Original article

Homocysteine activates vascular smooth muscle cells by DNA demethylation of platelet-derived growth factor in endothelial cells

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Abstract

Hyperhomocysteinemia (HHcy), as an independent risk factor of atherosclerosis, facilitates endothelial dysfunction and activation of vascular smooth muscle cells (VSMCs). However, little is known about the crosstalk between endothelial cells (ECs) and VSMCs under HHcy. We investigated whether homocysteine (Hcy) activates VSMCs by aberrant secretion of mitogen platelet-derived growth factors (PDGFs) from ECs in human and in mice. In this study, we found that increased Hcy level did not affect VSMC activity in 24 hrs until the concentration reached 500 μM. In contrast, Hcy at 100 μM significantly promoted proliferation and migration of VSMCs co-cultured with human ECs. This effect was partially reversed by pretreatment with a PDGF receptor inhibitor. Hcy concentration-dependently upregulated the mRNA level of PDGF-A, -C and -D but not PDGF-B in ECs. Hcy reduced the expression and activity of DNA methyltransferase 1, demethylation of PDGF-A, -C and -D promoters and enhanced the binding activity of transcriptional factor SP-1 to the promoter. Hcy upregulation of PDGF was confirmed in the aortic intima of mice with HHcy. Multivariate regression analysis revealed HHcy was a predictor of increased serum PDGF level in patients. Thus, Hcy upregulates PDGF level via DNA demethylation in ECs, affects cross-talk between ECs and VSMCs and leads to VSMC activation.

1. Introduction

Accumulating evidence has strongly suggested that hyperhomocysteinemia (HHcy) is an independent risk factor of atherosclerosis [1,2]. The underlying mechanism accounting for HHcy-accelerated atherosclerosis involves a complex series of events, including dysfunction of endothelial cells (ECs) and accelerated proliferation or migration of vascular smooth muscle cells (VSMCs) [3–5]. A number of studies have suggested that homocysteine (Hcy) triggers oxidative or ER stress in ECs but activates VSMCs via aberrant regulation of cyclin A and resistin, protein kinase C or ERK-MAPK signaling [6–8]. However, little is known about the crosstalk between ECs and VSMCs under HHcy. In particular, the optimal concentration (about 500 μM) of Hcy used in vitro for mitogenic activity of VSMCs is far beyond the clinically relevant range (>15 μM) [5,8], which further highlights the importance of cell–cell communication in vivo.

Recent compelling evidence has indicated that Hcy epigenetically regulates targets such as estrogen receptor α, fibroblast growth factor 2 (FGF2), cyclin A, p53 and p21, by inhibiting DNA methylation, which in turn favors early atherosclerosis [9]. However, how Hcy affects the crosstalk between ECs and VSMCs by epigenetic regulation is largely unknown.

Platelet-derived growth factors (PDGFs), of 4 isoforms (A, B, C, D), are strong mitogens for VSMCs and are constitutively or inducibly expressed in vessels. PDGFs regulate multiple pathophysiological events, from cell proliferation and migration, extracellular matrix accumulation and production of pro- and anti-inflammatory mediators, to tissue permeability and homodynamic. They have been implicated in various vascular pathophysiological processes such as atherosclerosis, restenosis, fibrosis and angiogenesis [10–12]. Previous studies have suggested that HHcy amplifies the mitogenic response of VSMCs to PDGF-BB. As well, site-specific methylation of PDGF-A or -B promoter has been reported in cancer cells [13,14]. In addition, recent study has shown that folate...
supplementation, which affects Hcy metabolism and alleviates HHcy formation, limits the aggressiveness of glioma by remethylation of PDGF-B [15]. Therefore, we hypothesized that HHcy, at a clinically relevant concentration, affects the cross-talk between ECs and VSMCs and leads to vascular dysfunction through DNA demethylation of PDGFs in ECs in vitro and in vivo.

2. Methods

2.1. Patients

We collected 30 serum samples from patients (mean age 61.20±12.54 years) with HHcy when they were first admitted to the First Affiliated Hospital of Shantou University Medical College. All patients were at high risk of cardiovascular events, including atherosclerosis, hypertension, aneurism, stroke, heart failure and Parkinson’s disease but not cancer, liver trauma or inflammation. The serum Hcy levels were detected before treatment (Supplemental Table 1).

