Homocysteine promotes human endothelial cell dysfunction via site-specific epigenetic regulation of p66shc

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1. Introduction

Abundant epidemiologic evidence demonstrates that an elevated plasma level of homocysteine (Hcy), a sulfur-containing amino acid formed during the metabolism of methionine, is an independent risk factor for atherosclerotic vascular disease. Hyperhomocysteinaemia is associated with vascular endothelial dysfunction. Homocysteine modulates cellular methylation reactions. P66shc is a protein that promotes oxidative stress whose expression is governed by promoter methylation. We asked if homocysteine induces endothelial p66shc expression via hypomethylation of CpG dinucleotides in the p66shc promoter, and whether p66shc mediates homocysteine-stimulated endothelial cell dysfunction.

Keywords
Homocysteine • p66shc • Epigenetic • CpG methylation • Endothelium

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The mechanisms by which HHcy induces endothelial dysfunction are likely to be many, and include oxidative inactivation of nitric oxide (NO) and reactive oxygen species (ROS) generation when it is auto-oxidized to homocysteine in the plasma. A growing body of evidence also suggests that Hcy-stimulated change in methylation of DNA and proteins may mediate its effects on endothelial cell phenotype. Cellular methyltransferases catalyze the transfer of the methyl group from S-adenosylmethionine (SAM) to substrates, among which are dC bases in CpG dinucleotides, leading to the formation of S-adenosylhomocysteine (SAH), which inhibits the transmethylation reaction. HHcy increases the cellular pool of SAH, decreasing SAM/SAH ratio. This mechanism has been implicated in Hcy-induced p21ras-mediated inhibition of endothelial cell growth. In addition, hypomethylation of the cyclin A gene locus, and consequent decrease in cyclin A expression, has been shown to play a part in Hcy-induced inhibition of endothelial cell growth. Furthermore, elevated SAH levels are associated with DNA hypomethylation in endothelial cells.

P66shc is a protein that belongs to the shcA family of adaptor proteins. It promotes oxidative stress within cells and tissues. Cells lacking p66shc have reduced levels of oxidants, and mice deficient for p66shc have an extended lifespan, protected against age-associated endothelial dysfunction and high-fat diet-induced atheromas, and exhibit increased endothelial bioavailability of NO and lower vascular oxidant levels. Similarly, down-regulation of p66shc increases endothelial NO synthase activity and improves endothelium-dependent vasorelaxation. P66shc expression varies among cell types and tissues. Modification of p66shc protein by phosphorylation affects its stability. In addition, methylation of CpG dinucleotides in the p66shc promoter regulates its transcription. Moreover, methylation of the p66shc promoter is decreased in patients with end-stage renal disease who have elevated plasma Hcy.

Recognizing the importance of DNA methylation in regulating p66shc expression and the impact of Hcy and its metabolites on cellular methylation reactions, we hypothesized that Hcy-induced oxidative stress and inflammatory gene expression in human endothelial cells are mediated, in part, by p66shc. Thus, we investigated the possibility that Hcy promotes oxidative stress and an inflammatory phenotype in human endothelial cells by epigenetically inducing p66shc expression.

2. Methods
Detailed methods are provided in the Supplementary material online. Brief descriptions are provided below.

2.1 Cells and transfections
Human umbilical vein endothelial cells (HUVECs), HEK 293, U937, and HCT116 cells were used in all experiments. Cell transfections were performed with Lipofectamine2000. Wild-type and DNA methyltransferase (DNMT1)−/−, DNA methyltransferase 3b (DNMT3b)−/− HCT116 cells were provided by Bert Vogelstein.

2.2 Adenoviral constructs
The AdLaCZ and Adp66shcRNAi adenoviruses have been described previously.

2.3 Antibodies and immunoblotting
Anti-shcA (Becton Dickinson) and anti-intercellular adhesion molecule-1 (ICAM-1) (Santa Cruz Biotechnology) antibodies were purchased and used in the standard SDS–PAGE and immunoblotting protocols with appropriate peroxidase-conjugated secondary antibodies. Chemiluminescent signal was developed using Super Signal West Pico or Femto substrate (Pierce), and blots were imaged and band densities quantified with a Gel Doc 2000 Chemi Doc system with Quantity One software (Bio-Rad).

