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In Vitro and In Vivo
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Homocysteine Upregulates Soluble Epoxide Hydrolase in Vascular Endothelium In Vitro and In Vivo

Donghong Zhang, Xina Xie, Yequeen Chen, Bruce D. Hammock, Wei Kong, Yi Zhu

Rationale: Hyperhomocysteinemia is a risk factor of atherogenesis. Soluble epoxide hydrolase (sEH) is a major enzyme that hydrolyzes epoxyeicosatrienoic acids and attenuates their cardiovascular protective effects. Whether homocysteine (Hcy) regulates sEH and the underlying mechanism remains elusive.

Objective: To elucidate the mechanism by which Hcy regulates sEH expression and endothelial activation in vitro and in vivo.

Methods and Results: Hcy treatment in cultured human endothelial cells dose-dependently and time-dependently upregulated sEH mRNA and protein. Hcy increased the expression of adhesion molecules, which was markedly reversed by inhibiting sEH activity. Hcy-induced sEH upregulation is associated with activation of activating transcription factor-6 (ATF6). Bioinformatics analysis revealed a putative ATF6-binding motif in the promoter region of the sEH gene, which was found at a methylation site. Site-directed mutagenesis and chromatin immunoprecipitation assays demonstrated that Hcy treatment or ATF6 overexpression promoted ATF6 binding to the promoter of sEH and increased its activity. Results of methylation-specific polymerase chain reaction revealed that the ATF6 binding site on the sEH promoter was partially methylated and was demethylated with Hcy. siRNA knockdown of ATF6 or Sp1 blocked and ATF6 overexpression and DNA methyltransferase inhibitor mimicked the effect of homocysteine on sEH upregulation. In vivo, immunofluorescence assay revealed elevated expression of sEH and adhesion molecules in the aortic intima of mice with mild hyperhomocysteinemia, which was attenuated by sEH deletion or inhibition.

Conclusion: ATF6 activation and DNA demethylation may coordinately contribute to Hcy-induced sEH expression and endothelial activation. Inhibition of sEH may be a therapeutic approach for treating Hcy-induced cardiovascular diseases. (Circ Res. 2012;110:00-00.)

Key Words: activating transcription factor 6 | demethylation | endothelial cells | homocysteine | soluble epoxide hydrolase

Hyperhomocysteinemia (HHcy) is a well-known independent risk factor for cardiovascular disease, but the underlying mechanisms remain unclear. Endothelial dysfunction plays a major role in the vascular pathology associated with homocysteine (Hcy). Oxidative stress, endoplasmic reticulum (ER) stress, inflammation, telomerase inactivation, cell apoptosis, and epigenetics regulation are involved in this process. Accumulating data suggest that ER stress is the major process linking the level of Hcy with apoptosis and inflammation. ER stress is associated with development of Hcy-induced atherosclerotic lesions in apolipoprotein E−/− mice. Increased intracellular Hcy content could increase the expression of several ER stress-response genes, including GRP78, GRP94, Herp, RTP, and GADD153, which are involved in ER stress-induced cell death, for further evidence of a mechanism involved in Hcy-induced cell dysfunction and programmed death. The specific conversion of elevated Hcy to S-adenosyl homocysteine (SAH) further inducing DNA hypomethylation represents another major mechanism. Clinical and animal model studies revealed that DNA hypomethylation can modulate atherosclerosis-related gene transcription and their protein function. Sharma et al reviewed the literature for 135 genes that
Non-standard Abbreviations and Acronyms

