

Homocysteine-impaired angiogenesis is associated with VEGF/VEGFR inhibition

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
 - 3.1. Cell culture
 - 3.2. Cell proliferation assay
 - 3.3. Cell viability assay
 - 3.4. Cell cycle analysis
 - 3.5. Cell migration assay
 - 3.6. Matrigel assay
 - 3.7. Zebrafish embryos angiogenesis assay
 - 3.8. Quantitative real time PCR for mRNA expression analyses
 - 3.9. Western blot analysis
 - 3.10. Statistical methods
4. Results
 - 4.1. Hcy inhibited HUVEC proliferation and viability
 - 4.2. Hcy induced HUVEC cell cycle G1/S arrest
 - 4.3. Hcy inhibited HUVEC migration
 - 4.4. Hcy suppressed tube formation
 - 4.5. Hcy inhibited the vessel formation in zebrafish embryo
 - 4.6. Hcy inhibited expression of VEGF, VEGFR1 and VEGFR2
 - 4.7. Hcy decreased ERK1/2 and Akt expression in HUVEC
5. Discussion
6. Acknowledgment
7. References

1. ABSTRACT

This study investigated the effects of homocysteine (Hcy) on angiogenesis in cultured human umbilical vein endothelial cells (HUVEC) and zebrafish embryos. We found that Hcy (50 micro mol/L) significantly decreased cell numbers, viability, and induced a G1/S arrest in HUVEC in the presence of adenosine (Ade, 50 micro mol/L). Hcy, in combination with Ade, reduced migration and suppressed tube-like formation on Matrigel in HUVEC. Further, Hcy reduced subintestinal vessel formation in zebrafish embryos. Interestingly, Hcy-induced inhibitory effects on cell growth, migration, tube-like formation, and vessel formation in HUVEC and zebrafish embryos were abolished by the supplement of recombinant VEGF (10ng/ml). Finally, Hcy in combination with Ade reduced the mRNA levels of VEGF, VEGFR-1, VEGFR-2, and attenuated protein levels of VEGF, ERK1/2 and Akt. The present study suggests that Hcy inhibits angiogenesis, and that the mechanism anti-angiogenic effects of Hcy may be through VEGF/VEGFR, Akt, and ERK1/2 inhibition.

2. INTRODUCTION

Hyperhomocysteinemia (HHcy) is an established independent and significant risk factor for cardiovascular disease (CVD) and can be classified as moderate (15 to 30μmol/L), intermediate (30 to 100μmol/L), and severe (>100μmol/L) (1, 2). Several mechanisms including endothelial cell (EC) damage and dysfunction (3-5), dyslipidemia (6), alterations in vessel structure (7, 8), and stimulation of vascular smooth muscle cell proliferation (9) have been proposed to explain the cardiovascular changes associated with HHcy.

The integrity of vascular endothelium is crucial for physiologic function. In response to injury, ECs undergo an active compensatory process involving proliferation, migration, and angiogenesis to reestablish homeostasis.

Homocysteine (Hcy) has been reported to inhibit angiogenesis *in vitro* and *in vivo* (10-12). The anti-angiogenic effect of Hcy occurs at very high concentrations

of Hcy (1-200 mmol/L), which is higher than the concentration of Hcy related CVD. The molecular and biochemical basis by which HHcy inhibits angiogenesis is not clear. Our previous study has shown that HHcy-induced endothelial dysfunction was associated with increased Thr-495 phosphorylation of endothelial nitric oxide synthase(eNOS) through protein kinase C (PKC) activation and decreased Ser-1177 phosphorylation of eNOS through compromised vascular endothelial growth factor(VEGF)/Akt signaling (3, 5). It is reasonable to ascertain that the VEGF/Akt/eNOS pathway plays a crucial role in angiogenesis (13).

Recently, it was reported that the specific conversion of Hcy to *S*-adenosyl Hcy (SAH) represents another mechanism capable of perturbing ECs phenotype (14). SAH is a potent inhibitor of cellular methylation. Elevated levels of Hcy, in combination with adenosine (Ade), leads to increased levels of SAH and acts to inhibit cellular methylation. Hypomethylation may be a specific biochemical mechanism by which HHcy induces vascular injury (14-16).

In the present study, , we studied whether clinically relevant concentrations of Hcy would inhibit angiogenesis through hypomethylation. To test our hypothesis, we measured the effect of Hcy on angiogenesis by examining endothelial cell proliferation, migration, and vessel formation in the zebra fish model and cultured HUVEC at an intermediate concentration of Hcy (50μmol/L) with and without Ade (50μmol/L).

3. MATERIALS AND METHODS

3.1. Cell culture

Primary human umbilical vein endothelial cells (HUVEC) were cultured as previously described (5). Briefly, HUVEC were isolated from human umbilical veins after 0.25% trypsin (TBD, China) digestion and maintained on gelatin (Acumedia, Lansing, MI, USA) coated T25 flasks in M199 (Hyclone, USA) supplemented with 20% fetal bovine serum (FBS; Hyclone, USA), 50 ug/mL endothelial cell growth supplement (ECGS; BD, Bedford, MA, USA), and 50ug/mL heparin (DINGGUO, Beijing, China). Cultures were maintained at 37°C in a humidified 5% CO₂ incubator. HUVEC were identified by their typical cobblestone appearance and the presence of von Willebrand Factor (factor VIII). HUVEC from 2-4 passages were used for the experiment.

3.2. Cell proliferation assay

HUVEC proliferation was assessed by direct cell counting. Briefly, HUVEC were seeded in 24-well plates at 3×10^4 cells/well. Cells were incubated with PBS, 50μmol/L adenosine (Ade) (Sigma, St Louis, MO), or 50μmol/L DL-Hcy in the presence or absence of 50μmol/L Ade for 48h. 10μmol/L erythro-9- (2-hydroxy-3-nonyl)-adenine (EHNA) (Sigma, RBI), an Ade deaminase inhibitor that stabilizes Ade, were added in all the experiments. The addition of Ade served to enhance the conversion of Hcy to SAH. HUVEC were also incubated with PBS, 50μmol/L Ade, or 50μmol/L DL-Hcy in the presence or absence of 50μmol/L Ade in the presence of 10 ng/mL recombinant human

VEGF165 (R&D, USA) to examine whether VEGF could counteract the effects of Hcy on HUVEC proliferation. The cells were then trypsinized and cell number was determined by counting with a hemocytometer.