All patients have been provided written consent in this study, and the study protocol was approved by local ethics committees (Shantou University Medical College and the First Affiliated Hospital of Shantou University Medical College, China) and the procedures committees according to the Declaration of Helsinki and Good Clinical Practice guidelines.

2.2. Cell culture

Primary rat thoracic aorta SMCs and human umbilical vein ECs (HUVECs) were isolated and cultured as described [16,17]. All experiments involved primary cells at passages 2 to 6. HUVEC-C–C line (ATCC #CRL-1730) and T/G Human Aorta –VSMC (T/G HA-VSMC) line (ATCC #CRL-1999) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Highclone, USA).

2.3. Co-culture of ECs with VSMCs

HUVECs were co-cultured with rat VSMCs or T/G HA-VSMC as described [18] with modification. Briefly, HUVECs and VSMCs were cultured in M199 and DMEM with 10% fetal bovine serum in the lower or upper chamber, respectively, of a trans-well system with 0.4 μm aperture (Corning, New York, USA). After both cells were about 80–90% confluence, the upper chamber with rat VSMCs were inserted into culture plates and co-cultured with HUVECs in the lower chambers. After treatment with Hcy for 24 hr, VSMCs in the upper chamber were harvested.

2.4. VSMCs proliferation and migration assay

For cell proliferation analysis, 5000 per well of VSMCs were seeded into a 96-well plate in VSMC culture alone, or VSMCs and ECs were co-cultured with HUVECs in the lower or upper chambers. After treatment with Hcy for 24 hr, VSMCs in the upper chamber were harvested.

2.5. Quantitative real-time RT-PCR (qRT-PCR)

qRT-PCR was performed as we previously described [19]. Total cellular or tissue RNA was extracted by use of Trizol reagent (Invitrogen, USA), then underwent cDNA synthesis and PCR amplification with the PrimeScript™ Real-Time RT-PCR reagent kit (Takara Biotechnology [DALIAN] Co.), then performed with Applied Biosystems Prism® 7300. Expression of analyzed gene was normalized to that of the housekeeping gene β-actin. Primer sequences are in Supplemental Table 2.

2.6. siRNA transfection

RNA interference with small interfering RNA (siRNA) was used to knock down the expression of DNA methyltransferase 1 (DNMT1) or SP-1 in ECs. The siRNAs targeting DNMT1, SP-1 and scramble siRNA were from GenePharma Co. The siRNAs and vehicle were transfected into cultured HUV-EC-Cs line at 60% confluence by the Jet PEI method (Polyplus, San Marcos, CA) for 48 hr. To verify the siRNA effect, cells underwent qRT-PCR analysis with DNMT1 or SP-1 primer (Supplemental Table 2).

2.7. Chromatin immunoprecipitation (ChIP) assay

HUVECs were treated with 100 μM Hcy, 8 μM 5-aza-CdR or 100 μM 3-DZA for 24 hr. ChIP assay was performed as described [19–22]. In brief, HUVECs were cross-linked cell proteins to DNA, sonicated, and then underwent immunoprecipitation (IP) with polyclonal anti-SP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Normal IgG was used as an IP control, and the supernatant was an input control. Immunoprecipitated complexes were collected by adding salmon sperm DNA/protein A/G-agarose. The beads were then treated with RNase A and proteinase K. DNA was extracted and underwent PCR amplification of SP-1 binding sites on PDGF promoters with specific primers (Supplemental Table 2). The resulting DNA was resolved on 1.5% agarose gel and stained with ethidium bromide.

2.8. Methylation-specific PCR (MSP)

Tissue or cellular genome DNA was extracted with TakaRa MiniBEST Universal Genomic DNA Extraction Kit and modified with sodium bisulfite, purified and used as a template for PCR. The primers for MSP amplification were designed with the use of the bioinformatics program (http://www.urogene.org/methprimer/index1.html) and shown in supplemental Table 2. PCR products were separated on agarose gels, and bands were visualized by ethidium bromide staining for determining methylation patterns as we previously described [19]. All positive PCR products were ligated into the pGEM-T vector and confirmed by sequencing. The relative DNA abundance was quantified by densitometry with use of NIH Image J.