2.4 Monocyte-endothelial cell adhesion assay
Adhesion of U937 monocytic cells to HUVECs was quantified by microscopy.

2.5 P66shc promoter methylation in vitro
Plasmid DNA (p66shc promoter) of 100 μg was incubated with 100 U of Sss1 methylase.

2.6 Promoter-reporter assays
An 1141-bp p66shc promoter fused to firefly luciferase was employed. Renilla luciferase plasmid was used to correct for differences in transfections efficiency. Firefly and renilla luciferase luminescence were measured using the Dual Luciferase reporter kit.

2.7 Chromatin immunoprecipitation assay
ChiP was performed in HUVECs using a ChIP Assay Kit and a rabbit polyclonal antibody for Anti-acetyl-lysine 9,14 for Histone 3 (H3) with primers to amplify a 169-bp fragment of human p66shc promoter. Human β-actin was amplified from input chromatin.

2.8 Quantitative real-time PCR
Real-time PCR was performed using the Prism 7000 Sequence Detection System with the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit on total RNA using specific primer sequences for human p66shc, ICAM-1, and GAPDH.

2.9 Small interfering RNA
HUVECs were transfected with 30 pmol of commercial siRNAs targeting DNMT1 and DNMT3b using Lipofectamine2000.

2.10 DNMT activity
DNMT activity was measured in HUVEC lysates with EpiQuik DNMT Activity/Inhibition Colorimetric Assay Ultra Kit.

2.11 Plasma homocysteine measurement
Plasma free Hcy was measured with the Axi Homocysteine Enzyme Immunoassay (AXIS-SHIELD).

2.12 Detection and quantification of CpG methylation in HUVECs
Bisulfite-converted genomic DNA isolated from HUVECs was subjected to PCR amplification followed by cloning of the fragment into E. Coli. Clones were sequenced.

CpG methylation was quantified by methylation-specific real-time PCR using 100 ng of bisulfite-converted genomic DNA as the template and methylation-specific primers for CpG(4) and CpG(6,7) in the human p66shc promoter.

2.13 Quantification of CpG methylation in peripheral blood leucocytes
Genomic DNA was isolated from peripheral blood leucocytes of subjects in the clinical cohort. An amplicon of the human p66shc promoter was amplified from bisulfite-converted genomic DNA. CpG methylation was quantified in this amplicon using the Sequenom EpiTyper at the Roswell Park Cancer Institute DNA Microarray Facility, Buffalo, NY. All human samples were obtained in accordance with a protocol approved by the
University of Pittsburgh Institutional Review Board which conformed to the principles in the Declaration of Helsinki.

2.14 Hydrogen peroxide measurement
Hydrogen peroxide produced by live cells was measured in conditioned medium of HUVECs using the Amplex Red Hydrogen Peroxide Assay Kit.

2.15 Nitrite and nitrate measurement
Nitrate and nitrite were measured in conditioned medium of HUVECs using a fluorometric assay kit (Cayman Chemicals).

2.16 Site-directed mutagenesis
CpG mutants in the human p66shc promoter-reporter construct were created with commercially available QuickChange kits.

3. Results

3.1 Homocysteine stimulates p66shc transcription
To examine the relationship between Hcy and p66shc, we first determined whether Hcy leads to a change in p66shc expression in cultured human endothelial cells. P66shc expression in HUVECs, both at the protein (Figure 1A) and mRNA (Figure 1B) level, was stimulated in a dose-dependent fashion by Hcy. We then determined the effect of Hcy on p66shc promoter activity. A promoter-reporter construct consisting of 1141 bp of the human p66shc promoter was used. Hcy dose-dependently stimulated the activity of the human p66shc promoter in HEK 293 cells (Figure 1C). Thus, Hcy increases p66shc expression at the transcriptional level.

3.2 Homocysteine leads to hypomethylation of the p66shc promoter
Next, we asked if Hcy-induced p66shc transcription is mediated by hypomethylation of CpG dinucleotides in the p66shc promoter. To do this, we first determined if Hcy leads to a change in CpG methylation in the p66shc promoter. We examined methylation of seven CpG dinucleotides which are in proximity to the transcription start site (Figure 2A). These dinucleotides are differentially methylated and known to regulate p66shc transcription. Bisulfite sequencing of genomic DNA from HUVECs under resting conditions revealed detectable methylation of CpG dinucleotides 6 and 7 (CpG6,7), which are in close proximity to each other (Figure 2A). We then examined the effect of Hcy on methylation of CpG(6,7). Quantitative methylation-specific real-time PCR showed that treatment of HUVECs with Hcy resulted in hypomethylation of CpG(6,7) (Figure 2B). Therefore, two CpG dinucleotides in the human p66shc promoter are methylated in endothelial cells, and Hcy hypomethylates these dinucleotides.