3.3. Cell viability assay

Cell viability was assessed using the MTT assay according to manufacture recommendation (Sigma, St Louis, MO, USA). Briefly, HUVEC (1×10^4 cells/well) were plated onto 96-well plates and routinely incubated for 24h. Cells were then treated with assay media for 48h. MTT reagent was added for the last 4h of incubation. The supernatant was removed and dimethyl sulfoxide (DMSO) (Sigma, St Louis, MO, USA) was added to solubilize the formazan crystals. Absorbance was measured at 492 nm with an enzyme-linked-immunosorbent assay plate reader (Thermo, Boston, Massachusetts, USA)

3.4. Cell cycle analysis

To analyze the cell cycle, DNA content was measured by flow cytometry. HUVEC were seeded in 60 mm dishes at the density of 1.25×10^5 cells/dish. After treatment with the assay media for 24h, HUVEC were harvested, adjusted to a concentration of 1×10^6 cells/ml, and fixed in 70% ethanol at 4°C overnight. The fixed cells were washed twice with cold PBS, and then incubated for 30 min with RNase (8 mg/ml) and propidium iodide (10 mg/ml). The cell cycle was then assayed by flow cytometry (EPICS XL-MCL, Beckman Coulter, Florida, USA).

3.5. Cell migration assay

Cell migration was measured using a scratch injury assay. After 24 hours of assay media treatment, 90% confluent monolayer of HUVEC were wounded using a micropipette tip (100 μl yellow tip), gently rinsed with PBS, and then incubated in the same assay media. HUVEC migration was photographed using a live cells imaging system (Zeiss Axio Observer Z1, Germany) in 37 °C and 5% CO₂ humidified atmosphere. Live cells can be photographed every 5 minutes and the results can be exported in way of movies which provided intuitive cell motion images. Individual cell migration was quantified from 3 separate experiments measuring 10 cells per treatment using AxioVision microscope software (Zeiss, Germany) with an automatic and interactive tracking module as described in Starke *et al* (17). Parameters including total distance, straight distance, mean velocity, and tortuosity were analyzed.

3.6. Matrigel assay

To determine whether Hcy inhibited angiogenesis, we used the experimental in vitro Matrigel system (18). Matrigel Basement Membrane Matrix (BD, Bedford, USA) was thawed on ice at 4°C overnight and all pipettes and 96-well flat bottom plate were pre-cooled before use. Ninety-six-well plates were coated with 50 μl Matrigel per well. HUVEC were seeded at 4×10^4 cells per well in 100μl assay media. Each group was in triplicate. After 16h of incubation, tube-like structures were photographed using inverted microscope (IX71). The total tube length was quantified using Image J software (NIH, Bethesda, MD, USA).

Table 1. Primer sequences for Quantitative real-time PCR

Gene	F primer	R primer
VEGF	5'- TACCTCCACCATGCCAAGTG -3'	5'- GATGATTCTGCCCTCCTCCTT -3'
VEGFR1	5'- TCACTGCCACTCTAATTGTCAATGT -3'	5'- AAACGATGACACGGCCTTTT -3'
VEGFR2	5'- CAAGACAGGAAGACCAAGAAAAGAC -3'	5'- GGTGCCACACGCTCTAGGA -3'
β-actin	5'-AACCAGCAGAGATGACCCAGATCATGTTT -3'	5'-AGCAGCCGTGGCCATCTCTTGCTCGAAGTC -3'

Table 2. Cell cycle distribution of HUVEC (Mean±SD)

Group	G1 (%)	S (%)	G2/M (%)
PBS Control	59.9±4.2	20.3±1.0	19.9±4.4
Ade	56.5±8.6	29.3±11.8	14.2±3.7
Hcy	59.9±5.9	20.5±1.3	19.7±5.7
Ade+Hcy	73.1±9.3 *	12.2±6.0 *	14.6±4.1
Ade+Hcy+VEGF	62.8±3.8 #	21.6±6.6 #	15.8±7.1

*p<0.05 vs PBS control, Ade and Hcy, #P<0.05 vs Ade+Hcy

3.7. Zebrafish embryos angiogenesis assay

Transgenic (flk1:GFP) zebrafish embryos were generated by natural pair-wise mating, collected and maintained in 28.5°C incubator as described before (19). Healthy, limpid, and fluorescent embryos were picked out at 24 hours post-fertilization (hpf) and distributed into a 96-well microplate (3 embryos/well) containing Holt Buffer. Meanwhile, 50μmol/L Ade, 50μmol/L DL-Hcy, or 50μmol/L DL-Hcy in the presence of 10ng/mL VEGF165 were added to the culture media. PBS served as the control. Embryos were maintained in a 28.5°C incubator for an additional 48h, placed onto a glass slide, and photographed using fluorescence stereomicroscope. The overall length and branch points of the subintestinal vessels (SIVs) were quantified using Image J software. Anti-angiogenic effects were defined as the decrease of SIVs length and branch points (20).

3.8. Quantitative real time PCR for mRNA expression analyses

Total RNA was extracted from the HUVEC cells treated with the assay media for 24h. 500 ng of total RNA from each sample were used for cDNA synthesis using Prime Script RT reagent Kit (TaKaRa). cDNA was subsequently amplified with the SYBR Premix Ex Taq™ Kit (TaKaRa) in 8 Strip PCR tubes using the iQ5 instrument (Roche) as follows: 10 minutes at 95°C for enzyme activation followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 55°C, one second at 72°C for the amplification step, and 30 seconds at 40°C for the cooling step. Changes in the expression of target genes (VEGF, VEGFR1, and VEGFR2) were measured relative to the mean critical threshold (CT) values of β-actin gene. The primers used in the qRT-PCR evaluation were specific for every gene as previously reported (Table 1)(21, 22). 1μM of both primers were used for all genes.