2.9. DNA methyltransferase 1 (DNMT1) activity assay

HUVECs were treated with 50 or 100 μM Hcy for 24 hrs. DNMT1 activity was measured using EpiQuik™ DNA Methyltransferase Activity/Inhibition Assay Ultra kit (Fluorometric) (Epigentek, Brooklyn, NY). Nuclear extracts were isolated using the EpiQuik Nuclear Extraction Kit (Epigentek) and equal amounts of nuclear extract (5 μg) were applied for DNMT activity assay which was performed according to the manufacturer’s protocol. Fluorescence was determined using a
2.10. Western blot analysis

Hcy treated HUVECs were washed with PBS and lysed in RIPA buffer supplemented with protease inhibitor cocktail. The protein concentration was determined by use of the BCA protein assay kit (NovasyGen, China). Equal amounts of protein (80 μg) underwent 12% SDS-PAGE and were transferred to PVDF membrane (Millipore, Germany). Immunoblotting involved primary antibodies against DNMT1 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Fluorescent immunocomplexes were then detected using a Li-COR Odyssey (Li-Cor, Lincoln, NE) and the densities of the bands were quantified and normalized to that of β-actin by the use of the Scion Image software (Scion Corp., Frederick, MD).

2.11. ELISA analysis of PDGFs

Protein levels of PDGFs in sera from clinical patients or mice, and the medium of cultured HUVECs were determined by use of the PDGF Quantikine enzyme-linked immunosorbent assay (ELISA) Kit (Groundwork Biotechnology Diagnosticate or R&D Systems).

2.12. Animal model

Male 8-week old C57BL/6j mice (n = 8), were fed standard mouse chow diet with 2% (wt/wt) l-methionine (Sigma, USA). The control group (n = 8) received standard mouse chow. 4 weeks or 8 weeks later, all animals were anesthetized, blood samples were drawn, and serum was harvested for measuring the concentrations of total Hcy or PDGFs. Total Hcy level was measured by an enzymatic method with a small molecule capture technology according to manufacturer’s protocol (Beijing Strong Biotechnologies, Inc., Beijing, China). Briefly, all protein-bound Hcy was first transformed into its free form, through covalent and substrate reaction, circulation amplifier and produced ATP. Adenosine was immediately hydrolyzed into ammonia, which made NADH transforming into NAD under the role of glutamate dehydrogenase. Hcy concentration is positively related with NADH conversion rate. Aortic intima was nucleated for total DNA and genomic DNA extraction and qRT-PCR or MSP assay.

Treatment of laboratory animals and experimental procedures were approved by the Institutional Animal Care and the Use Committee of the University of Shantou in accordance with the Guide for the Care and Use of Laboratory Animals.

2.13. Immunohistochemical (IHC) and immunofluorescence staining

Mice were killed and thoracic aortas were fixed with paraformaldehyde, embedded in paraffin, and cut into 4-μm-thick cross sections as we previously reported [23]. IHC staining of sections involved the VECTORSTAIN ABC System (Vector Labs) with mouse primary antibody for PCNA. For immunofluorescence staining, tissue sections were incubated with rabbit anti-ICAM-1 and anti-VCAM-1 primary antibodies, then Cy3-labeled goat anti-rabbit IgG (H + L) and DAPI. Images were analyzed by confocal laser scanning microscopy (OLYMPUS, Japan).

2.14. Statistical analysis

Experiments were performed at least in triplicate. Data are expressed as mean ± SD. Data were analyzed by two-sided Student’s t test, Student–Newman–Keuls test, Dunnett multiple comparison test, or bivariate correlation or multivariate linear regression analysis as appropriate. P < 0.05 was considered statistically significant. All statistical analysis involved use of SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Hcy promotes proliferation and migration of VSMCs via HUVEC-produced PDGF

To test whether Hcy affects the cross-talk between ECs and VSMCs, we first compared the activation of VSMCs by Hcy in the presence or absence of HUVECs. Hcy had a weak mitogenic effect on proliferation or migration of primary cultured rat aortic VSMCs in the absence of HUVECs (Fig. 1). VSMCs were not activated until Hcy reached 500 μM, which agrees with previous reports [5,8,10]. In contrast, in the presence of HUVECs, Hcy at 100 μM significantly increased VSMCs growth (Fig. 1A). In accordance, Hcy at 100 μM greatly enhanced the migration of VSMCs by 1.7-fold, and migration distance by 1.5-fold (Figs. 1B and C). Similar results were also confirmed with human thoracic aortic VSMC line (T/G-VA/VMC) (Supplemental Fig. 1). These data strongly suggested that Hcy, by stimulating HUVECs, activated VSMCs.