3.3 CpG(6,7) mediate homocysteine-induced transcription of p66shc
To determine whether CpG(6,7) mediate the effect of Hcy on p66shc transcription, we created a p66shc promoter-reporter construct in which the cytosines of both these CpG dinucleotides were mutated to non-methylatable adenosines. Activation of this CpG(6,7)-deficient (CpG6,7 mut) p66shc promoter by Hcy was then compared with the wild-type p66shc promoter in HEK 293 cells. Compared with the wild-type promoter, activity of CpG(6,7)-deficient promoter was higher.

Figure 1 Homocysteine increases endothelial p66shc expression. (A) Homocysteine (Hcy) dose-dependently increases p66shc protein expression in human umbilical vein endothelial cells (HUVECs). HUVECs were challenged with Hcy (200 μM) for 8 h. *P < 0.05, **P < 0.01 compared with untreated cells (n = 3–4). (B) Hcy dose-dependently increases p66shc RNA expression in endothelial cells. *P < 0.05, **P < 0.01 compared with untreated cells. (n = 3). (C) Hcy stimulates p66shc promoter activity. Promoter activity was measured in HEK293 cells transfected with an 1141 bp p66shc (WT) promoter-reporter construct. *P < 0.05, **P < 0.01 (n = 3) compared with untreated cells.
under basal conditions (Figure 2C). Moreover, in contrast to the wild-type promoter, Hcy did not increase activity of the CpG(6,7)-deficient promoter, but paradoxically decreased it (Figure 2C). Therefore, CpG(6,7) mediate Hcy-induced up-regulation of human p66shc promoter activity.

### 3.4 Homocysteine stimulates p66shc promoter by inhibiting methylation of CpG(6,7)

Next, we asked whether hypomethylation of CpG dinucleotides, in general, and CpG(6,7) in particular, in the p66shc promoter is responsible for Hcy-stimulated promoter activity. To do this, the wild-type and CpG(6,7)-deficient p66shc promoters were first methylated in vitro with the bacterial Sss1 methyltransferase. The effect of Hcy on activity of these methylated promoter-reporter constructs was then determined in HEK 293 cells. Methylation with Sss1 led to a decrease in basal activity of the wild-type promoter (Figure 2D). However, in contrast to the unmethylated wild-type promoter, Hcy had no effect on the Sss1-methylated promoter (Figure 2D). Moreover, although basal activity of the wild-type promoter was significantly diminished by in vitro methylation, that of the CpG(6,7)-deficient promoter was not (Figure 2E). The lack of effect of Hcy on activity of the promoter which is methylated in vitro indicates that Hcy does not stimulate p66shc promoter activity in cells by promoting de-methylation, but rather by inhibiting methylation. In addition, the
lack of effect of in vitro methylation on activity of the CpG(6,7)-deficient promoter shows that these CpG dinucleotides confer sensitivity of the promoter to methylation.

### 3.5 Homocysteine promotes histone 3 acetylation on the p66shc promoter

Methylation of CpG dinucleotides is often associated with changes in the chromatin at promoter regions. CpG hypomethylation is closely linked with histone acetylation which leads to an open chromatin and gene transcription. Therefore, we asked whether Hcy affects the acetylation of histones on the promoter. In HUVECs, Hcy increased acetylation of histone 3 (H3) on lysines 9 and 14 (Figure 2F), a signature epigenetic mark that indicates an open transcriptionally active chromatin.

Thus, hypomethylation of CpG dinucleotides in the p66shc promoter by Hcy is associated with hyperacetylation of H3 on the p66shc promoter, consistent with an open transcriptionally active chromatin at that locus.

### 3.6 DNA methyltransferase 3b mediates effect of homocysteine on p66shc expression

DNMTs induce CpG and non-CpG methylation, and are inhibited by S-adenosylhomocysteine (SAH), a metabolite of Hcy. Therefore, we asked whether inhibition of DNMTs play a part in Hcy-induced up-regulation of p66shc. In endothelial cells pre-treated with the pan-DNMT inhibitor 5-AZA, Hcy did not induce p66shc (Figure 3A). We also used a genetic approach to examine the role of DNMTs, utilizing the colon cancer cell line HCT116 in which the two principal DNMTs in adult cells and tissues, DNMT1 and DNMT3b are both knocked out. In contrast to wild-type HCT116 cells, Hcy did not induce p66shc.
expression in HCT116 DNMT1/3b (Dickinson) --/-- cells (Figure 3B and C). Thus, Hcy induces p66shc through its effect on DNMTs.