3.9. Western blot analysis

HUVEC were treated with assay media for 48 h. Western blot procedures were carried out as described previously (5). Briefly, whole cell lysates from each sample were taken after normalizing for equal protein concentration, and were analyzed with specific antibodies against VEGF (1:1000; Abcam, Cambridge, UK), Akt (1:1000; CST, USA), and ERK1/2 (1:1000; CST, USA). Incubation with monoclonal GAPDH antibody (1:10000; Sigma, USA) was carried out as the loading control. The blots were detected on Kodak X-Omat film by enhanced

chemiluminescence (Applygen Technologies, Beijing, China). Quantification of band intensity was carried out using Image J software (NIH, Bethesda, MD, USA).

3.10. Statistical methods

All data were from at least three independent experiments. Results were expressed as the mean ± SD. Statistical comparison among multiple groups was

performed by one-way ANOVA followed by LSD test using the SPSS 17.0 software. A *p*<0.05 was considered to be statistically significant.

4. RESULTS

4.1. Hcy inhibited HUVEC proliferation and viability

The effect of Hcy on HUVEC proliferation was evaluated by both direct cell counting and the MTT assay. In the presence of Ade, treatment with Hcy significantly decreased cell number and viability of HUVEC, whereas exposure of HUVEC to Hcy alone did not significantly affect cell number and viability when compared to PBS control (Figure 1A, B). Thus, Hcy in combination with Ade may inhibit HUVEC proliferation. In addition, we observed that the supplement of recombinant VEGF165 significantly improved Hcy-inhibited HUVEC proliferation and cell viability (Figure 1A, B).

4.2. Hcy induced HUVEC cell cycle G1/S arrest

The DNA content of HUVEC was measured to determine the cell cycle distribution after treatment with the assay medium. The exposure of HUVEC to Hcy in combination with Ade significantly increased the percentage of cells retained in the G1 phase of the cell cycle when compared to PBS control (from 59.9% to 73.1%; *P*<0.05). In addition, there was a concomitant decrease in cells entering the S phase (Table 2 & Figure 1C), which represents a G1/S arrest that is induced by Hcy in combination with Ade. No significant change was observed in HUVEC treated with Hcy alone. The supplement of recombinant VEGF165 significantly improved Hcy-inhibited HUVEC cell cycle transition (Table 2 & Figure 1C).

4.3. Hcy inhibited HUVEC migration

We investigated the role of Hcy in HUVEC migration using a scratch wound assay and a live cells imaging system. As shown in Figure 2, Hcy in combination

