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17β-Estradiol non-genomically induces vascular endothelial H_2S release by promoting phosphorylation of cystathionine γ-lyase

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Running Title: 17β-Estradiol induces H_2S release via non-genomic actions

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ABSTRACT

Estrogen exerts its cardiovascular protective role at least in part by regulating endothelial hydrogen sulfide (H_2S) release, but the underlying mechanisms remain to be fully elucidated. Estrogen exerts genomic effects, i.e. those involving direct binding of the estrogen receptor (ER) to gene promoters in the nucleus, and non-genomic effects, mediated by interactions of the ER with other proteins. Here, using human umbilical vein endothelial cells (HUVECs), immunological detection, MS-based analyses, and cGMP and H_2S assays, we show that 17β-estradiol (E2) rapidly enhances endothelial H_2S release in a non-genomic manner. We found that E2 induces phosphorylation of cystathionine γ-lyase (CSE), the key enzyme in vascular endothelial H_2S generation. Mechanistically, E2 enhanced the interaction of membrane ERα with the Gα subunit Gαi-2/3, which then transactivated particulate guanylate cyclase-A (pGC-A) to produce cGMP, thereby activating protein kinase G type I (PKG-I). We also found that PKG-Iβ, but

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not PKG-Iα, interacts with CSE, leading to its phosphorylation, and rapidly induces endothelial H_2S release. Furthermore, we report that silencing of either CSE or pGC-A in mice attenuates E2-induced aorta vasodilation. These results provide detailed mechanistic insights into estrogen's non-genomic effects on vascular endothelial H_2S release and advance our current understanding of the protective activities of estrogen in the cardiovascular system.

Introduction

The incidence and severity of cardiovascular disease (CVD) are significantly increased in postmenopausal women (1). Estrogen (E2) decline during women menopausal transition has long been thought to be the primary cause for elevated risk of CVD in postmenopausal women (2). Although epidemiological and experimental studies support the protective role of E2 in CVD, whether Estrogen Replacement Therapy (ERT) benefits to postmenopausal CVD remains controversial (3,4). The ''window of opportunity'' has therefore been proposed to explain the failure of clinical trials, which supports the view that ERT could be cardiovascular protective if started early at the onset of menopause (4). This hypothesis is supported by recent randomized clinical trials showing that women received ERT early after menopause exhibited significantly reduced risk of CVD (5,6). Further investigations are therefore required to fully understand the estrogen's cardiovascular effects and the underlying mechanisms.

It is well known that estrogen exerts its genomic and rapid non-genomic actions through binding to estrogen receptor (ER) ERα and ERβ, as well as the recently identified G-protein-coupled ER (GPR30) (7,8). The genomic effects of estrogen on cardiovascular function and disease have been well defined over the past years. A number of previous studies have shown that 17β-estradiol (E2) improves lipid profiles (9), inhibits proliferation and migration of vascular smooth muscle cell (10), induces vasorelaxation by increasing endothelial nitric oxide synthase (eNOS) protein expression and nitric oxide (NO) release (11,12), accelerates endothelial repair by promoting endothelial proliferation and migration (13,14), and alleviates vascular inflammatory response by modulating functions of immune cells (15). More recently, evidence has continued to accumulate that estrogen's non-genomic actions exert numerous long-term functions in a variety of cell types. For instance, non-nuclear-initiated actions of the ER is essential for the protective effects of estrogen against vascular injury, neointima formation, and cortical bone mass (16,17). Encouragingly, non-nuclear ER signaling promotes cardiovascular protection but not induces an uterotrophic response or promotes breast cancer tumor growth *in vivo* (18). Thus, the selective modulation of non-nuclear ER signaling may represent a promising avenue to protect CVD without increasing estrogen-related cancer risk.

Hydrogen sulfide (H_2S) is a well characterized gasotransmitters, which is mainly synthesized by cystathionine γ-lyase (CSE) from L-cysteine in vascular tissues (19). H_2S has been reported to induce vasorelaxation, stimulate endothelial cell-related angiogenic properties and protect against atherosclerosis (20-22). We have previously reported that E2 enhances endothelial CSE gene expression through ERα-Sp1 interaction with the binding sites in CSE gene promoter and increases

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endothelial H_2S release, which in turn promotes endothelial proliferation and migration (23). Interestingly, E2 also induces vasorelaxation by stimulating rapid endothelial H_2S release within minutes, while the detailed mechanisms remain unknown (24). In view of the importance of non-nuclear ER signaling functions in CVD, it is worthwhile to fully elucidate the underlying molecular mechanisms through which E2 rapidly stimulates H_2S release by vascular endothelial cells.

