knockdown of SORBS1 induced oxidative stress. The SOD2 expression was decreased in the cells transfected with SORBS1-siRNA compared with the cells transfected with NC-siRNA (Figure 6B, p<0.05). In addition, the mRNA level of SOD2 was also inhibited by SORBS1-siRNA (Figure 6B, p<0.05), indicating that knockdown of SORBS1 reduced anti-oxidative activity.

Discussion

Hcy has been proved to induce oxidative stress and cell injury in HUVECs[14,15]. Hcy treatment causes cell injury by inhibiting cell viability, inducing DNA damage, reactive oxygen species and secretory dysfunction of HUVECs[15]. Besides, Hcy also induces cell apoptosis in HUVECs through Endothelin b-receptor/NADPH oxidase-dependent pathways[16]. MDA is a kind of product of lipid peroxidation, and the MDA level is usually used to reflect the status of oxidative stress[17]. SOD is an important enzyme in antioxidant system, which protects cells from free radical damage[18]. There are two subtypes of SOD, SOD1 and SOD2; SOD1 is located in cytoplasm, while SOD2 is located in mitochondria[18]. The role of SOD2 is more important than SOD1 in oxidative stress[19]. Therefore, the MDA levels and SOD2 expressions were measured to evaluate the oxidative stress. The results showed that Hcy induced MDA level and reduced the SOD2 expression in HUVECs, indicating Hcy induced oxidative stress through increasing the product of lipid peroxidation and inhibiting the antioxidant system. There are many pharmacologic agents modulating oxidative stress, which are used for improving AS. Fc and antioxidant B12 have been proved to improve endothelial dysfunctions in patients with AS[20]. Fc and B12 have been reported[21, 22] to reduce the Hcy level in plasma and improve the vascular endothelium function. In the present study, the effect of Fc and B12 on Hcy-induced oxidative stress was evaluated. We found that the oxidative

Table II. Methylation levels of SORBS1 in cells with different treatments.

		Methylation of CpG island																				
Groups	**Clones**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Hcy	**1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
	2	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
	5	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Hcy+Fc	**1**	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hcy+Fc +B12	**1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Blank	**1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Notes: (+) methylated; (-) unmethylated.