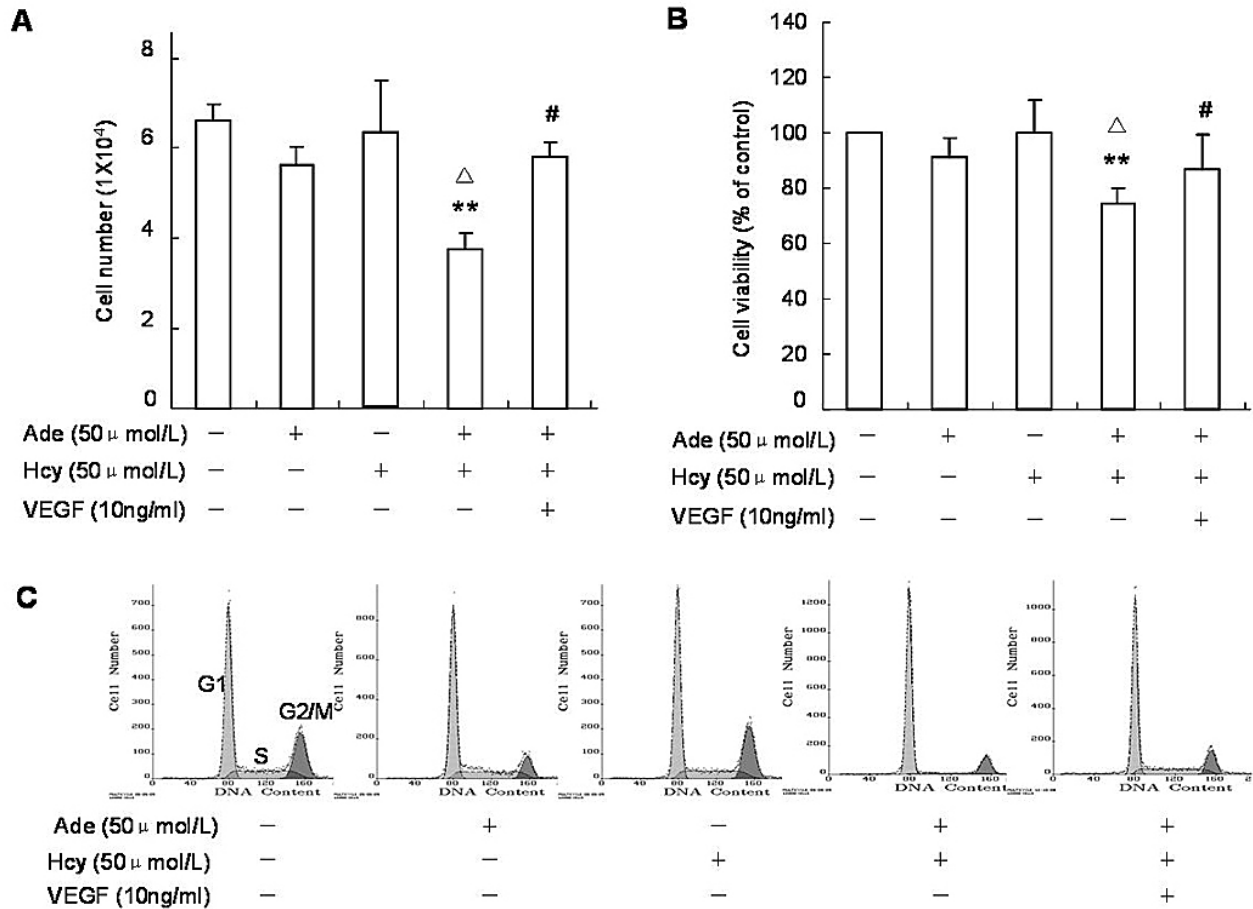


Figure 1. Effect of Hcy on HUVEC proliferation and cell cycle transition. A. Cell number counting. HUVEC were seeded in 24-well plates and treated with the assay media for 48h. The cells were then trypsinized and cell number was determined by counting with a hemocytometer. B. MTT analysis. HUVEC were seeded in 96-well plates and treated with the assay media for 48h. MTT was added for the last 4h. The supernatant was removed and DMSO was added to solubilize the formazan crystals. The absorbance at 492 nm was measured. C. Cell cycle graph from cytometry. HUVEC were seeded in 60 mm dishes and treated with the assay media for 24h before fluorescence-activated cell sorter analysis. D. Cell cycle graph from flow cytometry. ^{**} $p < 0.01$ vs PBS Control; $\geq p < 0.05$ vs Ade and Hcy; $\#p < 0.05$ vs Ade+Hcy.

with Ade significantly decreased HUVEC migratory straight distance, but not total distance when compared to PBS control. This decrease was associated with a loss of directionality. Hcy in combination with Ade also significantly increased the tortuosity of cells movement paths. However, Hcy alone did not impact the migration of HUVEC. The supplement of exogenous VEGF165 significantly improved migratory straight distance and decreased the tortuosity of HUVEC (Figure 2).

4.4. Hcy suppressed tube formation

We investigated the anti-angiogenesis ability of Hcy using the Matrigel tube formation assay. HUVEC were cultured on Matrigel, a solid gel of mouse basement membrane proteins, which easily align and form hollow tube-like structures. Figure 3A shows the representative micrographs. In HUVEC treated with Hcy and Ade, there are less tube-like structures in comparison to PBS control. Quantitative measurements confirmed that Hcy in

combination with Ade triggered a significant decrease in total tube length (Figure 3B). As anticipated, the simultaneous treatment of VEGF165 with Hcy significantly restored angiogenesis *in vitro*.

4.5. Hcy inhibited the vessel formation in zebrafish embryo

To study the effect of Hcy on angiogenesis *in vivo*, transgenic (flk1:GFP) zebrafish embryos, which express GFP in all vascular endothelial cells, were used. The development of zebrafish SIVs was visualized using fluorescence stereomicroscope. SIVs length and branch points number of SIVs were measured with Image J. The representative photos and quantitative analysis of zebrafish SIVs were showed in Figure 4. As expected, both the length and branch points number of SIVs were significantly decreased in zebrafish embryos treated with Hcy in combination with Ade when compared to PBS control, which indicates an inhibitory effect of Hcy plus Ade on angiogenesis of zebrafish embryo. However, Hcy alone

