Department of Pharmaceutics[1], The Third XiangYa Hospital, Central South University; Hunan Key Laboratory of Medical Epigenomics[2], Department of Dermatology, The Second Xiangya Hospital, Central South University, Hunan, Changsha, China

Homocysteine-induced hypermethylation of DDAH2 promoter contributes to apoptosis of endothelial cells

Su-Jie Jia[1], Yong-Quan Lai[1], Ming Zhao[2], Ting Gong[1], Bi-Kui Zhang[1]

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Bi-Kui Zhang, Department of Pharmaceutics, The Third XiangYa Hospital, Central South University, Hunan, Changsha, China. Tongzipo Road #138, Changsha 410013, China
zhbk@yahoo.cn

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Homocysteine (Hcy) could induce apoptosis of endothelial cells (ECs). Dimethylarginine dimethylaminohydrolase 2 (DDAH2) is recognized as a protective factor to improve the endothelial function. Defect of DDAH2 has been confirmed to be involved in the Hcy-induced dysfunction of endothelial NO system. This study was to determine whether Hcy could inhibit DDAH2 expression and induce apoptosis of ECs *via* increasing DNA methylation level of DDAH2 promoter and whether DNA methylation inhibitor 5-azacytidine (5-aza) could attenuate the effect of Hcy on ECs. Firstly, human umbilical vein endothelial cells (HUVECs) were treated by Hcy with or without 5-aza for 48 h. MTT assay showed that Hcy reduced the viability of HUVECs in a dose-dependent manner. The level of asymmetric dimethylarginine (ADMA) and the apoptosis rate of HUVECs treated with Hcy at 1.0 mM were increased significantly compared with that of control. Moreover, we found that mRNA level of DDAH2 was down-regulated and DNA methylation level of DDAH2 promoter was increased significantly in HUVECs treated with Hcy, in concomitance with up-regulated protein level of DNA methyltransferase 1 (DNMT1). Furthermore, we also found that 5-aza could neutralize the effect of Hcy on ECs through up-regulating mRNA level of DDAH2 and inducing demethylation of DDAH2 promoter. In conclusion, hypermethylation of DDAH2 contributes to Hcy induced apoptosis of ECs. Modulating DNA methylation status of DDAH2 promoter may be a potential strategy for treatment of endothelial dysfunction.

1. Introduction

Homocysteine (Hcy) is an amino acid derived from the metabolic demethylation of dietary methionine. As an independent risk factor for atherosclerosis (AS) (Bao et al. 2010), Hcy decreases endothelial nitric oxide (NO) production and impairs endothelium-dependent vasodilation (Heil et al. 2004). Importantly, Hcy can induce endothelial cells (ECs) apoptosis, which causes dysfunction of ECs and play an important role in the pathogenesis of AS (Bao et al. 2010). Presently the mechanisms of Hcy-induced ECs apoptosis are still not well understood.

The specific conversion of Hcy to S-adenosyl Hcy (SAH) represents one of mechanisms capable of perturbing ECs phenotype (Dayal et al. 2001; Wang et al. 1997). Altered methylation patterns associated with increased levels of SAH have been reported to modulate protein functions and the transcriptional activities of GC-rich promoters (Cedar 1988; Clarke 1993). Methylation of CpG islands within promoter or enhancer regions suppresses the transcription of genes (Newell-Price et al. 2000). Previous studies showed that Hcy could induce aberrant hypermethylation modifications of several genes such as FGF2, ERα, PPARα/γ and ApoE to decrease their expression, contributing to AS (Chang et al. 2008; Huang et al. 2009; Yi-Deng et al. 2007; Yideng et al. 2008).