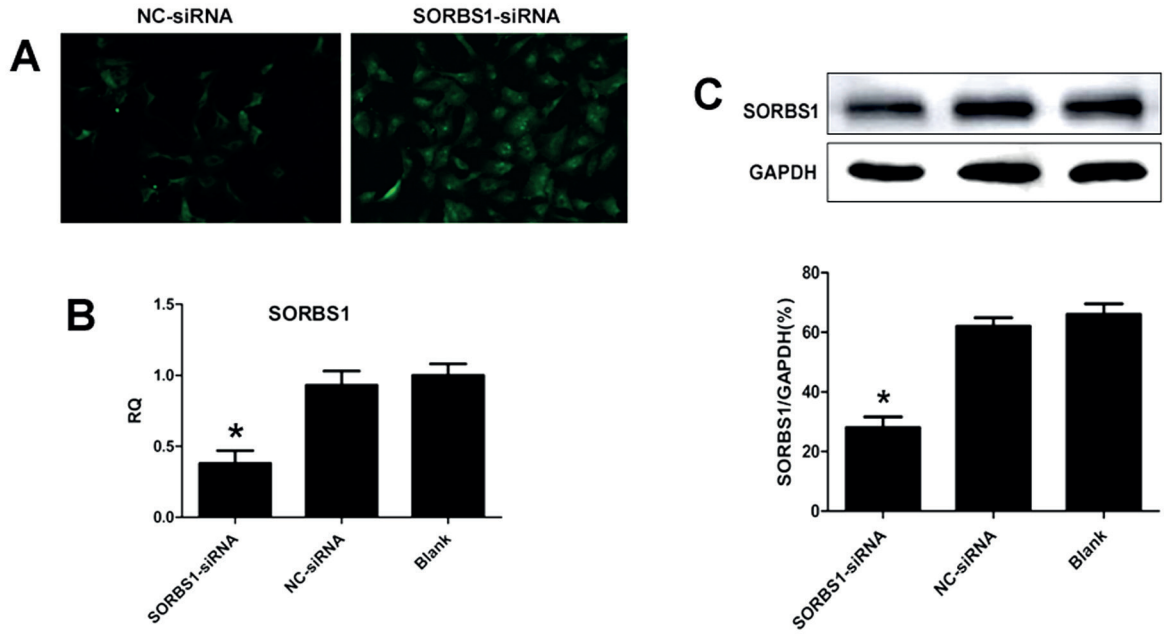

Figure 5. SORBS1 gene was successfully knocked down in HUVECs. To investigate the role of SORBS1 in oxidative stress, the SORBS1 was knocked down by transfection with SORBS1-siRNA. ***(A)*** The transfected cells were examined under a fluorescence microscope. ***(B)*** The mRNA levels of SORBS1 were detected by RT-PCR. ***(C)*** The protein levels of SORBS1 were detected by Western blot. *p<0.05 vs. Cells transfected with NC-siRNA.

stress induced by Hcy was attenuated by Fc and B12. Nitric oxide (NO) is one of the endothelium dependent vasodilatation factors which can regulate blood vessel tension and protect cells against

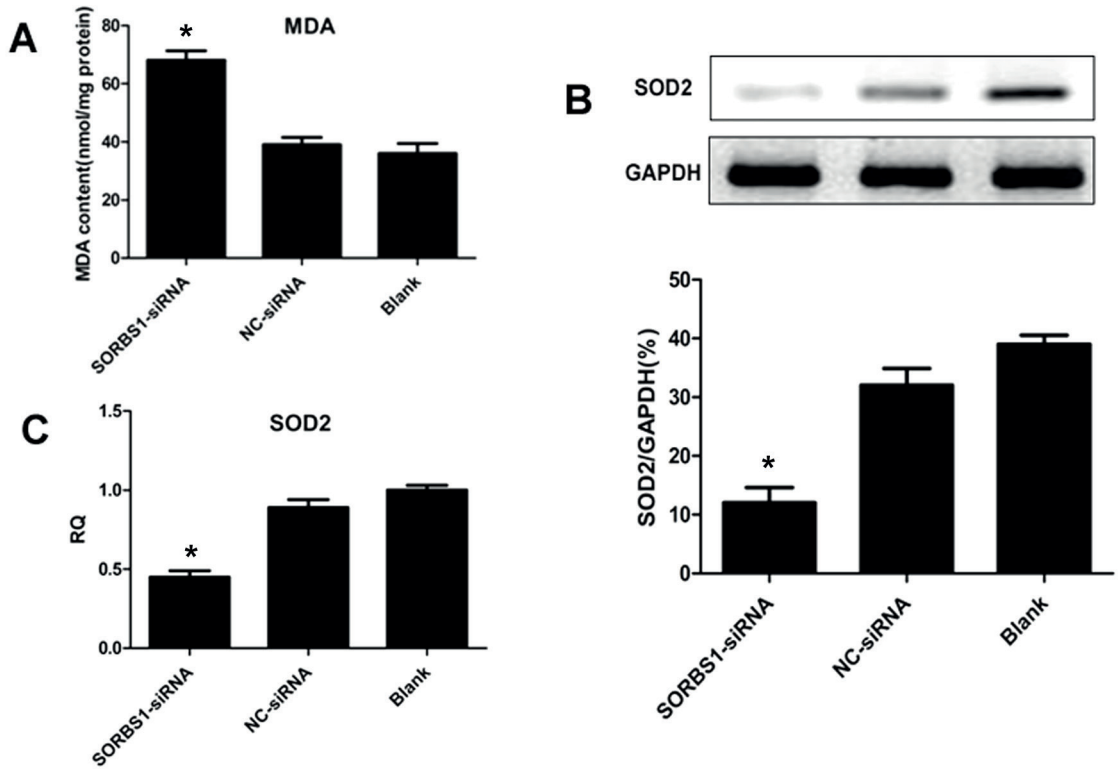

Figure 6. Knockdown of SORBS1 induced oxidative stress and reduced anti-oxidative activity. ***(A)*** The MDA level was measured using thiobarbituric acid (TBA) method. ***(B)*** The protein levels of SOD2 were detected by Western blot. ***(C)*** The mRNA levels of SOD2 were detected by RT-PCR. *$p<0.05$ vs. Cells transfected with NC-siRNA.