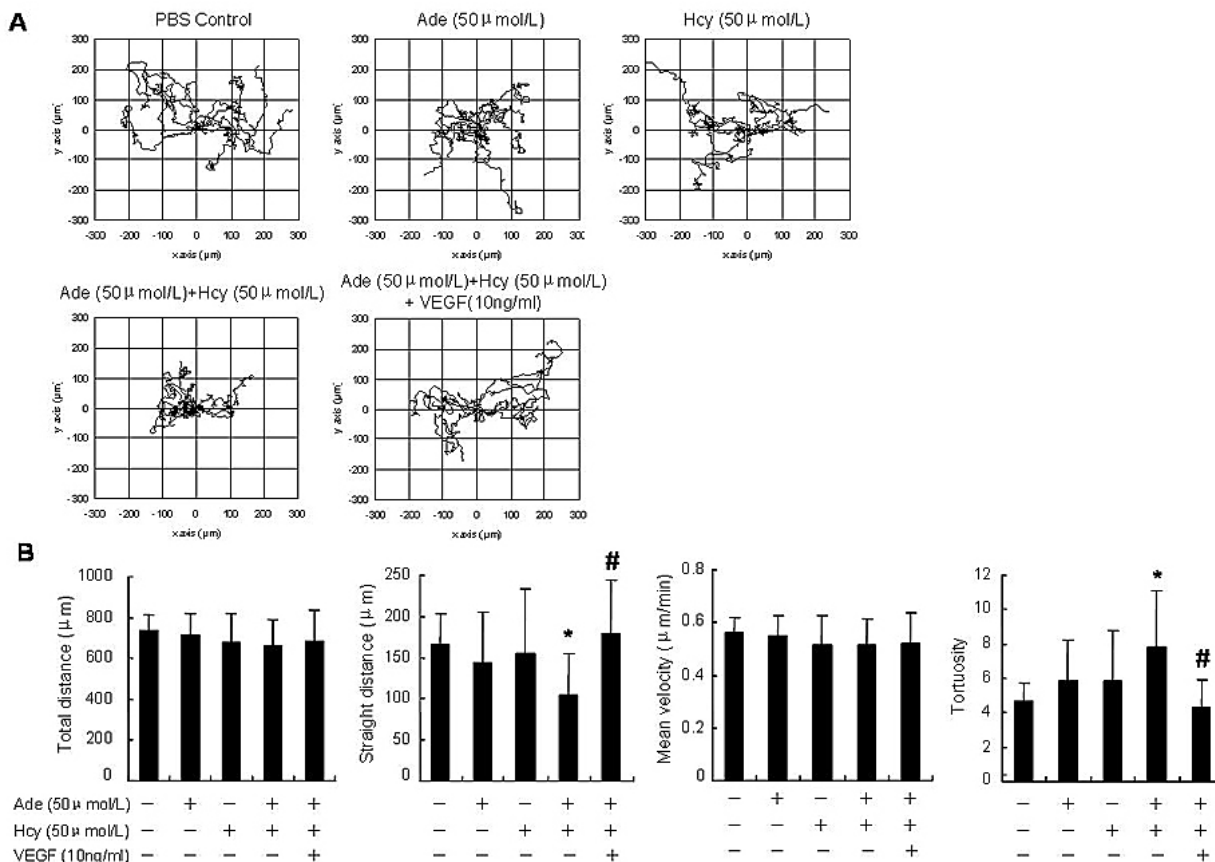


Figure 2. Effect of Hcy on HUVEC migration. HUVEC were incubated with the assay media for 24h and wounded by scratching with a micropipette tip. Cells migration was monitored for the next 22h using a live cells imaging system. 10 cells for each group were randomly marked, and the cells movement paths were tracked. A. Representative trajectory plots of HUVEC migrating into a wounded area for 22 hours. The starting point of each cell trajectory is plotted at the center of the graph and the wounded area is to the left or right of zero on the x-axis (5 cells for each direction). B. Quantitative analysis of migration parameters. * $p < 0.05$ vs PBS control; # $p < 0.05$ vs Ade+Hcy.

resulted in only a slight impairment of SIV formation in a fraction of embryos. The supplement of VEGF165 significantly counteracted the anti-angiogenic effect of Hcy on zebrafish embryos (Figure 4).

4.6. Hcy inhibited expression of VEGF, VEGFR1 and VEGFR2

In order to identify the molecular targets of the anti-angiogenic effect of Hcy on HUVEC, we measured mRNA expression of VEGF, VEGFR1 and VEGFR2 by real-time PCR and protein expression of VEGF by Western blot analysis. Treatment of HUVEC with Hcy plus Ade significantly down regulated mRNA expression of VEGF, VEGFR1, and VEGFR2 when compared to PBS control (Figure 5A). In contrast, treatment of HUVEC with Hcy alone did not result in significant change of VEGF, VEGFR1, and VEGFR2 mRNA expression. (??)Protein levels of VEGF were also significantly decreased by Hcy plus Ade (Figure 5B).

4.7. Hcy decreased ERK1/2 and Akt expression in HUVEC

To explore signaling pathways mediating the anti-angiogenic effect of Hcy, we also examined the expression of ERK1/2 and Akt. Figure 6 shows that in comparison to PBS control, protein levels of ERK and Akt were attenuated in the presence of Hcy and Ade.

5. DISCUSSION

In the present study, we demonstrated that Hcy (50 $\mu\text{mol/L}$) in combination with Ade (50 $\mu\text{mol/L}$) significantly inhibited HUVEC proliferation, migration, tube formation, and angiogenesis in the embryos of zebrafish. Moreover, we observed that the anti-angiogenic effects of Hcy in combination with Ade was associated with VEGF/VEGFR inhibition and a decrease of Akt and ERK1/2.

HHcy is a well established risk factor of CVD (1, 23). Endothelial dysfunction and damage is an initiated and determined event in the development of CVD. It has been

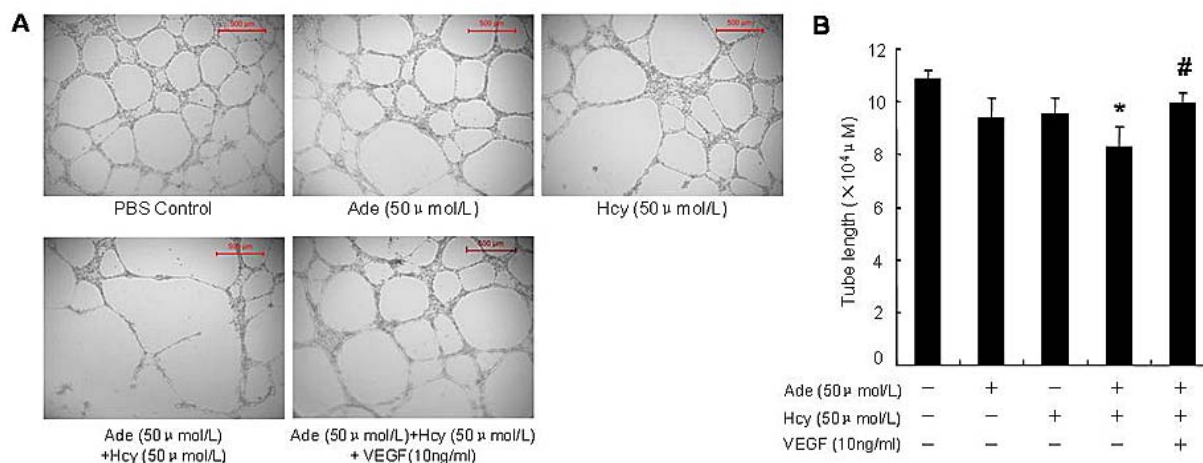


Figure 3. Effect of Hcy on angiogenesis *in vitro*. HUVEC were seeded into the 96-well plate coated with matrigel at a density of 4×10^4 /well with 100 μ l assay media. Each group was in triplicate. After 16h, tube-like structure was photographed. A. Representative photographs of tube-like structure. B. Quantitative analysis of total tube length. * $p < 0.05$ vs PBS control; # $p < 0.05$ vs Ade+Hcy.

reported that Hcy suppresses nitric oxide (NO) production and impairs endothelium-dependent vasodilatation (24-26). Oxidation has been recognized as the primary biochemical mechanism responsible for Hcy pathogenesis(27). Nagai *et al* observed that Hcy inhibited angiogenesis by preventing EC proliferation and migration(12). However, very high concentrations of Hcy (1 to 10 mmol/L) was used in their experiments. Our study uses HUVEC and zebrafish embryos model to delineate the mechanisms of how physiological relevant concentration of Hcy (50 μ mol/L) combined with Ade inhibit angiogenesis. Hcy may interact with Ade, a normal constituent of all body fluids, to become SAH, a potent inhibitor of cellular methylation. Wang *et al* demonstrated that Hcy increased cellular SAH concentration and exerted highly selective inhibitory effects on cyclin A transcription and EC growth through a hypomethylation related mechanism, which blocked cell cycle progression and endothelium regeneration(28, 29).