Increased level of the circulating asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase (NOS) inhibitor, has been shown to contribute to apoptosis of ECs in AS (Jiang et al. 2006). Dimethylarginine dimethylaminohydrolase (DDAH), the key enzyme for degradation of ADMA, plays an important role in modulating the level of ADMA (Palm et al. 2007). DDAH2 predominates in tissues expressing endothelial NOS, and impairment of DDAH2 activity and/or expression rather than DDAH1 is involved in the elevation of plasma ADMA in ECs of AS (Fiedler et al. 2009). Previous study showed High CG content was enriched in the promoter region of DDAH2 gene, which was regulated by DNA methylation modification (Zhang et al. 2007). However, up to now, there is no study exploring whether Hcy can cause apoptosis of ECs through inducing hypermethylation of DDAH2 promoter and inhibiting DDAH2 expression.

In this study, we found that Hcy induced apoptosis of ECs and the elevation of ADMA level, accompanying with hypermethylation of DDAH2 promoter and reduced DDAH2 expression, which could be inhibited by DNA methylation inhibitor 5-azacytidine (5-aza). These data suggest that Hcy induces apoptosis of ECs by increasing DNA methylation level of DDAH2 promoter and inhibiting DDAH2 expression, which may be a novel target for AS treatment.

2. Investigations and results

2.1. Effects on cell viability of Hcy with different concentrations

MTT assay showed that the viability of HUVECs was decreased by Hcy (from 0.1 mM to 30 mM) in a dose-dependent

Table 1: Effects of Hcy on cell viability in HUVECs

Concentration of Hcy (mM)	Cell viability (OD value)
0	0.821 ± 0.009
0.1	0.751 ± 0.015
0.3	0.704 ± 0.028
1.0	0.668 ± 0.029 *
3.0	0.594 ± 0.029 *
10.0	0.424 ± 0.026 *
30.0	0.279 ± 0.015 *

Cells were incubated with various concentrations of Hcy. All data are presented as mean ± SEM of five independent experiments ($^{*}P < 0.05$ *vs.* control).

manner compared with solvent control (Table 1). The significant decreased viabilities were observed in 1 to 30 mM Hcy treated groups. To avoid from toxicity effect of Hcy, 1 mM was chosen in following experiments, which has also been reported by other group (Lee et al. 2005).

2.2. 5-Aza can resist the apoptosis of HUVECs and elevated ADMA level induced by Hcy

According to a flow cytometric assay, we found that the level of ADMA and the apoptosis rate of HUVECs were increased significantly after incubation with 1 mM Hcy for 48 h compared with the solvent control group. However, the increased ADMA level and apoptosis of HUVECs induced by Hcy can be inhibited significantly when HUVECs were treated simultaneously by 1 mM Hcy and 5 μM 5-aza simultaneously (Fig. 1 and 2).

2.3. Hcy down-regulated DDAH2 expression and 5-aza attenuated its inhibition

The levels of DDAH2 mRNA expression were measured using RT-qPCR. All of the values were normalized with β-actin, which was served as the internal control. After treatment with 1 mM Hcy for 48 h, DDAH2 mRNA expression was inhibited significantly. However, no significant change of DDAH2 mRNA level was observed in HUVECs treated by 1 mM Hcy with 5 μM 5-aza simultaneously (Fig. 3).

2.4. Hcy induced DNA hypermethylation of DDAH2 promoter, which can be inhibited by 5-aza

To investigate whether aberrant DNA methylation might contribute to the decreased expression of DDAH2, we measured the DNA methylation level of DDAH2 promoter in HUVECs treated by Hcy by MS-PCR. According to analysis of CpG island