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<th>Non-standard Abbreviations and Acronyms</th>
<th>Full Form</th>
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<tr>
<td>3-DZA</td>
<td>3-deazadenosine</td>
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<tr>
<td>5-aza-CdR</td>
<td>5-aza-2’ deoxycytidine</td>
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<tr>
<td>AEBSF</td>
<td>4-(2-aminoethyl) benzenesulfonyl fluoride</td>
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<tr>
<td>ATF6</td>
<td>activating transcription factor-6</td>
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<td>EC</td>
<td>endothelial cell</td>
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<td>EET</td>
<td>epoxyeicosatrienoic acid</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>Hcy</td>
<td>homocysteine</td>
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<td>HHcy</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<tr>
<td>ICAM-1</td>
<td>intercellular cell adhesion molecule-1</td>
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<tr>
<td>MSP</td>
<td>methylation-specific polymerase chain reaction</td>
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<tr>
<td>SAH</td>
<td>S-adenosyl homocysteine</td>
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<tr>
<td>sEH</td>
<td>soluble epoxide hydrolase</td>
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<tr>
<td>TUPS</td>
<td>1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoro-methoxy-phenyl)-urea</td>
</tr>
<tr>
<td>UPRE</td>
<td>unfolded protein response element</td>
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<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
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modulated the blood level of Hcy or that were regulated by elevated levels of Hcy. The dysregulation of these genes and their respective pathways involved in the development of atherosclerosis could affect cell function by oxidative and ER stress.

Recently, soluble epoxide hydrolase (sEH) functioning to enzymatically hydrolyze epoxyeicosatrienoic acids (EETs) and other fatty acid epoxides to their respective diols has attracted great interest as a potential therapeutic tool in the treatment of Hcy-induced cardiovascular diseases.

Given that Hcy-induced increase in sEH expression could contribute to endothelial activation, Western blot analysis, inhibitors of signal transduction pathways, adenovirus infection, chromatin immunoprecipitation assay, immunohistochemical and immunofluorescence staining, transfection of siRNA, reporter gene assays, and methylation-specific polymerase chain reaction, are described in the Online Supplemental Methods and all polymerase chain reaction primers used are in Online Table I.

### Statistical Analysis

Results are expressed as means±standard deviation from at least three replicates for each experiment. Statistical analysis involved two-tailed Student t test, one-way ANOVA, Student-Newman-Keuls test, and Dunnett multiple comparison test, as appropriate. *P*<0.05 was considered statistically significant. All statistical analysis involved use of SPSS 16.0 (SPSS, Chicago, IL).

### Results

**Hcy Upregulated sEH Expression and Endothelial Activation in Human ECs**

Elevated Hcy content causes vasculature injury and atherosclerosis by inducing endothelial activation and dysfunction. The sEH hydrolyzes EETs, which decreases the protective role of EETs. We first examined the effect of Hcy on sEH expression in ECs. Human umbilical vein ECs (HUVECs) were treated with Hcy (25–200 μmol/L) for 24 hours or with 50 μmol/L Hcy for various times. Real-time quantitative reverse-transcriptase polymerase chain reaction and Western blot analysis revealed that Hcy dose-dependently and time-dependently upregulated the mRNA and protein levels of sEH, Hcy at 50 μmol/L significantly upregulated sEH expression at both mRNA and protein levels, with peak expression at 200 μmol/L (Figure 1A, C), and Hcy at 50 μmol/L upregulated sEH beginning at 24 hours and lasting for at least 72 hours (Figure 1B, D). In parallel, Hcy increased the expression of VCAM-1 and ICAM-1, markers of endothelial activation, in a dose-dependent manner (Figure 1E, F). Hcy-induced sEH upregulation was confirmed in human aortic endothelial cells (Online Figure IA, B).

**EETs and sEH Inhibitor Prevented Hcy-Induced Endothelial Activation**

Given that Hcy-induced increase in sEH expression could reduce the amount of EETs in cells, we measured the levels of EETs and the ratio of EETs to DHETs in HUVECs. The Hcy decreased the levels of 14,15-EET and the ratio of 14,15-EET to 14,15-DHET, which could be reversed by treatment with the sEH inhibitor 1-((methylsulfonyl-piperidin-4-yl)-3-(4-trifluoro methoxy-phenyl)-urea (TUPS; 1 μmol/L). Further, Hcy-induced VCAM-1 upregulation and ICAM-1 upregulation were reversed by pretreatment with TUPS or 14,15-EET (100 nmol/L) 1 hour before Hcy stimulation (Figure 2C–F and Online Figure IC–E). Therefore, sEH induction may contribute to Hcy-induced endothelial activation and dysregulation of sEH, as well as its inhibition, may be a therapeutic tool in the treatment of Hcy-induced cardiovascular diseases.
induced endothelial activation, and inhibition of sEH activity can prevent the effect of Hcy, at least in part, through the increased protective effect of EETs and possibly other epoxylipides in HUVECs.