To identify the specific DNMT in endothelial cells which mediates the effect of Hcy on p66shc expression, we used a selective knockdown strategy in HUVECs. Hcy-induced p66shc expression was not affected in HUVECs in which DNMT1 was knockdown with siRNA (Figure 3D), whereas knockdown of DNMT3b abrogated the effect of Hcy on up-regulation of p66shc (Figure 3D and E). Thus, DNMT3b has a role in Hcy-stimulated p66shc expression.

3.7 P66shc mediates homocysteine-stimulated increase in reactive oxygen species, and decrease in nitric oxide, produced by endothelial cells

Vascular oxidative stress is one mechanism through which Hcy induces vascular dysfunction.28 Because p66shc is a pivotal protein in the regulation of intra-cellular reactive oxygen species (ROS) levels, we examined the role of p66shc in mediating Hcy-stimulated ROS in endothelial cells. Hcy-stimulated hydrogen peroxide (H$_2$O$_2$), a diffusible ROS generated by cells, was measured and compared in control cells (infected with a control inert AdLacZ adenovirus), and cells in which p66shc was knocked down with an adenovirus encoding a shRNA for p66shc (Adp66shcRNAi). In comparison with control cells (infected with a control inert AdLacZ adenovirus), and cells in which p66shc was knocked down with siRNA (Figure 3B and C). Thus, p66shc mediates the increase in H$_2$O$_2$ stimulated by Hcy in endothelial cells.

HHcy is also associated with a decrease in bioavailable NO.12 Moreover, p66shc inhibits endothelial NO synthase-derived NO production.31 Therefore, we asked if p66shc plays a part in Hcy-induced decrease in endothelial NO. Hcy-stimulated change in nitrite and nitrate, metabolites of NO generated by cells, was measured and compared in HUVECs infected with AdLacZ or Adp66shcRNAi. Hcy-induced decrease in nitrate and nitrate was markedly inhibited in cells infected with Adp66shcRNAi when compared with those infected with AdLacZ (Figure 4B), indicating that inhibition of endothelial NO by Hcy is also mediated by p66shc.

3.8 P66shc mediates homocysteine-stimulated ICAM-1 expression

Vascular oxidative stress is associated with up-regulation of inflammatory genes, including expression of intercellular adhesion molecule 1 (ICAM-1) on the endothelium.29 Moreover, HHcy leads to vascular inflammation30 and up-regulation of endothelial adhesion molecules.31,32 Therefore, we asked if p66shc, which mediates Hcy-stimulated oxidative stress in endothelial cells, also mediates Hcy-stimulated expression of ICAM-1. Hcy increased endothelial expression of ICAM-1 both at the protein (Figure 4C) and mRNA (Figure 4D) levels. Furthermore, Hcy-induced ICAM-1 expression was blunted in endothelial cells in which p66shc was knocked down with Adp66shcRNAi when compared with cells infected with control AdLacZ (Figure 4E). Thus, up-regulation of ICAM-1 by Hcy in endothelial cells is dependent, in part, on p66shc.

3.9 P66shc mediates homocysteine-stimulated adhesion of monocytes to endothelial cells

Up-regulation of adhesion molecules on endothelial cells leads to infiltration of inflammatory leucocytes into the vessel wall and is one of the hallmarks of a dysfunctional endothelium associated with HHcy.33 Therefore, we asked if p66shc is responsible for Hcy-stimulated adhesion of leucocytes to endothelial cells. We examined the effect of Hcy on adhesion of the monocytic cell line U937 to HUVECs. Similar to that observed with the proto-typical inflammatory cytokine tumour necrosis factor-α (TNF-α), Hcy stimulated the adhesion of U937 cells to HUVECs (Figure 4F). Furthermore, in HUVECs in which p66shc expression was knocked down with Adp66shcRNAi, Hcy-stimulated adhesion of U937 cells was significantly diminished (Figure 4G). Thus, p66shc mediates the adhesion of monocytes to endothelial cells exposed to Hcy.