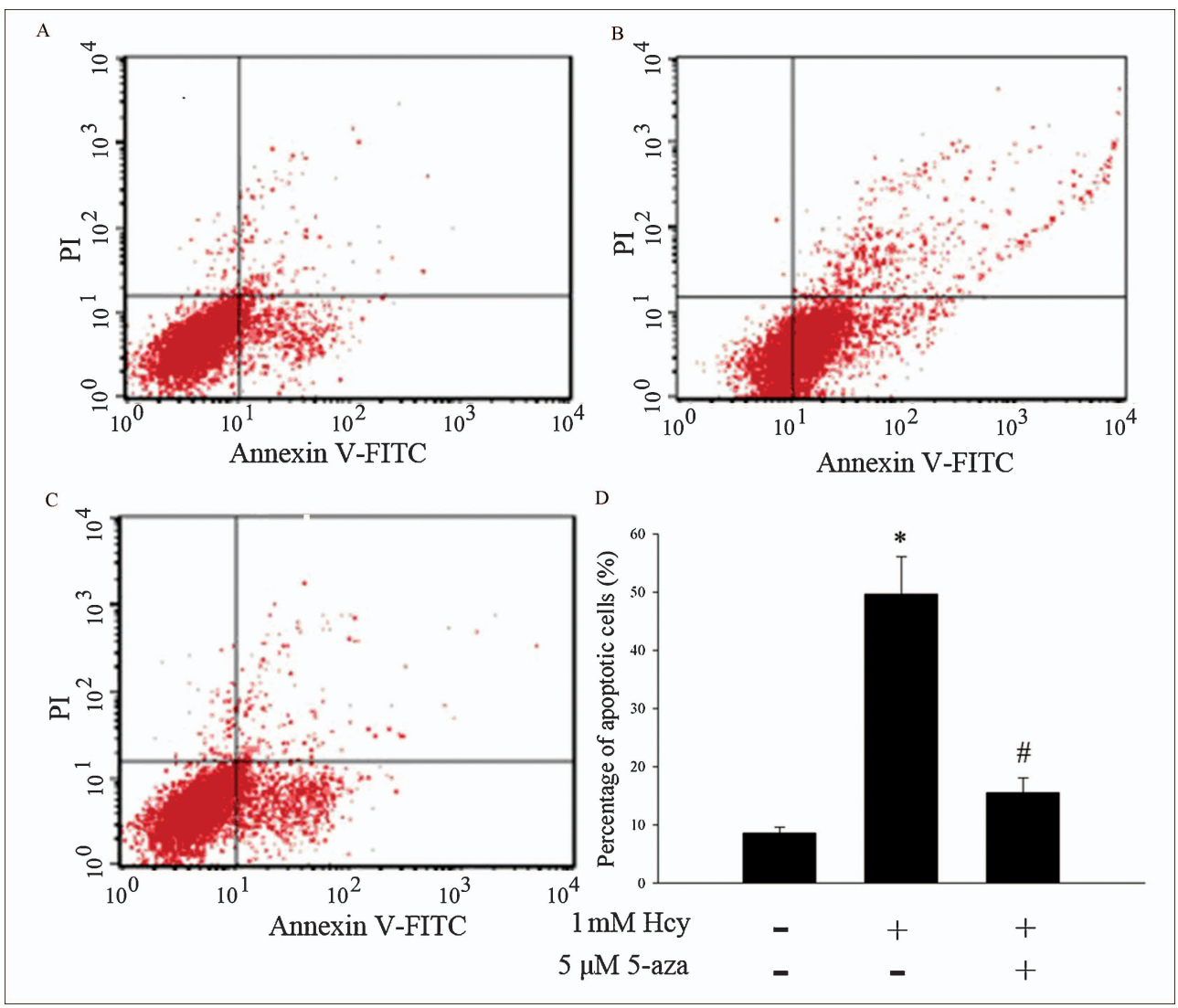

Fig. 1: Hcy induced apoptosis of HUVECs, which was attenuated by 5-aza. HUVECs were treated with DMSO (negative control, A) and 1 mM Hcy without 5-aza (B) or with 5-aza (C), and then were analyzed by flow cytometry. Bar graph showing the percentage of apoptotic cells in HUVECs. The Data expressed as mean ± SEM of five independent experiments ($^{*}P < 0.05$ vs.control; $^{\#}P < 0.05$ vs. Hcy-treated group).