Because PDGF is a key factor involved in VSMC behavior, we next asked whether PDGF mediates the effect of Hcy-stimulated ECs on VSMC activation. Interestingly, pretreatment with a PDGF receptor inhibitor (0.25 μM, PDGF Tyrrosine Kinase Inhibitor III [EMD Millipore, Germany]) attenuated the Hcy-stimulated HUVEC-VSMC cross-talk, including the proliferation and migration of rat-VSMCs, to about 40%, respectively (Fig. 1D). The similar effects of PDGF receptor inhibitor was also observed in Hcy-stimulated human EC-VSMC co-culture system (Supplemental Fig. 1). Therefore, PDGF participated in Hcy-enhanced proliferation or migration of VSMCs.

3.2. Hcy transcription upregulates PDGF-A, -C and -D in HUVECs

Next we asked whether the stimulatory effect of Hcy was due to increased expression of PDGF by ECs. First, we detected the relative expression of the 4 members of PDGF in HUVECs and rat VSMCs. Hcy for 24 hr concentration-dependently, from 25 to 200 μM, enhanced the mRNA levels of PDGF-A, -C and -D but not PDGF-B in HUVECs, with no effect of 100 μM Hcy in VSMCs. In addition, the mRNA levels of other mitogens, such as vascular epithelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor 2 (IGF-2), and transforming growth factor β 1 (TGF-β1) were not affected by 100 μM Hcy. Of note, the mRNA level of FGF2 was reduced by Hcy in HUVECs (Figs. 2A–C). In accordance, Hcy also concentration-dependently increased the secretion of PDGFs from HUVECs (Fig. 2D).

3.3. Hcy reverses the methylation of PDGF promoter in HUVECs

Because Hcy can globally repress DNA methylation and activate various target genes, we next asked whether Hcy affects the DNA methylation of PDGFs in ECs. We used a bioinformatic program, Methyl Primer Express Software v1.0, to analyze the sequence characteristics of the 5′-flanking region (−2000 to +1000 bp) of human PDGF genes. CpG-rich regions were defined as stretches of DNA with GC content > 60% and ObsCpG/ExpCpG > 0.60. The multiple CpG sites within the PDGF-A, -B, -C and -D promoter regions are shown in Fig. 3A, which indicated potential epigenetic regulation by Hcy. Next, we used specific inhibitors of methylation transferase, 5-aza-CdR or 3-deazadenosine (3-DZA), in HUVECs. Similar to Hcy, 5-aza-CdR or 3-DZA treatment greatly increased the mRNA expression of PDGF-A, -C and -D but not PDGF-B as shown (Fig. 3B). Interestingly, Hcy and 3-DZA had a synergistic effect on inducing PDGF-A, -C and -D (10.2-, 9.5- and 13.4-fold, respectively).

Furthermore, the CpG islands within the promoter of PDGFs showed SP-1 binding sites, which is critical for transcriptional regulation of PDGF. MSP assay of SP-1 binding sites revealed that Hcy time-dependently promoted the demethylation of PDGF-A, -C and -D but not PDGF-B promoters (Fig. 3C). As a positive control,
treatment with the methylation transferase inhibitors, 5-aza-CdR or 3-DZA, also inhibited the DNA methylation of PDGF-A, -C and -D promoters (Fig. 3D).

To explore the consequence of demethylation of CpG islands by Hcy on SP-1 binding to the PDGF promoters, ChIP analysis was performed with primers targeting SP-1 binding sites. Compared with the control, Hcy, 5-aza-CdR or 3-DZA demethylation of the SP-1 binding site within the promoter region indeed enhanced SP-1 binding activity to the PDGF-A, -C and -D promoters (Supplemental Fig. 2A). Consistently, siRNA used to knock down SP-1 in HUV-EC-C
line could inhibit Hcy-induced upregulation of PDGF-A, -C and -D and VSMC migration and proliferation in the co-culture (Supplemental Fig. 2B–E).