In the present study, we showed that E2 rapidly induced phosphorylation of CSE, the key enzyme for vascular endothelial H_2S generation and enhanced H_2S release in a non-genomic manner. We also found that pGC-A/cGMP/PKG signaling was required for E2's non-genomic effects on vascular endothelial H_2S release. Our findings provide a novel mechanistic insights into estrogen's non-genomic effects on vascular endothelial H_2S release and advances our current understanding of estrogen's cardiovascular protective actions.

Results

E2 rapidly induced H_2S release by vascular endothelial cells in a non-genomic manner

We measured H_2S release by HUVECs exposed to various concentrations of E2 for 15 min. E2 significantly stimulated H_2S release with concentrations as low as 1nM and this induction peaked at 10nM (Figure 1A). E2 at a physiological concentration (10nM) resulted in a significant increase in H_2S release after 5 min, and this increase reached maximum after 15 min (Figure 1B). Comparable changes in H_2S release was also observed in HAECs treated with 10nM E2 (Figure 1C).

To confirm whether E2-induced H_2S release was dependent on its non-genomic pathway, we incubated HUVECs with E2 and protein synthesis inhibitor cycloheximide (CHX) or transcription inhibitor actinomycin D (Act D). As expected, Act D treatment efficiently prevented the transcription of ICAM1 and PFKB3 (Figure S1A, B), while CHX treatment significantly blocked the translation of VCAM1 in HUVECs (Figure S1C). Interestingly, both Act D and CHX treatment had no effect on E2-induced H_2S release (Figure.1D). The cell-impermeant E2-BSA compound is commonly used to identify E2's rapid actions that occur on the outer surface of the cell membrane. We also observed that E2-BSA significantly increased H_2S release in a similar manner with E2 (Figure 1E, F).

We found that ER antagonist ICI182780 significantly reduced E2-stimulated H_2S release and this effect was not inhibited by GPR30 antagonist G15 (Figure 1G). Consistently, GPR30 agonist G1 alone had no effect on H_2S release (Figure 1G). Interestingly, G15 itself significantly induced H_2S release (Figure 1G).

E2 enhanced endothelial H_2S release through rapid phosphorylation of CSE

H_2S is primarily produced by CSE in vascular tissues and its phosphorylation is critical for endothelial H_2S production (25). Short-term treatment with E2 did not alter CSE expression, but rapidly induced phosphorylation of serine and threonine residues of CSE, which reached maximum around 15 min in HUVECs (Figure 2A). Comparable changes in phosphorylation of serine and threonine residues of CSE was also seen in HAECs treated with 10nM E2

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(Figure 2B). Previous reports have reported that phosphorylation of CSE Ser377 plays an important role in regulating endothelial H_2S release (26). In order to address whether anti-Phospho-(Ser/Thr)Phe used in the present study can recognize phosphorylation of CSE Ser377, we performed a mass spectrometry analysis to examine anti-Phospho-(Ser/Thr)Phe immunoprecipitated samples from HEK293 cells. Potential phosphorylation sites of CSE was predicted using NetPhos 3.1 Server (Figure 2C). Interestingly, the residue serine 56 was found to be phosphorylated (Figure 2D) rather than serine 377.

E2 stimulated phosphorylation of CSE and endothelial H_2S release through PKG-Iβ

It has been previously shown that cGMP-dependent protein kinase G (PKG) phosphorylates the serine residue in CSE (26,27). In line with this, E2-induced Thr/Ser phosphorylation of CSE was significantly reduced in the presence of 10μM the PKG inhibitor KT5823 (Figure 3A). Moreover, E2-induced H_2S production by HUVECs was also dramatically abolished by KT5823 (Figure 3B). The specificity of KT5823 is concentration dependent, with 10μM of this inhibitor may also inhibits PKC activity. To determine whether E2-induced phosphorylation of CSE was also involved with PKC, a specific PKC inhibitor Bisindolylmaleimide I (BIMI) was used. The results showed E2-induced phosphorylation of CSE and endothelial H_2S release was not attenuated in the presence of 5μM BIMI (Figure 3C, D). These data suggest that E2-induced phosphorylation of CSE and endothelial H_2S release through activation of PKG.