3.10 Plasma homocysteine inversely correlates with methylation of CpG(6,7) in peripheral blood leucocytes

Finally, we searched for an association between Hcy and CpG methylation in the p66shc promoter in human subjects. P66shc promoter CpG methylation was quantified in genomic DNA of peripheral blood leucocytes, and Hcy was measured in the plasma, of patients with coronary artery disease who presented for a heart catheterization. There was a significant difference in plasma Hcy when comparing patients with the highest and lowest tertiles of CpG(6,7) methylation (Figure 5A). This difference was specific to CpG(6,7) methylation, as there was no difference in plasma Hcy when comparing patients with the highest and lowest tertiles of methylation of CpG(5), or of mean methylation of all CpG dinucleotides in the p66shc promoter (Figure 5B and C). Clinical (including renal function) and demographic characteristics in patients with the highest and lowest tertiles of CpG(6,7) methylation were similar (Figure 5D). Thus, the highest and lowest levels of CpG(6,7) methylation in peripheral blood leucocytes show a significant inverse relationship with plasma Hcy in humans.

4. Discussion

The CpG dinucleotides in the p66shc promoter hypomethylated in response to Hcy lie after the transcription start site, and are highly methylated in cells in which p66shc is silenced.24 The transcriptional activators and repressors that bind to this region of the promoter have not been identified. Nevertheless several transcription factors including Sp1 have putative binding sequences24 in this region of the promoter. Sp1-mediated gene transcription is known to be modulated by CpG hypermethylation,34 and interestingly, Sp1 is one of several transcription factors that physically interacts with DNMT3a/b.35 This raises two equally plausible possibilities: (i) DNA-binding factors such as Sp1 that bind to this region of the p66shc promoter recruit DNMTs to silence gene expression or (ii) transcription factors (such as Sp1) inducing p66shc expression recruit DNMTs to the promoter, which counteract the effect of these factors, thus modulating p66shc expression. The former scenario would be reminiscent of CpG methylation of the p16INK4A locus by DNMT3a/b facilitated by the haematopoietic transcription factor PU.1.36
Our data indicate that DNMT3b silencing abrogates up-regulation of p66shc by Hcy, whereas down-regulation of DNMT1 does not. This observation does not necessarily show that Hcy (or more accurately SAH) preferentially targets one class of DNMTs over another but more likely reflects the specificity of DNMTs for target gene promoters. Such specificity could be achieved by preferential binding of a class of DNMTs to DNA-binding factors (such as binding of Sp1 to DNMT3a/b but not DNMT1) which have binding elements on the promoter of the specific gene. Moreover, nucleotides flanking CpGs in the p66shc promoter may also confer
Homocysteine stimulates endothelial cell ROS production, ICAM-1 expression, and adhesion of monocytes to endothelial cells, and decreases endothelial cell NO by a p66shc-mediated mechanism. (A) p66shc mediates Hcy-induced oxidative stress. HUVECs were infected with an adenovirus encoding a shRNA for p66shc (Adp66shcRNAi) or the inert E. coli LacZ gene (AdLacZ) for 24 h and hydrogen peroxide levels were measured in conditioned media (*$P < 0.05$, compared with AdLacZ, $n = 3$). (B) p66shc mediates Hcy-induced decrease in endothelium-derived NO. HUVECs were infected with Adp66shcRNAi or AdLacZ for 24 h. The NO metabolites nitrite and nitrate were measured in conditioned media of cells in the presence and absence of Hcy (50 $\mu$M for 24 h). Decrease in nitrate and nitrate with Hcy, normalized to HUVECs protein, is shown (**$P < 0.01$, $n = 4$). (C and D): Hcy increases endothelial ICAM-1. ICAM-1 (C) protein and (D) RNA was measured in endothelial HUVECs challenged with Hcy (8 h). RNA was normalized to GAPDH RNA (**$P < 0.05$, ***$P < 0.01$ compared with untreated cells, $n = 3$). (E) P66shc mediates Hcy-induced ICAM-1. HUVECs infected with Adp66shcRNAi or AdLacZ were challenged with Hcy for 8 h. ICAM-1 protein expression was normalized to $\beta$-actin (***$P < 0.05$ and #P = NS compared with untreated cells, $n = 3$). All immunoblots shown are representative of three independent experiments. (F) Hcy and TNF-$\alpha$ induce U937 monocyte adhesion to HUVECs. HUVECs treated with Hcy and TNF-$\alpha$ for 8 h were incubated with U937 monocyte cells for 30 min. Representative photomicrographs of U937 cells adherent to HUVECs (top) and quantification of adherent cells (bottom) is shown. (**$P < 0.05$ compared with non-treated cells, $n = 3$). (G) P66shc mediates Hcy-induced adhesion of monocytes to HUVECs. HUVECs infected with Adp66shcRNAi or AdLacZ were challenged with Hcy for 8 h and incubated with U937 cells for 30 min. Representative photomicrographs of U937 cells adherent to HUVECs (left) and quantification of adherent cells (right) are shown. (**$P < 0.05$ compared with AdLacZ-infected cells, $n = 3$).