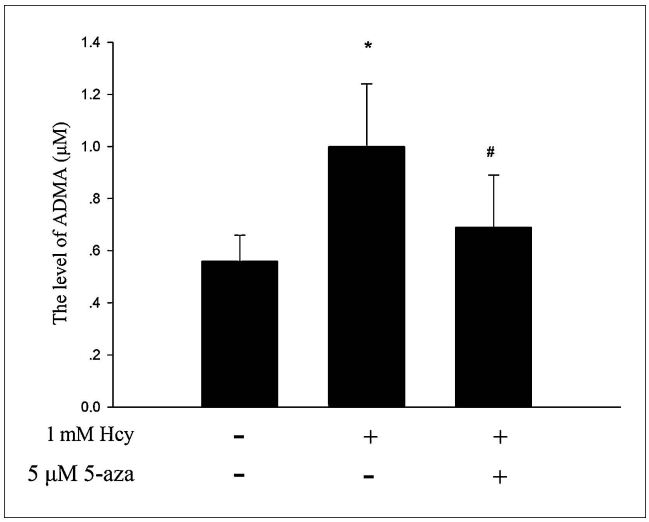

Fig. 2: 5-aza prevented Hcy induced elevation of ADMA level in conditioned medium of HUVECs. HUVECs were treated by 1 mM Hcy with or without 5 μM 5-aza. Negative control was treated by vehicle (DMSO). Values were expressed as mean ± SEM of five independent experiments ($^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. Hcy-treated group).

using an online program (http://www.urogene.org/methprimer), we found that two CpG islands were located in the promoter of DDAH2 gene from −1000 bp to + 500 bp of the transcription start site (TSS). The results from MS-PCR showed that the methylation level of the promoter region of DDAH2 gene was increased significantly in HUVECs treated with Hcy (1.0 mM, 48 h) compared with the solvent control group. In contrast, DDAH2 promoter still remains hypomethylated in HUVECs treated by 1 mM Hcy and 5 μM 5-aza simultaneously (Fig. 4).

2.5. DNMT1 protein level was increased in HUVECs treated with Hcy, which was reversed by 5-aza

As DNA methyltransferase, DNMT1 plays a critical role in maintaining DNA methylation. To investigate the mechanism of hypermethylation induced by Hcy, we measured DNMT1 protein level in HUVECs treated by 1 mM Hcy. Figure 5

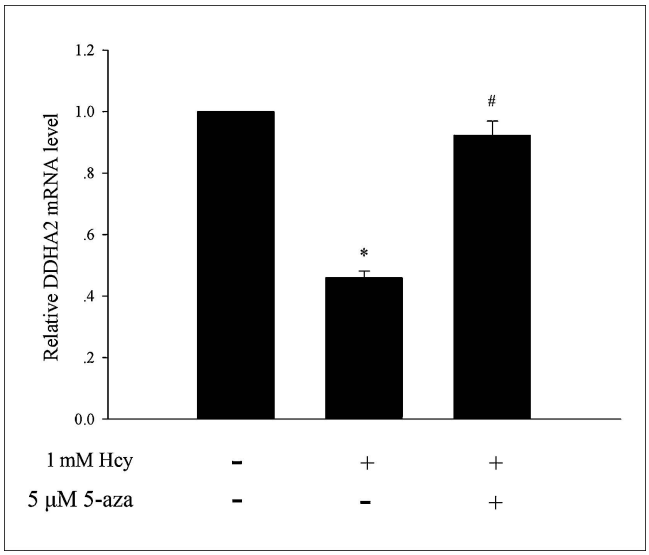

Fig. 3: DDAH2 mRNA level was down-regulated by Hcy (1mM), which was attenuated by 5-aza (5 μM). Bar graphs showing relative mRNA expression of DDAH2 normalized to GAPDH. HUVECs were treated by 1mM Hcy with or without 5 μM 5-aza. Negative control was treated by vehicle (DMSO). Values were expressed as mean ± SEM of five independent experiments ($^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. Hcy-treated group).