**ATF6 Pathway Involved in Hcy-Induced sEH Expression in HUVECs**

The Hcy can alter the cellular redox state and induce ER stress. To determine whether ER stress plays a role in Hcy-upregulated sEH expression, we detected markers of ER stress with an ER inducer, thapsigargin, used as a control. A high concentration of Hcy (200 µmol/L) increased the protein expression of GRP78, JNK, and caspase-12, which was associated with increased sEH expression and activity (Figure 3A and Online Figure IIA, B). Three ER stress inhibitors, taurine, serine protease inhibitor 4-(2-aminoethyl) benzene-sulfonyl fluoride (AEBSF), and salubrinal, were reported to have a protective effect against Hcy-induced ER stress, respectively. We found the Hcy increased mRNA level of GRP78 and sEH attenuated by taurine and AEBSF but not salubrinal (Figure 3B and Online Figure IIC). Immunofluorescence staining revealed that taurine and AEBSF blocked both thapsigargin-induced and Hcy-induced nuclear translocation of ATF6 and upregulation of sEH (Figure 3C), so activation of ATF6 is involved in Hcy-induced sEH expression.

To further study the effects of ATF6 on sEH expression, we examined the overexpression of ATF6 and siRNA knockdown of endogenous ATF6a, a potent transcriptional factor of ER stress-response genes. The mRNA levels of GRP78 and sEH in HUVECs were significantly higher with adenovirus-mediated overexpression and nuclear translocation of N-terminal ATF6 (Ad-ATF6(N)) for 24 hours than with Ad-GFP–infected control (Figure 3D and Online Figure IIIA). Also, Western blot analysis revealed increased protein levels of sEH in Ad-ATF6(N)–infected HUVECs (Online Figure IIIB). Further, in HUV-EC-C cells (ATCC CRL-1730), a human umbilical vein vascular endothelium cell line, siRNA knockdown of ATF6a (to 11.35%) attenuated Hcy-induced sEH expression at protein level (Online Figure IIID) and mRNA level, with no change in basal levels of GRP78 and sEH (Figure 3E).

**ATF6 Binding Site on the sEH Promoter Contributed to the Induction of sEH in ECs**

ATF6 was reported to bind directly to the unfolded protein response element (UPRE; TGACGTGG/A) of target genes and to activate the transcription of UPR genes such as GRP78 in rodents and X-box binding protein 1 in humans, in which the G flanking the “TGACGT” is critical for ATF6-specific binding. The human sEH promoter region has multiple transcription factor binding sites, including sites for AP-1 and SP1, as we reported. To determine whether ATF6 can directly regulate the promoter activity...
of sEH, we used bioinformatics analysis. Sequence analysis revealed one UPRE-like region “GGGCGTGG” within −279 to −286 bp upstream of the transcription initiation site of the sEH promoter region in humans, 43 to 50 bp in mouse, and 63 to 70 bp in rat (Figure 4A), which suggest a common regulatory mechanism of ATF6 on sEH in rodents and humans. Interestingly, nucleotide “GGGCG” in the UPRE-like region on the human sEH promoter is a methylated site by a bioinformatics analysis (http://www.urogene.org/methprimer/index1.html), so a multimechanism including ER stress and DNA methylation could be involved in sEH gene regulation.