Figure 5 Plasma homocysteine is inversely associated with p66shc promoter methylation in peripheral blood leucocytes in patients with coronary artery disease. (A) Plasma homocysteine concentrations in subjects with the highest and lowest tertiles of p66shc promoter CpG(6,7) methylation in peripheral blood leucocytes ($P = 0.03$, $n = 40$). (B) Plasma homocysteine concentrations in subjects with the highest and lowest tertiles of p66shc promoter CpG(5) methylation in peripheral blood leucocytes ($P = 0.52$, $n = 37$). (C) Plasma homocysteine concentrations in subjects with the highest and lowest tertiles of total p66shc promoter CpG methylation in peripheral blood leucocytes ($P = 0.36$, $n = 38$). Each box plot shows the distribution of plasma homocysteine from 25 to 75th percentile, and the lines inside the boxes denote the medians. Whiskers denote the intervals between the 5 and 95th percentiles, with dots representing data points outside these percentiles. (D) Baseline clinical and demographic characteristics, % CpG(6,7) methylation, and plasma homocysteine, of subjects with the highest and lowest tertiles of CpG(6,7) methylation in peripheral blood leucocytes.

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Each box plot shows the distribution of plasma homocysteine from 25 to 75th percentile, and the lines inside the boxes denote the medians. Whiskers denote the intervals between the 5 and 95th percentiles, with dots representing data points outside these percentiles. (D) Baseline clinical and demographic characteristics, % CpG(6,7) methylation, and plasma homocysteine, of subjects with the highest and lowest tertiles of CpG(6,7) methylation in peripheral blood leucocytes.

Figure 4 Homocysteine stimulates endothelial cell ROS production, ICAM-1 expression, and adhesion of monocytes to endothelial cells, and decreases endothelial cell NO by a p66shc-mediated mechanism. (A) p66shc mediates Hcy-induced oxidative stress. HUVECs were infected with an adenovirus encoding a shRNA for p66shc (Adp66shcRNAi) or the inert E. coli LacZ gene (AdLacZ) for 24 h and hydrogen peroxide levels were measured in conditioned media ($^{*}$ $P < 0.05$ compared with AdLacZ, $n = 3$). (B) p66shc mediates Hcy-induced decrease in endothelium-derived NO. HUVECs were infected with Adp66shcRNAi or AdLacZ for 24 h. The NO metabolites nitrite and nitrate were measured in conditioned media of cells in the presence and absence of Hcy (50 $\mu$M for 24 h). Decrease in nitrate and nitrate with Hcy, normalized to HUVECs protein, is shown (***$P < 0.01$, $n = 4$). (C and D): Hcy increases endothelial ICAM-1. ICAM-1 (C) protein and (D) RNA was measured in endothelial HUVECs challenged with Hcy (8 h). RNA was normalized to GAPDH RNA (**$P < 0.05$, ***$P < 0.01$ compared with untreated cells, $n = 3$). (E) P66shc mediates Hcy-induced ICAM-1. HUVECs infected with Adp66shcRNAi or AdLacZ were challenged with Hcy for 8 h. ICAM-1 protein expression was normalized to $\beta$-actin (***$P < 0.05$ and #P = NS compared with untreated cells, $n = 3$). All immunoblots shown are representative of three independent experiments. (F) Hcy and TNF-$\alpha$ induce U937 monocyte adhesion to HUVECs. HUVECs treated with Hcy and TNF-$\alpha$ for 8 h were incubated with U937 monocyte cells for 30 min. Representative photomicrographs of U937 cells adherent to HUVECs (top) and quantification of adherent cells (bottom) is shown. (**$P < 0.05$ compared with non-treated cells, $n = 3$). (G) P66shc mediates Hcy-induced adhesion of monocytes to HUVECs. HUVECs infected with Adp66shcRNAi or AdLacZ were challenged with Hcy for 8 h and incubated with U937 cells for 30 min. Representative photomicrographs of U937 cells adherent to HUVECs (left) and quantification of adherent cells (right) are shown. (**$P < 0.05$ compared with AdLacZ-infected cells, $n = 3$).
specificity with respect to what DNMTs target them. In this regard, it is noteworthy that DNMT3a/b have specificity for sequences flanking the CpG dinucleotide, with RCpGY being the preferred sequence (R = purine, Y = pyrimidine). The sequence flanking CpG(6) (ACpGT) matches this preferred sequence.