As the process of angiogenesis involves coordinated ECs proliferation, we investigated the effect of Hcy on HUVEC proliferation and cell cycle transition. Previous studies have reported that Hcy disrupted the growth of ECs and exhibited delayed progression through the cell cycle at the G1/S transition (21, 29). Similarly, we found that Hcy combined with Ade induced an obvious G1/S cell cycle arrest and significantly inhibited cell proliferation in HUVEC, whereas Hcy alone did not. In addition, the supplement of recombinant VEGF165 significantly improved Hcy-inhibited HUVEC proliferation and cell cycle transition.

Angiogenesis encompasses many of the steps including cell proliferation and migration(30). We therefore examined the effect of Hcy on HUVEC migration using a scratch wound assay. Using the live cells imaging system and software, the results can be exported in way of movies which provided intuitive cell motion images and thus cell movement paths can be tracked over time. By using this

method of tracking, we are able to witness the most visible and direct evidence of migration. We found that Hcy plus Ade significantly decreased migratory straight distance, but not total distance, and that this decrease was associated with a loss of directionality. The movement paths of HUVEC treated with Hcy plus Ade exhibited a significantly higher degree of tortuosity. ECs migration is a prerequisite for angiogenesis. Thus, the inhibition of HUVEC migration indicates impaired angiogenesis ability. We also found that HUVEC migration rescue was achieved with exogenous VEGF165 supplementation.

Organization of ECs into a network of tubes is a late event during angiogenesis. We found that Hcy used in combination with Ade inhibited the formation of tube-like structures on Matrigel, which is an indicator of angiogenesis *in vitro*(18). Moreover, we observed the inhibitory effect of Hcy on angiogenesis *in vivo* in transgenic (flk1:GFP) zebrafish embryos where VEGFR is expressed in all ECs(31). Blood vessel patterning is highly characteristic in the developing zebrafish embryo, and the SIVs can be visualized microscopically as a primary screen for compounds that affect angiogenesis(32). Our results showed that Hcy in combination with Ade inhibited the growth of SIVs in zebrafish embryos. Treatment of embryos with exogenous VEGF165, in addition to Hcy plus Ade, significantly improved angiogenesis in zebrafish embryos. Taken together, our present study and previous data predict that Hcy in combination with Ade might primarily exert its anti-angiogenic effect via a hypomethylation mechanism; the impacts of Hcy alone might be injurious through oxidative mechanism at high concentrations. Future research is needed to explore the direct relationship between Hcy-induced hypomethylation and angiogenesis.

Several growth factors have been shown to be crucial for angiogenesis. The well-known angiogenic factor VEGF promotes EC proliferation, migration, and tube