To elucidate the role of this motif in response to ATF6, we used plasmids of the human sEH promoter sEH-1000-Luc, as well as deletion and ATF6 binding-site point mutation constructs, for transient transfection. The promoter activities of these constructs were evaluated in EA.Hy926 cells, a human endothelial cell line for better transfection efficiency, with or without Hcy or Ad-ATF6(N). As shown in Figure 4B, the sEH promoter activity of sEH-1000-Luc was increased approximately 4.3-fold with Hcy treatment for 24 hours, which was reversed in part by pretreatment with AEBSF or mutated ATF6 site (Mu283) in the transfection. Ad-ATF6(N) infection significantly increased sEH promoter activity on the wild-type plasmid but lost the responsiveness on transfection with the mutation plasmid Mu283 (Figure 4C). Further, AEBSF decreased Hcy-induced sEH promoter activity (from 7.1-fold to 3.1-fold) for sEH-330-Luc constructs containing ATF6 binding sites (−279 to −286 bp) but had no effect on sEH-256-Luc (Figure 4D), which also suggested that the UPRE-like motif plays critical roles in the regulation of sEH by ATF6 and Hcy. Chromatin immunoprecipitation assay further confirmed that Ad-ATF6(N) and Hcy increased ATF6 binding to the sEH promoter (−343 to −144 bp) as compared with controls. AEBSF could partially block the Hcy-induced binding of ATF6 to the UPRE-like region (Figure 4E). The negative control was reported previously.18

Hcy-Induced DNA Demethylation of ATF6 Binding Site Facilitated the ATF6-Induced sEH Upregulation

We previously reported that DNA demethylation could increase the binding of SP1 to its motifs in the sEH promoter and induce sEH expression.18 Further bioinformatics analysis revealed DNA methylation sites in the UPRE-like region on the sEH promoter. To study the role of DNA methylation in Hcy-induced sEH expression, we used siRNA knockdown of ATF6α or SP1 level in HUV-EC-C cells, which was decreased to 11.4% and 38.7%, respectively. Compared with their corresponding siRNA controls, siRNA knockout of ATF6α or SP1 in HUV-EC-C cells could partially attenuate Hcy-induced sEH expression at both mRNA and protein levels, whereas knockdown of both ATF6α and SP1 completely inhibited the effect of
was upregulated by 4.1-fold (3-DZA), or Ad-ATF6(N) infection, the mRNA level of sEH was increased 1.9 times or 8.0-fold (si-ATF6) or scramble siRNA control (si-Ctrl) for 48 hours (E). *P<0.05 and **P<0.01 vs Ad-GFP infection or si-Ctrl transfection; *P<0.05 vs Hcy. Data are mean±SD from at least three independent experiments. Beta-actin was an internal control.

Hcy (Figure 5A and Online Figure IVA). When HUVECs were treated with DNA methyl transferase inhibitors 5-aza-2'-deoxycytidine (5-aza-CdR) or 3-deazadenosine (3-DZA), or Ad-ATF6(N) infection, the mRNA level of sEH was upregulated by 4.1±1.9 times, 4.3±1.1 times, or 4.9±0.5 times, respectively. Ad-ATF6(N) and 3-DZA or 5-aza-CdR treatment combined increased sEH mRNA level by 9.0±1.8 times or 8.0±1.3 times (Figure 5B), which was consistent with sEH protein expression (Online Figure IVB). Thus, both ATF6 and SP1 contributed to Hcy-induced sEH expression via DNA demethylation.

To determine the changes in DNA methylation status with Hcy treatment, HUVECs underwent methylation-specific polymerase chain reaction after treatment with Hcy (200 μmol/L) for 24 hours. As shown in Figure 5C, the methylation of the ATF6 binding site was increased in Hcy-treated ECs, compared with control. The DNA methylation rate for the identified methylated site within the UPRE-like region on the sEH promoter was decreased from 73.4% to 33.2% with Hcy treatment. Similar effects were also found with 3-DZA or 5-aza-CdR, for a decrease to 24.4% and 27.7%, respectively (Figure 5D). Also, a lower concentration of Hcy (50 μmol/L) could time-dependently (from 24–72 hours) promote changes in DNA methylation of the site on the sEH promoter (Figure 5E). Chromatin immunoprecipitation assay further confirmed that DNA demethylation by Hcy, 3-DZA, and 5-aza-CdR increased ATF6 or SP1 binding to the sEH promoter (∼343 to −144 bp) as compared with the phosphate-buffered saline control (Figure 5F). Therefore, the ATF6 binding site (UPRE-like region) on the sEH promoter was partially methylated with Hcy, and Hcy switched the binding site from methylated to unmethylated to facilitate ATF6 binding and promoter activation.