3.4. Hcy inhibits DNA methyltransferase 1 (DNMT1) expression

DNA methylation involves DNMTs, which include 4 key molecules, 1, 3a, 3b and methyl CpG binding protein 2 (Mecp2). We therefore measured the relative expression of these transferases in HUVECs in response to Hcy. The basal level of DNMT1 and Mecp2 was high in HUVECs, with barely detectable levels of DNMT3a and 3b (Fig. 4A). With 100 μM Hcy stimulation, the expression of DNMT1 mRNA was significantly lower (about 25%) than that of the control. In contrast, DNMT 3a, DNMT 3b or Mecp2 expression was not significantly altered (data not shown). As well, 3-DAZ and 5-aza-CdR, two specific DNMT inhibitors, reduced the expression of DNMT1 at mRNA levels, by 35% and 30%, respectively. Further, the protein expression and activity of DNMT1 was also decreased with Hcy treatment at 100 μM for 24 hr (Figs. 4C and D). Consistently, specific knock down of DNMT1 by siRNA in HUV-EC-C line, resulted in a 4.4-, 3.6- and 4.2-fold increase in mRNA levels of PDGF-A, -C and -D, respectively (Figs. 4E and F), when Hcy did not show further adding effect. Thus, DNMT1 repression may mediate Hcy-induced DNA demethylation of PDGF promoters and their subsequent upregulation in ECs.

3.5. HHcy upregulated PDGF-A, -C and -D in mouse aortic intima via DNA demethylation

C57 mice fed a diet supplemented with the 2% methionine for 4 to 8 weeks showed a mild to a moderate HHcy (27.6 ± 4.5 or 61.5 ± 31.4 vs. 5.2 ± 1.3 μM in the control group, both P < 0.01). Similar to our in vitro study, mRNA levels of PDGF-A, -C and -D, but not PDGF-B, were significantly increased in the HHcy mouse aortic intima. Accordingly, total PDGF production was higher by 3-fold in the mild or moderate HHcy aortic intima as compared with control mice (1991.3 ± 943.3 vs. 664.3 ± 293.4 pg/mL in 4 weeks; 2346.69 vs. 787.87 pg/mL in 8 weeks) (Figs. 5A and B). To further confirm the epigenetic regulation of PDGF by HHcy in mouse aortic intima in vivo, we used MSP analysis to
determine SP-1 binding sites. We found 86.0%, 78.2% and 73.2% methylated SP-1 binding sites in PDGF-A, -C and -D promoters in control mice. In contrast, mild HHcy significantly reduced the methylated status to 52.2%, 36.7% and 33.0%, respectively (P<0.05), which is consistent with our in vitro observations. A similar result was also found in the moderate HHcy mice, compared with their corresponding control (Fig. 5C). To study the effect of HHcy on the injury of vasculature, we found that PCNA staining increased in the aortic media of HHcy mice. However, we did not observe apparent media thickness in the aorta of the mice. Immunostaining also revealed increased protein levels of VCAM-1 and ICAM-1, markers of endothelial dysfunction, in the aortic endothelium of HHcy mice (Fig. 5D), as we previously reported [23].

3.6. HHcy positively correlated with increased serum PDGF level in patients

To extend our hypothesis to clinical patients, we analyzed serum levels of Hcy and PDGF in 52 patients with high risk of for cardiovascular events. The mean serum Hcy level ranged from 1.2 to 86.8 μM and PDGF level from 256 to 4775.2 pg/mL. Multivariate linear regression revealed a positive association of PDGF secretion and Hcy concentration with adjustment for leukocyte and platelet count, mean platelet volume, platelet distribution width and plateletcrit level (unadjusted R = 0.444, P = 0.001; adjusted R = 0.472, P = 0.002; Fig. 6A). The secretion of PDGF concentration was higher for patients with moderate or intermediate than normal Hcy level (1341.8±681.4 and 2083.6±1116.2 vs. 2784.3±1126.1 pg/mL, P = 0.036 and 0.001) (Fig. 6B), which suggests that HHcy may be associated with increased PDGF production.