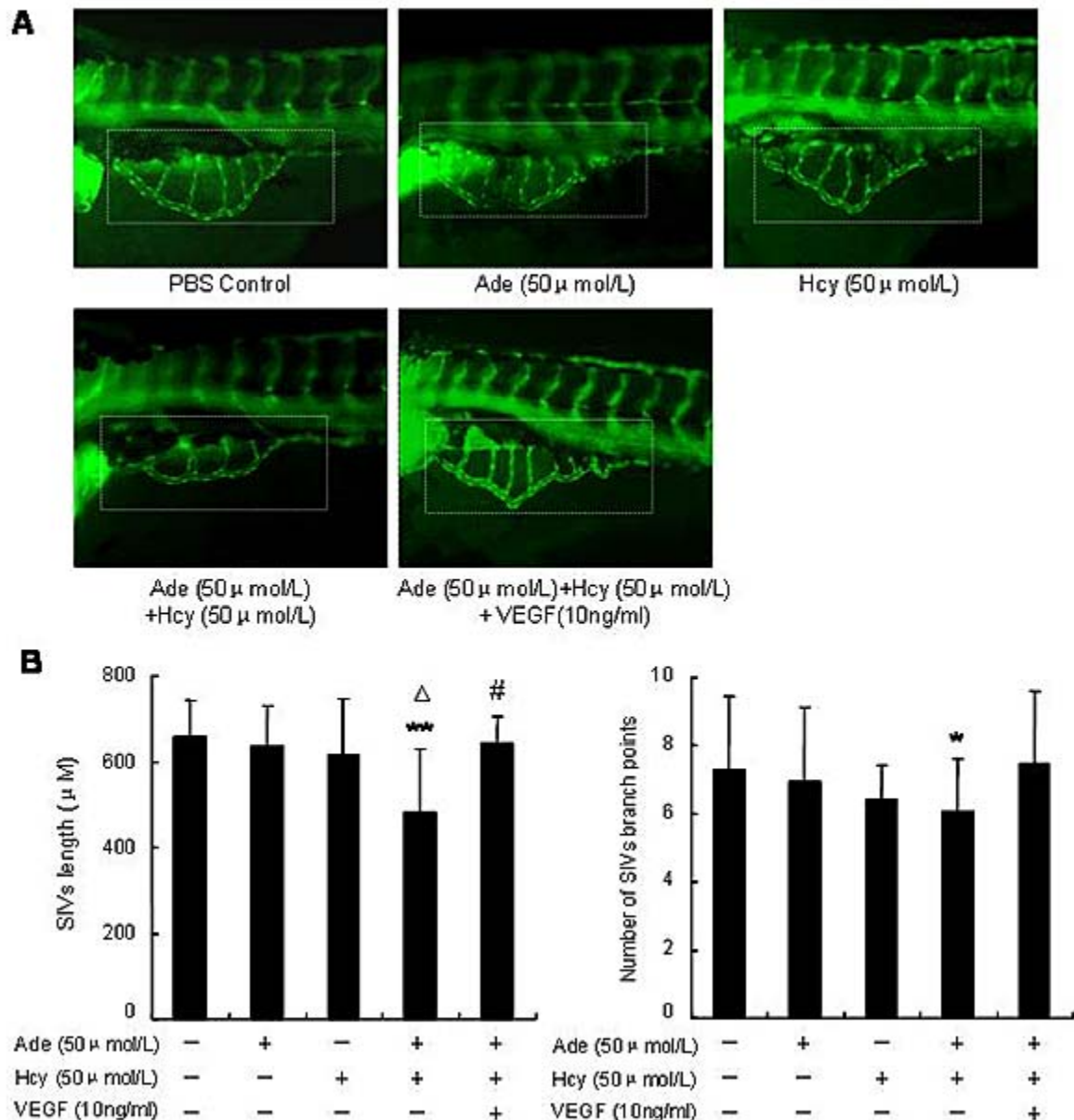


Figure 4. Effect of Hcy on angiogenesis *in vivo* in zebrafish embryos. A. Representative photographs of zebrafish SIVs. The SIVs of zebrafish embryos are indicated by white rectangle. B. Quantitative analysis of overall length and branch points number of zebrafish SIVs. * $p < 0.05$ and ** $p < 0.01$ vs PBS control; $\geq p < 0.05$ vs Ade and Hcy; # $p < 0.01$ vs Ade+Hcy.

formation (33, 34). Both VEGF, which is expressed in the vicinity of sprouting vessels, and its receptor, VEGFR2/Flk-1/KDR, are required for angiogenesis (35, 36). The subsequent stimulation of VEGFR results in a molecular cascade leading to EC mitosis and migration into the perivascular space (37). We examined the effect of Hcy on the expression of VEGF and VEGFR. Proteomic analysis indicated that Hcy inhibited microvascular ECs angiogenesis partly by decreasing VEGF (38). Chang (21) reported that, in combination with Ade, Hcy reduced VEGF expression in a dose and time-dependent manner and reached a significantly reduction when Hcy was raised to

500 μ mol/L in bovine aortic ECs and human coronary arterial ECs. Results from our experiments showed that Hcy in combination with Ade significantly decreased VEGF mRNA and protein expression in HUVEC. We speculate that this difference may be due to 1) VEGF protein levels were examined after Hcy treatment for 24h and 48h in Chang's study and the present experiments respectively; 2) different methods were used in these experiments. Intracellular VEGF levels were assayed by ELISA in Chang's study, and by real-time PCR and Western blot in the present study; and 3) different kinds of ECs were used in Chang's study compared to the present

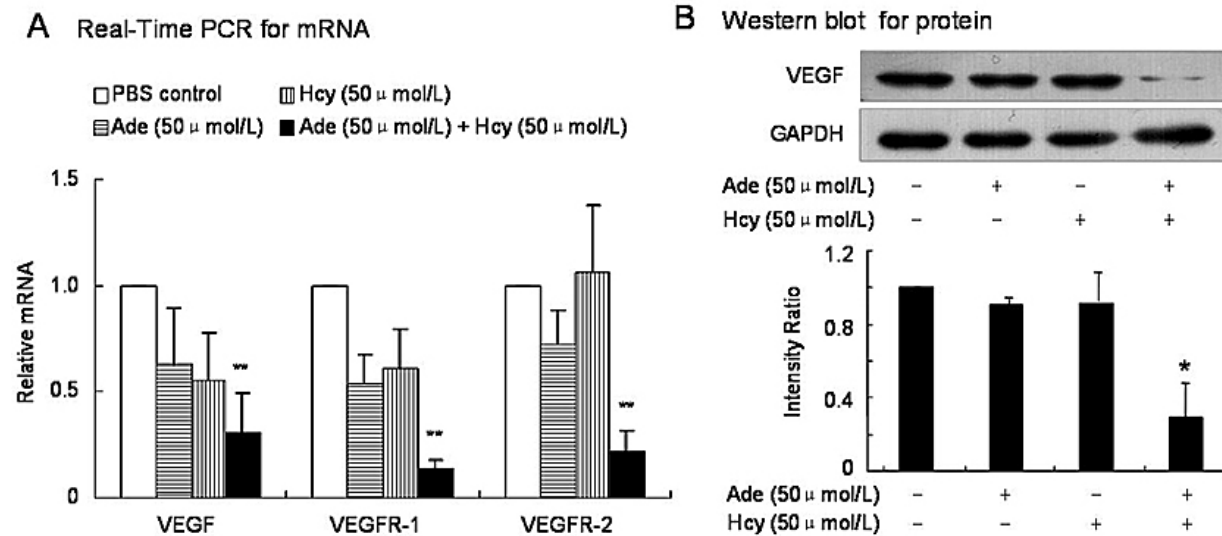


Figure 5. Effect of Hcy on the expression of VEGF, VEGFR1 and VEGFR2 in HUVEC. A. Measurement of mRNA by real time PCR. HUVEC were treated with the assay media for 24h and total RNA was subjected to quantitative real-time PCR analysis with specific primers for VEGF, VEGFR1 and VEGFR2. Values are mean \pm SD (relative to PBS-treated samples) after normalization to beta-actin. ** p <0.01 vs PBS control. B. Measurement of protein by Western blot. HUVEC were treated with the assay media for 48h and VEGF was detected by Western blot. Representative blots (upper) and quantitative analysis of VEGF compared to GAPDH (lower) were given. * P <0.05 and ** p <0.01 vs PBS control.