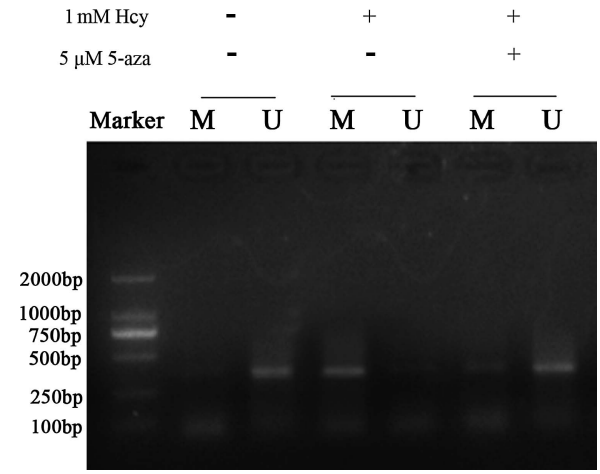

Fig. 4: 5-aza prevented DDAH2 promoter from DNA hypermethylation induced by Hcy. Examples of MSPCR analysis of the DDAH2 promoter region. The resulting products were analyzed by gel electrophoresis. A visible product in the (M) lane indicates that DDAH2 is methylated; a visible product in the (U) lane indicates that the DDAH2 gene is unmethylated in the corresponding DNA. Lane 1 is DNA marker. Lane 2 and 3 are the products of PCR of negative control. Lane 4-7 show PCR products of DNA from HUVECs treated by Hcy (1 mM) with (+/+) or without (+/−) 5-aza (5 μ M).

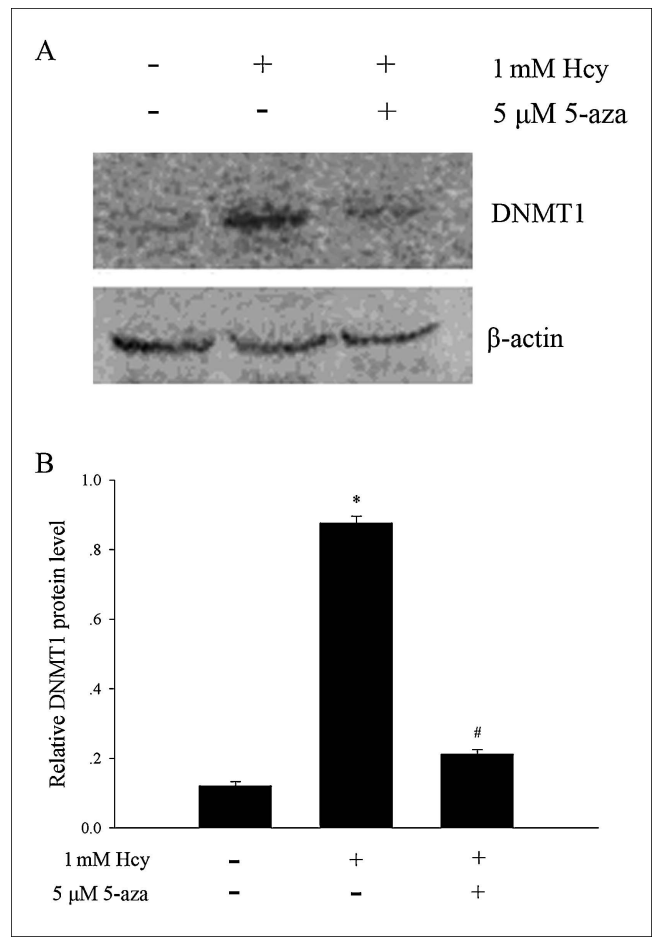

Fig. 5: DNMT1 protein level was increased in HUVECs treated with Hcy, which was reversed by 5-aza. The protein expression of DNMT1 was detected by western blot. Data expressed as mean ± SEM of five independent experiments. Means ± SEM were plotted as percentages relative to the untreated control which was set to 1 (100 %). ($^{*}P < 0.05$ vs.control; $^{\#}P < 0.05$ vs. Hcytreated group). A: Representative results of western blot. B: statistic analysis of band intensity. Y axis shows the relative protein level of DDAH2 normalized to ß-actin in HUVECs treated by negative control (DMSO) and 1mM Hcy with or without 5μM 5-aza.

shows that DNMT1 protein expression was up-regulated significantly in HUVECs treated by 1 mM Hcy compared with controls. However, no significant change of DNMT1 protein level was observed in HUVECs treated by 1 mM Hcy and 5 μM 5-aza.