4. Discussion

Epidemiological studies have strongly suggested HHcy as an independent risk factor of coronary artery disease and stroke, although the underlying mechanism is not completely understood. In particular, compelling evidence has indicated that HHcy aggravates post-injury neointima formation in vivo and enhances VSMC activation in vitro.
Fig. 4. Hcy upregulated PDGs expression by inhibiting DNMT1 expression and activity. The basal relative mRNA levels of DNMT-1, 3a, 3b and Mecp2 in untreated HUVECs were measured by quantitative RT-PCR (A). DNMT1 mRNA expression were examined in HUVECs treated with Hcy (100 \( \mu \)M), 3-DZA (200 \( \mu \)M), 5-aza-CdR (8 \( \mu \)M) or Hcy plus 3-DZA for 24 hr (B). DNMT1 protein expression and activity in HUVECs was examined by western blot and piQuik DNA Methylation-1 Activity/Inhibition Kit treated with Hcy, respectively (C and D). DNMT1 siRNA and si-scramble were transfected into the cultured HUV-EC-Cs line for 48 hr. qRT-PCR analysis of mRNA expression of DNMT1 for 24 hr (B). DNMT1 protein expression and activity in HUVECs was examined by western blot and piQuik DNA Methyltransferase 1 Activity/Inhibition Assay Ultra Kit treated with Hcy, respectively (C and D). DNMT1 siRNA and si-scramble were transfected into the cultured HUV-EC-Cs line for 48 hr.


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[24]. However, the concentration of Hcy used in vitro is far above the clinically relevant plasma Hcy level, which indicates the importance of cell–cell communication in the in vivo microenvironment. In the current study, we show that Hcy activates VSMCs by interfering in EC-VSMC crosstalk at least in part by DNA demethylation of PDGF-A, -C, -D in ECs (Supplemental Fig. 3).

The functions of VSMCs, including differentiation, proliferation, migration and contraction, are fine-tuned by adjacent ECs. In general, vascular ECs are more susceptible to the high levels of Hcy microenvironment. The first defense of vessels against HHcy is ECs. As well, compared with VSMCs, ECs have lower transport capacity and higher affinity for Hcy, which may result in increased retention of Hcy [25]. Here, we showed that Hcy, with low direct mitogenic effect on VSMCs, greatly enhanced VSMC activation by EC stimulation. Previous study showed that Hcy decreases endothelium-dependent vasorelaxation in porcine arteries [26]. As well, short-term exposure to Hcy depresses rat aortic contractility by an endothelium-dependent mechanism [27]. Our study provides the experimental evidence that Hcy affects the cross-talk of ECs and VSMCs and therefore causes vascular dysfunction.

PDGFs are a family of disulfide-bonded homo- and heterodimers encoded by 4 genes, PDGF-A, -B, -C, and -D, that activate cells by binding to homo- or heterodimers of 2 PDGF receptor proteins. Data from multiple studies support the original hypothesis that the PDGF family may mediate atherosclerosis and other cardiovascular disease [12]. The genomic organization of the PDGF genes is similar, and they share common mechanisms of gene regulation controlled by the zinc finger transcription factors Egr1/Ets-1 and SP-1 [28]. Also, the promoters of PDGFs have affinity for overlapping GC-rich binding sites in the proximal region [21,29–32]. Nevertheless, existing data suggest divergent cellular localization and roles of different PDGF ligands and receptors in physiological or pathophysiological states [12]. For example, after myocardial infarction, PDGF-A and -D levels are significantly increased in the infarcted myocardium, and cells expressing PDGF-A and -D were primarily ECs, macrophages and myofibroblasts, with PDGF-B and -C expression reduced in the infarcted heart [33]. Recent study also showed that PDGF-A, -C, and -D but not PDGF-B increased TGF-β1 and chronic rejection in rat cardiac allografts [34]. Here, we identified that Hcy stimulated the production of PDGF-A, -C, and -D but not -B in ECs, which highlights the temporal and special distribution of PDGF members. Clarifying the distinct effects of PDGF ligands may be helpful for developing therapies to target disease but avoid physiological side effects. bFGF and FGF2 pathways in ECs may also contribute to atherosclerosis. bFGF could promote proliferation of VMSC [35]; FGF2 and PDGF-BB...
had a cross-talk in VSMCs in which PDGF-BB upregulated FGFR1, mediating proliferation signals triggered by FGF2 [36]. However, levels of bFGF and PDGF-BB did not change, when FGF2 was decreased by the treatment of Hcy in present study. Thus, Hcy affect EC-VSMC crosstalk seems largely dependent on PDGFs pathway.