Elevated plasma SAH is associated with decreased DNMT3a/b, but not DNMT1, expression in peripheral blood cells. However, it is not universally accepted that Hcy leads to hypomethylation by decreasing DNMT expression. Reports indicate that Hcy in fact increases expression of both DNMT1 and DNMT3a/b. Our studies also showed a modest up-regulation of endothelial cell DNMT3b with Hcy (Supplementary material online, Figure S1), despite a decrease in cellular DNMT activity (Supplementary material online, Figure S2) and CpG(6,7) methylation.

The promoter region of p66shc does not have a true CpG island as defined by the generally accepted criteria (regions >200 bp with G + C content >0.5 and the observed/expected CpG ratio >0.65). Nonetheless, there is ample evidence that functional CpG methylation occurs in non-CpG islands, and CpG methylation does silence p66shc in some cancer cells including the myelomonocytic U937 cell line. Unlike U937 cells, p66shc is expressed in HUVECs, and this may, in part, explain markedly lesser promoter methylation in HUVECs compared with U937 cells (Supplementary material online, Figure S3). Regardless of basal expression, Hcy decreased CpG(6,7) methylation in U937 cells as well (Supplementary material online, Figure S3). It is important to note, however, that even though the p66shc promoter is not heavily methylated in HUVECs, demethylation of only a few CpGs could affect gene expression, as has been shown for the oestrogen receptor-β gene in endothelial cells, and for the tumour suppressor p53.

Our analysis showed a significant difference in plasma Hcy levels when comparing the highest and lowest tertiles of CpG(6,7) methylation. We also found a similar inverse association between the highest and lowest quartiles of CpG(6,7) methylation (Supplementary material online, Figure S4). Importantly, this difference in plasma Hcy was only evident when examining methylation of CpG(6,7), and not other CpGs in the p66shc promoter, speaking to a targeted effect of Hcy on these dinucleotides. However, when analyzing plasma Hcy of our entire cohort, we could not find a significant negative correlation (r = −0.228, P = 0.0875) with p66shc CpG(6,7) methylation. This could be due to lack of statistical power offered by the small size of our cohort (n = 57). Lack of significant correlation across the entire cohort could also be attributed to the relatively narrow range of plasma Hcy in this cohort (2.04–22.3 μM), with only a minority of patients (21%) having plasma Hcy levels considered to be elevated (>12 μM) which reflects epidemiologic data for the prevalence and magnitude of HHcy in coronary artery disease. In contrast, a study which used a cohort with end-stage renal disease, in which plasma Hcy was appreciably higher and in a broader range (7.3–49.4 μM) than in our cohort, did manage to demonstrate a significant negative correlation with p66shc promoter methylation.

Even though Hcy decreases methylation of CpG(6,7) in the p66shc promoter, this finding does not necessarily indicate that Hcy leads to CpG hypomethylation in all genes, or in intergenic regions. On the contrary, HHcy is associated with global hypomethylation in blood monocytes. In endothelial cells as well, Hcy increases methylation of specific CpG dinucleotides in the fibroblast growth factor-2 (FGF-2) gene, inhibiting its transcription. This effect is not unique to FGF-2, as there are many other examples of Hcy-stimulated CpG hypermethylation. We too found that in HUVECs, unlike hypomethylation of CpG(6,7), Hcy hypermethylates CpG(4) in the p66shc promoter (Supplementary material online, Figure S5). Hcy-induced decrease in activity of the p66shc promoter which is not methylatable on CpG(6,7) (Figure 2C) could be explained by such hypermethylation of CpG dinucleotides other than CpG(6,7). Thus, Hcy may not only target specific CpG dinucleotides in a particular promoter, but its effect on methylation status of those dinucleotides may be gene- and site-specific.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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References