There are two major types of PKG in vascular cells, including PKG-Iα and PKG-Iβ. siRNA-mediated silencing of PKG-Iβ, but not PKG-Iα, significantly attenuated E2-induced phosphorylation of CSE (Figure 3E, F). In agreement, E2-induced H_2S release was significantly abolished by PKG-Iβ siRNA but remained unchanged in the presence of PKG-Iα siRNA (Figure 3G). E2-induced phosphorylation of CSE and H_2S released was also abolished by PKG-Iβ siRNA rather than PKG-Iα siRNA in HAECs (Figure 3H, I).

We also overexpressed PKG-Iα or Iβ to examine their effects on CSE phosphorylation and H_2S release (Figure 4A, B). Overexpression of PKG1β dramatically induced Thr/Ser phosphorylation of CSE with or without E2 (Figure 4D), while overexpression of PKG-Iα had no effects (Figure 4C). Basal or E2-induced H_2S release was not altered by overexpression of PKG-Iα, while overexpression of PKG-Iβ significantly enhanced H_2S production in the absence or presence of E2 (Figure 4E).

To test if there is a direct interaction between PKG and CSE, we performed immunoprecipitation assay. Enhanced interaction of PKG-Iβ and CSE was observed after E2 treatment, while the interaction of PKG-Iα and CSE remained unchanged (Figure 4F). Taken together, these data indicate that E2 stimulated phosphorylation of CSE and H_2S production through enhancing the interaction between PKG-Iβ and CSE.

E2-induced H_2S release was dependent on elevated cGMP level

Since PKG is a cGMP dependent protein kinase, we investigated whether E2-induced H_2S production is associated with cGMP level. E2 significantly increased

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cGMP levels after 5-30 min (Figure 5A). As expected, PET-cGMP (membrane permeant analogues of cGMP) treatment induced ser239 phosphorylation of its major substrate vasodilator-stimulated phosphoprotein (VASP) and phosphorylation of CSE (Figure 5B). Similar to E2, PET-cGMP rapidly induced H_2S production by HUVECs after 15min when used at a concentration of 0.1nM and reached maximum at a concentration of 10 nM (Figure 5C). These data revealed that E2-induced H_2S release was dependent on elevated cGMP level.

E2 enhanced cGMP and induced endothelial H_2S production mainly through pGC-A

cGMP is generated from guanosine triphosphate (GTP) by guanylyl cyclases (GCs). Two different forms of GCs exist in mammals including the soluble (sGC) and the particulate GC (pGC) (28). As expected, sGC agonist BAY41-2272 (BAY) significantly increased VASP phosphorylation and this effect was stronger than E2 (Figure 6A). However, sGC inhibitor NS2028 slightly reduced E2-induced phosphorylation of VASP (Figure 6A), indicating that sGC is not the principle enzyme for E2's effect on cGMP signaling.

sGC is the only conclusively proven receptor for NO and E2 is a well characterized stimulator of endothelial NO (29,30). To exclude the possible role of NO-sGC pathway in E2 induced cGMP production, NO donor sodium nitroprusside dehydrate (SNP) and NO synthase inhibitor NG-Nitro-L-arginine (L-NNA) were used. SNP induced a stronger VASP phosphorylation than E2 (Figure 6B), which manifests the activation of NO-sGC pathway is sufficient to activate PKG. Notwithstanding, L-NNA didn't alter E2-induced VASP phosphorylation (Figure 6B). As a result, E2-induced endothelial H_2S release was slightly but not significantly attenuated by NS2028 and L-NNA (Figure 6C). These data support that NO-sGC pathway is not involved in E2-induced cGMP/PKG activation.

Since pGC-A (equivalent to natriuretic peptide receptor A, NPR-A) is the main isoform of pGC expressed in vascular endothelial cells (31), we next evaluated the role of pGC-A in E2-induced cGMP signaling and H_2S release. Transfection with pGC-A siRNA significantly reduced E2-induced phosphorylation of VASP and CSE, and endothelial H_2S release (Figure 6D-F). In HEACs, silence of pGC-A also inhibited phosphorylation of VASP and CSE, and endothelial H_2S release that induced by E2 (Figure 6G, H). Conversely, pGC-A ligand atrial natriuretic peptide (ANP) induced phosphorylation of VASP and CSE in a time-dependent manner (Figure 6I). Taken together, these data show that pGC-A is critical in E2-induced cGMP/PKG/CSE signaling pathway and H_2S production.