**HHcy Upregulated sEH Expression in Aortic Intima In Vivo**

We next determined the pathophysiological relevance of Hcy-induced sEH expression and endothelial activation in vivo by establishing an HHcy model in C57BL/6J and sEH−/− mice with 2% (wt/wt) L-methionine in a chow diet for 4 weeks. We also used the sEH inhibitor TUPS (20 mg/L in drinking water, HHcy+TUPS) for treatment. The mean plasma level of total Hcy was significantly elevated in mice fed L-methionine (HHcy group) as compared with controls (27.6±4.5 versus 5.2±1.3 μmol/L; P<0.001; Figure 6A). The TUPS treatment and sEH gene deletion did not change the elevated plasma Hcy levels in HHcy mice. Real-time reverse-transcriptase polymerase chain reaction revealed a 2.6-fold increase in sEH mRNA level in the aortic arteries of HHcy mice as compared with controls (Figure 6B). Immunostaining also revealed increased expression of sEH in the aortic endothelium of HHcy mice (Figure 6C). Although TUPS did not alter the expression of sEH, the increased protein levels of VCAM-1 and ICAM-1 were decreased with TUPS in aortic intima of HHcy mice and in sEH−/− mice, as detected by immunofluorescence staining (Figure 6C). Therefore, in-
hibition of sEH activity or sEH gene deletion could protect against Hcy-induced aortic endothelial activation and injury in vivo. With longer treatment of L-methionine in C57BL/6J mice for 8 weeks, the mean plasma level of total Hcy was increased up to 61.5 ± 9.26 μmol/L, and sEH mRNA increased 6.5-fold as compared with the control group (Figure 6D, E). Furthermore, mouse aortas showed partial methylation of the ATF6 binding site on the sEH promoter. Methylation-specific polymerase chain reaction analyses revealed the methylation level up to 73% in the control group but decreased to 47% and 23% in HHcy mice for 4 and 8 weeks, respectively (Figure 6F). Thus, our in vitro and in vivo data suggested that sEH upregulation by both ER stress and DNA demethylation plays an important role in Hcy-induced endothelial activation.

Discussion

An optimal level of EETs has several beneficial effects on cardiovascular homeostasis, including hyperpolarizing vascular smooth muscle cells, dilating coronary arteries, and suppressing adhesion molecules. An imbalance in the metabolism of EETs by increased sEH expression or activity may lead to impaired vascular protection. Our previous studies showed that AP-1 or SP1 activation participated in sEH transcription upregulation, which contributes to hypertension and cardiac hypertrophy. Here, we defined the role of sEH in Hcy-induced endothelial dysfunction and the underlying mechanism. Our novel findings include the following: (1) Hcy transcription upregulates sEH expression in human ECs through ER stress and the ATF6 pathway; (2) Hcy-induced DNA demethylation enhances ATF6 binding to the sEH promoter and increases sEH expression; (3) ATF6 mediates Hcy-induced sEH expression via the synergistic effect between DNA demethylation and ER stress; and (4) increased sEH expression plays an important role in Hcy-induced endothelial activation, which can be prevented by inhibition of sEH enzyme activity.
Previous studies reported that exogenous cytochrome P450 epoxygenases increased human EC survival and protected bovine aortic ECs against cytokine/lipopolysaccharide-induced apoptosis by mitogen-activated protein kinase and PI3K/Akt signaling pathways. However, the levels of sEH expression and endogenous EETs with insults such as HHcy on endothelial injury were unclear. Our results reveal the mechanism of Hcy-regulated sEH expression in ECs and show that sEH inhibition could reverse, in part, the Hcy-induced increase in expression of the endothelial activators VCAM-1 and ICAM-1 in vitro and in vivo. Because 14,15-EET also could reduce the Hcy-induced increase in expression of adhesion molecules, increased sEH expression may accelerate the degradation of EETs, thereby losing the protective effect of EETs on ECs. Moshal et al reported that Hcy treatment downregulated CYP2J2 protein expression and reduced the generation of EETs. We and others have demonstrated that high levels of Hcy and thapsigargin, an ER stress inducer, cause ER stress and increase the expression of UPR genes, including GRP78, JNK, Caspase12, and p-eIF2α, in human ECs.