AS[23, 24]. The release of NO is reduced during vascular endothelial dysfunction, leading to the development of AS[23]. eNOS is the key enzyme for the synthesis of endothelial NO[23]. In the present work we observed the effect of Hcy on eNOS expression. The results showed that eNOS expression was significantly decreased by Hcy induction. After Fc and B12 treatment, the eNOS expression was significantly increased, indicating that Hcy may reduce the release of NO and result in vasodilation dysfunction. ICAM-1 is a type of intercellular adhesion molecule and belongs to the immunoglobulin superfamily[25]. The ICAM-1 is low-expressed in endothelial cells under physiological condition; when cells are under oxidative stress, the NF-κB is activated, leading to the overexpression of ICAM-1 and improvement of neutrophil adhesion, finally resulting in inflammation[26]. In the present study, Hcy significantly induced the ICAM-1 expression in HUVECs, indicating that Hcy caused oxidative and inflammatory injury. Treatment with Fc and B12 alleviated the injury induce by Hcy. Previous studies[27] proved that genomic DNA methylation levels are decreased in the development of AS. It has been proved that the decreased genomic DNA methylation levels might be the early marker of AS progression or the reason for the formation of atherosclerotic plaque[9]. Therefore, DNA methylation is closely related with AS, and might be the mechanism of AS[9]. In this work, we also found that genomic DNA methylation levels were significantly decreased in the cells treated with Hcy. However, the mechanism was still unclear and needed to be clarified. Besides, we also found that treatment with Fc and B12 attenuated the inhibition effect of Hcy on DNA methylation, suggesting that Fc and B12 may be useful for preventing the development of AS. SORBS1 is an adaptor protein, which belongs to SOHO family[28]. SORBS1 has been found to suppress tumor metastasis and improve the sensitivity of cancer to chemotherapy drug[29]. Researches proved that SORBS1 was associated with cardiovascular and cerebrovascular diseases. It is reported that 2 specific single nucleotide polymorphisms (SNPs) of SORBS1 were associated with the prevalence of hypertension[30, 31]. SORBS1 is an important regulator of insulin signaling pathway, which majorly participates in the insulin signal transduction[32]. Dysfunction of insulin signaling pathway may result in insulin resistance

(IR)[33,34]. It has been reported that the activation of the oxidative-inflammatory loop plays a major role in the pathogenesis of IR[35]. The findings provide evidence that SORBS1 is associated with oxidative and inflammatory responses. However, the role of SORBS1 in AS is unclear. Our findings indicated that methylation level of SORBS1 in Hcy treatment group was higher than that of control group. The results indicated that Hcy caused IR and induced cell injury through inhibiting SORBS1 expression via regulating SORBS1 methylation. Besides, knockdown of SORBS1 induced oxidative stress and reduced anti-oxidative activity in HUVECs. The findings indicated that Hcy induced SORBS1 methylation in HUVECs, causing the dysfunction of insulin signaling pathway, thus resulting in IR and finally induced cell injury. On the other hand, Hcy induced SORBS1 methylation in HUVECs, thus down-regulated the SORBS1 expression, which may further induce oxidative stress and reduce anti-oxidative activity in HUVECs. In addition, methylation level of SORBS1 was decreased in the cells treated with Fc and B12, suggesting that Fc and B12 might be a potential agent for the Hcy induced AS.

Conclusions

We evaluated the mechanism of Hcy induced oxidative stress in HUVECs. The results indicated that Hcy induced oxidative stress in HUVECs via regulating methylation of SORBS1. Besides, the protective effect of Fc and B12 on Hcy induced oxidative stress was also investigated. We found that Fc and B12 treatment attenuated the oxidative stress and inflammation induced by Hcy in HUVECs. The findings indicated that Fc and B12 might be effective agents for the treatment of Hcy induced AS.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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