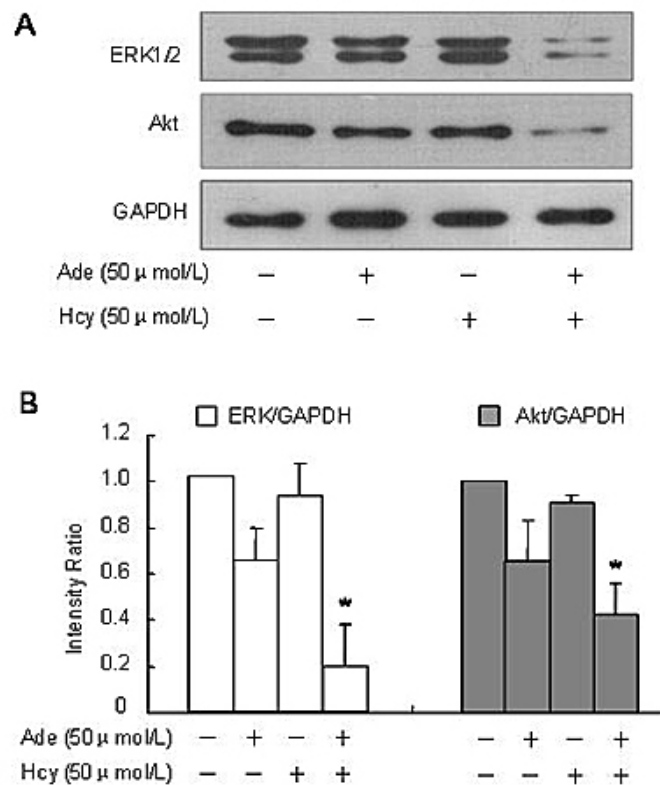


Figure 6. Effect of Hcy on ERK1/2 and Akt expression in HUVEC. HUVEC were treated with the assay media for 48 h. ERK1/2 and Akt were detected by Western blot analysis. A. Representative blots and B. quantitative analysis of ERK and Akt compared to GAPDH. * p <0.05 vs PBS control.

experiments. Arterial ECs (bovine aortic ECs and human coronary arterial ECs) were used in Chang's research and vein ECs (HUVEC) were used in our present study. Different cells may have different reactions in response to Hcy. Actually, Maeda *et al* observed that Hcy induced VEGF expression and secretion in macrophages indicates that Hcy is involved in vascular inflammation, and therefore, may contribute to atherosclerotic plaque progression(39). Roybal *et al* demonstrated that Hcy increases VEGF expression in a human retinal pigmented epithelial cell line (ARPE-19)(40). We observed that Hcy inhibited VEGF expression in ECs. Furthermore, we found that both *in vitro* and *in vivo* anti-angiogenic effect of Hcy in combination with Ade were abolished by the supplement of recombinant VEGF165. Because exogenous VEGF supplementation to Hcy and Ade significantly improved all parameters of evaluation including HUVEC proliferation, migration, tube formation, and angiogenesis in zebrafish embryo, we believe that the observed decreases in VEGF expression induced by Hcy plus Ade are biologically relevant. Hcy may exert its anti-angiogenic effect via the inhibition of VEGF transcription.

The mitogen-activated protein kinase/ERK1/2 cascade, which can be activated by VEGF, plays an important role in angiogenesis(37, 41). Srinivasan *et al* recently demonstrated that ERK1 and ERK2 coordinated ECs proliferation and migration during angiogenesis(41). ERK activation is involved in proliferation, migration, and morphogenesis of ECs induced by pro-angiogenic factors. Inactivation of ERK effectively inhibited *in vitro* angiogenesis. Numerous studies have implicated Akt-dependent signaling pathways in the regulation of angiogenesis(42, 43). VEGF is an important regulator in ECs function and exerts this effect through ERK and Akt signaling pathway. Angiogenic inducers have been shown to increase the angiogenic process by activating angiogenic signal mediators such as ERK and Akt, as well as by up-regulating pro-angiogenic gene expression including VEGF (43-45). We observed that Hcy in combination with Ade significantly decreased the protein levels of ERK1/2 and Akt as determined by Western blot analysis. Thus, the suppression of ERK1/2 and Akt might be responsible for the anti-angiogenic effect of Hcy *in vitro* and *in vivo*.

In summary, the present study revealed that clinically relevant concentrations of Hcy in combination with Ade inhibited angiogenesis both *in vitro* and *in vivo*. We proposed the hypothesis that elevated Hcy in combination with Ade may lead to decreased angiogenesis by hypomethylation, given the propensity for Hcy to convert to SAH. The anti-angiogenic effect of Hcy may be associated with inappropriate gene expression of VEGF/VEGFR, ERK1/2, and Akt by shifting the cellular methylation potential. The characteristics and key links involved in the proposed pathogenic mechanisms still need to be investigated. Our findings pose challenges for future studies as angiogenesis plays a crucial role in different physiological and pathological processes, such as embryonic development, wound repair, tumor growth, and atherosclerosis. Understanding further the mechanism by

which the anti-angiogenic effect of Hcy occurs will lead to the development of novel therapies.

6. ACKNOWLEDGEMENT

Quan Zhang and Qian Li contributed equally to this work. This work was supported by National Natural Science Foundation of China (Grant No. 30600250), Natural Science Foundation of Guangdong province of China (Grant No. 10151008901000146), the Fundamental Research Funds for the Central Universities (Grant No. 10ykpy33) and Scientific Research Funds for the Returned Overseas Chinese Scholars from the Ministry of Personnel (2006,164#). The authors have no competing financial interests to disclose regarding this manuscript.

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Abbreviations: HUVEC: Human Umbilical Vein Endothelial Cells; HHcy: hyperhomocysteinemia; CVD: cardiovascular disease; ECs: endothelial cells; eNOS: endothelial nitric oxide synthase; PKC: Protein Kinase C; VEGF: vascular endothelial growth factor; SAH: S-adenosyl Hcy; Ade: adenosine; DMSO: Dimethyl sulfoxide; SIVs: subintestinal vessels

Key Words: Homocysteine, Adenosine, Zebrafish, Angiogenesis, VEGF

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