We showed that Hcy demethylated promoters of PDGFs, which may be mediated by inhibiting DNMTs. All mammalian DNMTs use S-adenosylmethionine (SAM) as a source of methyl groups and transfer the methyl groups to the 5' position of cytosine residues that are immediately followed by guanine (CpG dinucleotides). The most abundant is DNMT1 [37], which preferentially methylates hemimethylated DNA, recognized as the "maintenance methyltransferase". In contrast, DNMT3a or 3b is known as the "de novo methyltransferase." Changes in the activity or expression of DNMTs can have a relatively specific outcome in terms of site-specific methylation and regulation of specific genes in selected cells or tissues. Wang and colleagues reported

**Fig. 5.** PDGF expression and DNA methylation of promoters in aortic intima of HHcy mice. C57 mice were fed standard chow with or without 2% methionine for 4 and 8 weeks (n = 8). (A) Serum levels of PDGF measured by ELISA. (B) qRT-PCR analysis of mRNA levels of PDGF-A, B, C and D mRNA in the aortic intima, normalized to the expression of β-actin. *P<0.05, **P<0.01, vs. standard diet group. (C) MSP analysis of PDGF-A, -C and -D promoter methylation in aortic tissue by ethidium bromide staining. The histogram represents the ratio of DNA methylation to total methylation and unmethylation of gene promoters. DM: DNA marker, M: methylated, U: unmethylated. (D) Representative immunohistochemical staining for PCNA or confocal images of immunofluorescence staining for VCAM-I and ICAM-I (red) and nuclei (blue) in cross sections of aortas from control and HHcy mice.
that treatment with 50 μM of Hcy for 48 hrs inhibited the activity of DNMT1 but not DNMT3, and adenovirus-overexpressed DNMT1 could reverse the Hcy-mediated repression of DNA synthesis and EC growth [7]. In current study, we found that treatment with 100 μM Hcy for 24 hrs could inhibit the activity and expression of DNMT1 in HUVECs. The inhibitory effect of Hcy on DNMT1 is not clear, but possibly through a product feedback inhibition mechanism by elevated levels of Hcy and accumulation of SAH [7]. Also, it might relate with aberrant DNA methylation (global genome hypomethylation and specific gene hypermethylation). It was reported a unique epigenome of the human placenta includes down-regulation of DNMT1 with concomitant hypomethylation of other DNMT gene [38]. We observed that PDGF-A, -C and -D but not PDGF-B were significantly increased with DNMT1 knockdown. Thus, DNMT1 was believed to be one of the major targets of Hcy-induced hypomethylation in ECs. Our data support that the inhibitory effect of Hcy on DNMT1 is likely mediated by increased levels of S-adenosylhomocysteine in ECs via a product feedback inhibition mechanism.

Our HHcy mouse models showed that both the mRNA and protein levels of PDGF-A, -C and -D elevated in the aortic intima, and their methylation status were reduced. The increased expressions of PCNA in aortic media, and ICAM-1 and VCAM-1 in the intima indicated the activation of ECs and potential proliferation of VSMCs in mice fed l-methionine. However, we did not observe apparent media thickness or neointima formation in these HHcy mice. These results suggest that short-term moderately elevated Hcy may not be enough to initiate the vascular remodeling, but it may enhance the effects of other stimuli, such as cytokines, balloon injury [39] and lipid metabolites [40].

In experimental and clinical studies, Hcy led to the tendency to thrombosis via platelet activation in patients with severe HHcy [41,42]. Given that the PDGFs are stored in platelet alpha-granules and are released with platelet activation, a limitation of the present study is the lack of information about changes in platelet-activation biomarkers in patients. However, we found no difference in leukocyte or platelet factors in patients with normal Hcy levels and HHcy (Supplemental Table 1). We may have recruited patients with relatively lower HHcy. As well, we recruited a small number of patients.

In summary, Hcy-induced DNA hypomethylation largely through decreasing DNMT1 expression could result in the upregulation and paracrine section of PDGFs from ECs and further increase the proliferation and migration of VSMCs. Our study provides a novel mechanism for HHcy-induced vascular dysfunction.

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Disclosure statement

None.
Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2012.07.010.

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