ATF6 is a member of the basic-leucine zipper family of transcription factors and is activated by cleavage from the ER membrane and translocation to the nucleus under ER stress. We found that ATF6 nuclear translocation contributed to Hcy-induced sEH induction; N-terminal ATF6 overexpression could mimic and the inhibitor of ATF6 activation (AEBSF), but not p-eIF2α inhibitor, could inhibit the effect of Hcy. That Hcy-induced GRP78 and sEH upregulation was reduced with siRNA knockdown also supported the ATF6 pathway involved in Hcy-induced sEH transcription upregulation. A consensus DNA binding sequence “TGACGTGG/A” for ATF6 in the rat or mouse GRP78 promoter and human X-box binding protein 1 promoter has been identified. In this study, we found a core sequence, “CGTGG,” on the sEH promoter in humans, rats, and mice (Figure 4A). The DNA binding of ATF6 and activity of sEH promoter were increased by ATF6(N) overexpression and Hcy treatment in the presence of the UPRE-like motif (eg, sequence of sEH-330 to 256 in human). This effect was significantly attenuated by mutation of ATF6 binding site and treatment with the ATF6 inhibitor AEBSF, which suggests that this UPRE-like motif contributes to the regulation of sEH by ATF6 as well as Hcy.

Notably, Hcy-induced sEH expression was partially inhibited by ATF6 inhibition, which indicates the involvement of other mechanisms such as DNA methylation or reactive oxygen species. We previously reported that...
concentrations. Only 16 genes related to atherosclerotic disease with elevated plasma level of Hcy (sEH, VCAM-1) and methyltransferase activity in the aortic intima of hyperhomocysteinemia (HHcy) mice. Male C57BL/6J or sEH−/− mice (6 weeks old) were fed standard chow diet with 2% (wt/ wt) L-methionine with or without 1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoro methoxy-phenyl)-urea (TUPS) in drinking water (20 mg/L/d) for 4 to 8 weeks. Mean total plasma homocysteine (Hcy) levels (A) and (B) sEH mRNA expression (mouse β-actin was an internal control) in aorta intima in four groups of mice (n=8) for 4 weeks. C, Representative immunohistochemical staining for sEH or confocal images of immunofluorescence staining for VCAM-1 and ICAM-1 (red) and nuclei (blue) in cross-sections of pectoral aortas from four groups of mice. The arrows indicate the endothelium. Positive immuno- staining of sEH, ICAM-1, and VCAM-1 in aortic intima was evaluated semiquantitatively. Mean total plasma Hcy levels (D) and (E) sEH mRNA expression in aorta intima detected in control and HHcy mice for 8 weeks. F, Methylation-specific polymerase chain reaction (MSP) analysis of methylation status of sEH promoter with a focus on ATF6 binding site in the aortic intima of the mice. Histogram represents the ratio of DNA methylation to total methylation and unmethylation on the sEH promoter. *P<0.05 and **P<0.01 vs control (Ctrl); #P<0.05 and ##P<0.01 vs Hcy treatment. Data are mean±SD. M, methylated; U, unmethylated.