3. Discussion

It is now widely accepted that increased plasma Hcy is associated with increased cardiovascular risk, independent of other AS risk factors (Dinavahi and Falkner 2004). Prolonged exposure of endothelial cells to Hcy impairs the production of nitric oxide and endothelium-dependent vasodilatation (Chao et al. 2000). Multiple reports have shown that Hcy induced apoptosis of ECs and cardiomyocytes through p38 MAPK signaling (Bao et al. 2009; Bao et al. 2010; Wang et al. 2012). In this study, our results showed that DDAH2 expression, as the key enzyme for degradation of ADMA, was down-regulated by Hcy, accompanying the increased ADMA level in ECs. Our previous study has demonstrated that ADMA, an endogenous inhibitor of NOS, can induce apoptosis of endothelial cells *via* elevation of intracellular oxidant production, which involves p38 MAPK/caspase-3-dependent signaling pathway (Jiang et al. 2006). Therefore, our finding suggests a novel mechanism whereby Hcy induces apoptosis of ECs through regulating the DDAH2-ADMA axis.

More and more evidence suggests that increases or decreases in DNA methylation are associated with the development of AS, and the methylation changes that occur in those processes may include the activation or inactivation of AS-related genes (Post et al. 1999; Turunen et al. 2009; Zhu et al. 2005). Previous reports showed that Hcy regulates the expression of multiple genes in different cell types through altering DNA methylation modification (Chang et al. 2008; Wang et al. 1997; Yi-Deng et al. 2007; Yideng et al. 2008; Zhang et al. 2007). Therefore, we postulated that Hcy down-regulated DDAH2 through regulating DNA methylation modification in the process of apoptosis of ECs. We first analyzed the promoter of DDAH2 gene and found that two CpG islands were enriched within 2 kb region upstream of TSS of DDAH2. In this study, we chose to detect the DNA methylation status of the CpG island nearby TSS, because it has been shown to be a methylation-sensitive region (Tomikawa et al. 2006).

Our data showed that aberrant DNA hypermethylation in the DDAH2 promoter region and inhibited DDAH2 expression occurred in apoptotic HUVECs induced by Hcy (1 mM), suggesting that DNA hypermethylation of DDAH2 promoter may be involved in the Hcy-induced apoptosis of ECs. To further clarify the mechanism of apoptosis of ECs induced by Hcy, DNA methylation inhibitor 5-aza was used in HUVECs treated by Hcy simultaneously. Our data showed that hypermethylation of DDAH2, down-regulation of DDAH2 expression and apoptosis of ECs induced by Hcy were attenuated by 5-aza. Moreover, we also found that the protein expression level of DNA methyltransferase 1 (DNMT1) was significantly elevated in HUVECs treated by Hcy, which is the key enzyme for maintaining DNA methylation modification (Rountree et al. 2001). Together, the above indicated that Hcy induced DNA hypermethylation modification of specific genes in ECs through increasing DNMT1 expression.

In summary, our data show that DNA hypermethylation status of DDAH2 promoter was induced in HUVECs treated by Hcy. DNA methylation inhibitor 5-aza can lead to demethylation of DDAH2 promoter, reactivation of DDAH2 transcript expression and inhibition of Hcy-induced apoptosis. These results suggest that DNA hypermethylation of DDAH2 promoter plays an important role in Hcy induced endothelial apoptosis. Our results provide new evidence for the epigenetic mechanism in the development of AS which could be a new target for the therapy of cardiovascular diseases.