E2 activated cGMP/PKG pathway and endothelial H_2S release through the enhanced interaction of ERα with Gαi2/3 and pGC-A

Gαi is important for E2's non-genomic signaling (32). We found that at resting stage, pGC-A interacted with ERα, Gαi-2 and Gαi-3 but not Gαi-1 (Figure 7A). E2 readily increased the interaction of ERα with Gαi-2 or Gαi-3 and pGC-A (Figure 7A). siRNA-mediated silencing of Gαi-2 or Gαi-3 significantly reduced E2-induced phosphorylation of VASP and Thr/Ser phosphorylation of CSE (Figure 7B-E). As a

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result, E2-induced endothelial H_2S release was almost completely abolished by Gαi-2 or Gαi-3 siRNA (Figure 7F, G). These data indicate that E2 enhanced the protein complex formation of ERα with Gαi2/3 and pGC-A.

To further confirm whether the interaction of Gαi with ERα is required for E2-induced non-genomic signaling and H_2S production, we transfected HUVECs with $ER\alpha^{D258A}$ vector, which prevents the interaction of Gαi with ERα (33). Indeed, overexpression of the $ER\alpha^{D258A}$ mutant prevented E2's non-genomic activation of classical p-ERK and p-Akt pathways (Figure 8A). In parallel, it prevented E2-induced PKG activation, as shown by the level of phosphorylated VASP (Figure 8A). Meanwhile, $ER\alpha^{D258A}$ mutant decreased E2-induced interaction of Gαi with ERα (Figure 8B), Thr/Ser phosphorylation of CSE and endothelial H_2S release (Figure 8C, D). These data confirm that the interaction of Gαi with ERα is required for E2-induced cGMP/PKG activation and endothelial H_2S release.

Knockdown of vascular pGC-A and CSE impaired E2-induced vasodilation

Having observed the important role of CSE and pGC-A in E2-induced endothelial H_2S release, we finally examined whether knockdown of CSE or pGC-A in mice can attenuate E2-induced vasodilation *in vitro*. Mice were accepted tail vein injection of control, pGC-A or CSE AAV-shRNA for 4 weeks and aortae were dissected out to detect vascular tension. AAV-mediated shRNA was efficiently delivered to the aorta, as confirmed by GFP^+ expression (Figure S2A). Our immunostaining and western results showed a successful silence of pGC-A and CSE protein in vascular endothelial cells (Figure S2B-D).

To preclude the effects of NO and PGI2, the arteries were incubated with NOS inhibitor L-NNA and COX inhibitor indomethacin for 30min to inhibit the production of NO and prostacyclin. Then the NO- and prostacyclin-resistant responses of all the three groups of aortic arteries to cumulative application of acetylcholine (Ach) were studied. Compared to control (Figure 9A), AAV-mediated pGC-A and CSE knockdown resulted in significantly attenuated Ach-induced vasodilation (Figure 9 B-D). In addition, we found that E2 induced vasodilation (Figure 9E), which was significantly reduced by sliencing of pGC-A and CSE expression (Figure 9 F-H). These data suggest that activation of CSE and pGC-A is important for E2-induced vasodilation *in vitro*.

Discussion

We have previously demonstrated that E2 attenuates atherosclerosis through up-regulation of vascular endothelial CSE protein expression and H_2S release (23). Besides, we found that E2 rapidly stimulates endothelial H_2S release, while the mechanism remains obscure (24). The current study extend our previous studies to disclose the underlying molecular mechanisms through which E2 rapidly induces endothelial H_2S release and vasodilation.

In the present study, CSE expression was not altered, but its phosphorylation was readily increased with short-term treatment of E2. A previous study has reported that vascular endothelial CSE is phosphorylated by taurolithocholic acid via Akt and cAMP-dependent PKA on serine resides, in particular Ser377, to enhance H_2S release

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(26). Our mass spectrometry results showed the anti-Phospho-(Ser/Thr)Phe antibody used in the present study only recognizes phosphorylation of CSE Ser56. However, its role in E2's rapid effect on H_2S release remains to be further investigated.