sEH-325 to −316 was an SP1 binding site, which was completely methylated in a hepatocellular carcinoma cell line. Our results reveal a mechanism of DNA methylation-dependent perturbation of SP1 binding to the sEH promoter as a cell type-specific epigenetic regulation of the sEH gene. Similarly, this phenomenon exists in human ECs. This site could be demethylated by Hcy and facilitate SP1 binding and sEH promoter activation. However, many other transcription factors also have CG-rich binding sites in their DNA recognition element. DNA methylation may interfere with the binding of SP1 to DNA and may repress gene transcription and expression. We found DNA methylation on the ATF6 binding site of the sEH promoter, as detected by methylation-specific polymerase chain reaction sequencing. The synergistic effects of ATF6 activation and DNA demethylation, as well as the combined effect of knockdown of both SP1 and ATF6, support that Hcy treatment demethylates the SP1 binding site and the ATF6 binding site, which largely increases SP1 and ATF6 binding to the sEH promoter and is an argument for its transcriptional regulation.

Patients with vascular disease, especially atherosclerosis, show disturbed global DNA methylation associated with elevated plasma level of Hcy (>75 μmol/L) and SAH concentrations. Only 16 genes related to atherosclerotic diseases have been reported to be regulated, at least in part, by DNA methylation. Given the important role of sEH in EET metabolism and HHcy in the development of atherosclerosis, we established a mild or moderate HHcy mouse model with plasma Hcy levels of 27.6 and 62.5 μmol/L, respectively. In this model, the expression of ER stress markers ATF6, GRP78, or caspase-12 (Online Figure V) and sEH was elevated in the aortic intima, and that of ICAM-1 and VCAM-1 was elevated in the intima, which suggests injury or activation of ECs in mice fed L-methionine. Because humans and rodents share high homology for the sEH promoter sequence, we found partial methylation of the ATF6 binding site on the sEH promoter also in the mouse, and the methylation status switch by HHcy may reflect a permission effect for full induction of sEH by Hcy. However, we established the role of sEH in Hcy-induced EC activation because the induction of VCAM-1 and ICAM-1 in the mouse aortic intima was largely attenuated by sEH inhibition and sEH gene deletion. Although we did not detect the plasma concentration of SAH and global methylation status in HHcy mice, Liu et al found the global DNA methylation status of the aortic tissue was lower in HHcy mice than in controls, which supports our results. However, plasma SAH concentrations were negatively associated with global DNA hypomethylation and DNA methyltransferase activity in the aortic tissue of the authors’ apolipoprotein E-deficient mice, which suggested that plasma SAH is a better biomarker of atherosclerosis than Hcy. The authors indicated that variations in the plasma SAH levels might reflect changes in the intracellular concentrations of SAH, which led to...
global DNA hypomethylation in the aortic tissue of their atherosclerotic mouse model. Our results suggest that both ER stress and DNA hypomethylation contribute to the athero-prone effect of Hcy, in which sEH transcription upregulation might play an important role and lead to endothelial dysfunction.

Conclusions
In conclusion, our study revealed that sEH could be transcriptionally upregulated by Hcy by both ER stress or ATF6 activation and DNA methylation in vitro and in vivo. As shown in Figure 7, in ECs, high levels of Hcy induce ER stress to activate ATF6; Hcy also hydrolyzes to SAH, which decreases the methylation status of Sp1 and ATF6 binding motifs on the sEH promoter, facilitates both ATF6 and Sp1 binding to the sEH promoter, and augments sEH expression. In turn, the increased sEH expression hydrolyzes EETs and reduces the protective effect of EETs, which increases the expression of VCAM-1 and ICAM-1 and activates ECs. Our results may reveal a novel mechanism of Hcy-induced endothelial injury that may contribute to the development of atherosclerosis. Our findings warrant further studies to uncover the molecular mechanism underlying the protective effect of sEH inhibition in clinical applications.

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Disclosures
None.