4. Experimental

4.1. Cell lines and culture

Human umbilical vein endothelial cells (HUVECs) were originally obtained from ATCC (CRL-2480). HUVECs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) fetal bovine serum. Cells were seeded at a density of 3×10^5/ml and were treated with Hcy (0.1, 0.3, 1, 3, 10, 30 mM) for 48 h. In other cases, HUVECs were treated with Hcy (1 mM) in the presence of or in the absence of 5-aza (5 μM) for 48 h.

4.2. MTT Cell viability assay

Cell viability was determined by the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Endothelial cells were seeded into 96-well culture plates at an optimal density of 5×10^3/ml. After 3–4 days of culture to 90% confluence, 20 μL of MTT solution (5 mg/mL MTT in phosphate buffer solution, pH 7.4) was added to each well and incubated at 37 °C for 4 h. The precipitate was dried in air and dissolved in 200 μl of DMSO. The optical density of each sample was immediately measured in microplate reader (ELX800, USA) at 490 nm.

4.3. Isolation of RNA and real-time quantitative reverse transcription -polymerase chain reaction (RT-qPCR)

Total RNA was isolated from HUVECs using the RNeasy mini kit (Qiagen, CA, U.S.A.). cDNA synthesis was performed using RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada) and 1 μg of total input RNA according to the manufacturer's instructions. RT-qPCR was performed using a Rotor-Gene3000 (Corbett Research, NSW, Australia) and mRNA levels were quantified using SYBR Premix Ex Taq^{TM} real-time PCR Kit (TaKaRa Biotech [Dalian] Co., China). GAPDH was also amplified and used as a loading control. The fold change was calculated using the formula $2^{-\Delta\Delta Ct}$. $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{internal control}})_{\text{treated}} - (Ct_{\text{target gene}} - Ct_{\text{internal control}})_{\text{untreated}}$. The following primers were used: DDAH2 5'-GGTGCTGGGAGGTAAACTGA-3'(Forward), 5'TCGCGTTCTCGTCTCCTATT-3' (Reverse); GAPDH: 5'-AACAGCCTCAAGATCATCAG-3 (Forward) 5'-GGATGATGTTCTG GAGAGCC-3' (Reverse).

4.4. Apoptosis assay by annexin V staining

HUVECs were seeded in 12-well plates and were treated with 1 mM Hcy in the presence or absence of 5-aza for 48 h, respectively. The cells were then collected and treated as the protocol of Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China) and the percentages of apoptotic cells were determined by a FACSCalibur flow cytometer (Becton Dickinson, CA, USA). Results were analyzed by Cell Quest Pro software (Becton Dickinson).

4.5. Determination of ADMA concentration

ADMA level was measured by high performance liquid chromatography as previously described (Jia et al. 2006).

4.6. Methylation-specific polymerase chain reaction (MS-PCR)

Genomic DNA was isolated from HUVECs with the use of a TIANamp Genomic DNA Kit (TIANGEN Biotech, BEI JING) according to the manufacturer's instructions. The reaction of genomic DNA (2 μg) with bisulfite using EpiTect® bisulfite kit (QIAGEN, Valencia, CA) converted all unmethylated cytosine residues to uracil. Bisulfite-treated genomic DNA was amplified using either a methylation-specific or an unmethylation-specific primer set. The CpG island close to TSS was analyzed. Sequences of the methylation-specific primers were 5'-TAGGGAATTTTGGAGTATTTGTTTC-3' (forward) and 5'-AAATCT-AACCGACCCTAACGAC-3' (reverse). Sequences of the unmethylation-specific primers were 5'-TAGGGAATTTTGGAGTATTTGTTTT-3' (forward) and 5'-CCAAATCTAACCAACCCTAACAA-3'(reverse). The cycling conditions were as follows: 10 min at 95 °C, and 36 cycles of 30 s at 96 °C, 20 s at 61 °C followed by 20 s at 72 °C.

4.7. Statistical analysis

Results are expressed as means ± SEM. Statistical analysis of these data were performed using unpaired Student's *t*-test. The significance level was chosen as $P < 0.05$.

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