References


SUPPLEMENTAL MATERIAL

Materials and Methods

Detailed Materials and Methods

Materials and Reagents

DL-Homocysteine (Hcy), thapsigargin (Tg), serine protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), salubrinal, sodium bisulfite, and 14,15-EET were from Sigma-Aldrich (St. Louis, MO, USA). Primary antibody for GRP78, caspase-12, sEH, β-actin, phosphorylated eIF2α, GAPDH, ATF6, polyperoxidase-anti-mouse/rabbit IgG and protein A/G PLUS-agrous were from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescent detection (ECL) reagents and Cy3-labeled goat anti-rabbit IgG (H+L) were from Beyotime (Beijing, China) and DAPI was from Vector Labs (Burlingame, CA).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated and maintained in M199 (Gibco, USA) with 20% fetal bovine serum (FBS; Highclone, USA). Primary human aortic endothelial cells (HAECs) were purchased from ScienCell (Carlsbad, CA) and maintained in Endothelial Cell Medium with 5% FBS. Experiments were performed with HUVECs or HAECs between 4 to 6 passages. Human endothelial cell lines EA.hy926 and Human umbilical vein endothelial cell line (HUVEC-C, ATCC# CRL-1730) were cultured in DMEM supplemented with 10% FBS. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. For experiments, subconfluent ECs (80-90% density) were incubated with phosphate buffered saline (PBS; control), Tg, Hcy, AEBSF, 14,15-EET or a specific sEH inhibitor 1-(1-methanesulfonyl-piperidin-4-yl)-3-(4- trifluoro-methoxy-phenyl)-urea (TUPS). 2,3

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

We used qRT-PCR as in our previous study.4 Total cellular or tissue RNA was extracted by the Trizol reagent method (Invitrogen, USA). The quantity and quality of RNA samples were determined by spectral absorption at 260 and 280 nm. The PCR primers used are in supplemental Table 1. mRNA levels were normalized to that of β-actin.

Western blot analysis

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 10% SDS-PAGE and were transferred to PVDF membrane (Millipore, Germany). Immunoblotting involved primary antibodies against GRP78, JNK, VCAM-1 and ICAM-1, caspase-12, sEH and β-actin. Corresponding secondary antibodies were applied and blots were developed by use of Super ECL Plus Detection Reagent (NovasyGen, China).

Site-directed mutagenesis, transient transfection and luciferase activity assay

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SUPPLEMENTAL MATERIAL

Detailed Materials and Methods

Materials and Reagents
DL-Homocysteine (Hcy), thapsigargin (Tg), serine protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), salubrinal, sodium bisulfite, and 14,15-EET were from Sigma-Aldrich (St. Louis, MO, USA). Primary antibody for GRP78, caspase-12, sEH, β-actin, phosphorylated eIF2α, GAPDH, ATF6, polyperoxidase-anti-mouse/rabbit IgG and protein A/G PLUS-agrous were from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescent detection (ECL) reagents and Cy3-labeled goat anti-rabbit IgG (H+L) were from Beyotime (Beijing, China) and DAPI was from Vector Labs (Burlingame, CA).

Cell culture
Human umbilical vein endothelial cells (HUVECs) were isolated and maintained in M199 (Gibco, USA) with 20% fetal bovine serum (FBS; Hyclone, USA). Primary human aortic endothelial cells (HAECs) were purchased from ScienCell (Carlsbad, CA) and maintained in Endothelial Cell Medium with 5% FBS. Experiments were performed with HUVECs or HAECs between 4 to 6 passages. Human endothelial cell lines EA.hy926 and Human umbilical vein endothelial cell line (HUV-EC-C, ATCC# CRL-1730) were cultured in DMEM supplemented with 10% FBS. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. For experiments, subconfluent ECs (80-90% density) were incubated with phosphate buffered saline (PBS; control), Tg, Hcy, AEBSF, 14,15-EET or a specific sEH inhibitor 1-(1-methanesulfonyl-piperidin-4-yl)-3-(4- trifluoro-methoxy-phenyl)-urea (TUPS). 2, 3

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