In vascular endothelial cells, PKG-Iα and PKG-Iβ are predominantly expressed (35,36). On the basis of our previous findings that PKG is critical for H_2S release (24), here we further identified that E2-induced phosphorylation of CSE and H_2S production is dependent on PKG-Iβ but not PKG-Iα. The substrate specificity of PKG-I depends on the different N-terminus of the two isoforms. For example, the isoform PKG-Iα specifically recognizes regulatory myosin phosphatase targeting subunit 1 or RhoA (37,38), while the IP3RI-associated cGMP kinase substrate (IRAG) is a specific substrate for PKG-Iβ (39). Our co-immunoprecipitation result further indicated that with E2 treatment, PKG-Iβ interacted with CSE, suggesting that CSE is a new specific substrate for PKG-Iβ.

PKG-I-knockout mice show an impaired response to NO-induced vasodilatation and vascular smooth muscle-specific PKG-Iα or PKG-Iβ expression can rescue vasodilatory response (40,41). The mechanisms include the inhibition of intracellular Ca^{2+}-release from sarcoplasmic/endoplasmic reticulum by PKG-Iβ or the activation of myosin light chain phosphatase by PKG-Iα (39,42). Although most work describe a vasodilatory effect of PKG-I on vascular smooth muscles, it was recently reported that the activation of endothelial PKG also leads to vasorelaxation and this effect is possibly related to potassium channel (43). Interestingly, H_2S is able to regulate endothelial conductance (IK(Ca)) and small conductance (SK(Ca)) potassium channels (44,45). From these points of view, our findings provide a new possible explanation to the vasodilatory effect of endothelial PKG-I, namely that PKG-Iβ interacts and activates CSE to produce H_2S, which then acts on potassium channels to induce vasodilation.

We have previously reported that E2 activates PKG in vascular endothelial cells, as indicated by VASP phosphorylation at Ser239 (46). Likewise, PKG is also found as the target of E2 in many other tissues, such as vascular smooth muscle cells, osteocytes, breast cancer cells (47-49). sGC is the only conclusively proven receptor for NO and E2 is able to enhance endothelial NO release (29, 30). As the important regulators in cardiovascular system, H_2S and NO interact with each other's biosynthesis and physiological response (50). NO increased CSE expression and H_2S generation from vascular tissues (51). Likely, H_2S increased eNOS phosphorylation and NO generation through the Akt pathway (52). In our study, sGC or NOS inhibitor didn't significantly alter E2-induced PKG activation and H_2S release, while the silence of pGC-A completely abolished E2's effects. This is consistent with others' data showing that E2-enhanced cGMP level was not altered by sGC inhibitor NS2028 in hepatocyte (53). Our work indicates that NO/sGC pathway does not involve in E2-induced H_2S release.

pGC-A is the trans-plasma membrane protein that is traditionally activated by ANP binding to its extracellular receptor portion. It plays an important role in cardiovascular system. For example, pGC-A knockout mice exhibited significant arterial hypertension

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and cardiac hypertrophy (54,55). E2 was able to augment pGC-A signaling by increasing ANP level (55,56). In addition to this, E2 acts at the extracellular face of the plasma membrane to stimulate pGC-A in a non-genomic manner in hepatocyte, but the detailed mechanism remain unexplored (53). Here we further revealed that membrane ERα recruited Gαi-2/3, which then transactivated pGC-A to produce cGMP. In agreement, it was shown that pGC-A activation was dependent on Gαi in *C. elegans* photoreceptor cells (57). Therefore, E2 can enhance pGC-A signaling via indirect regulation of its ligand ANP or direct transactivation of it in vascular endothelial cells.

We confirmed that ERα agonist 4′,4″,4‴-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT), but not ERβ agonist 2,3-bis-(4--hydroxyphenyl)-propionitrile (DPN), stimulated endothelial H_2S release, suggesting that ERα is the key receptor in this event (24). In this study, we extended and identified that ERα interacted with Gαi-2 and Gαi-3 to stimulate PKG and H_2S release. Indeed, ERα directly interacts with Gαi and mediates its non-genomic effects. The disruption of ERα-Gαi interaction prevented E2-induced nitric oxide synthase activation, re-endothelialization and protection against vascular injury (16,18,32). It was reported that endothelial Gαi, in particular Gαi-2, coupled to many G protein coupled receptors, such as α2-adrenergic receptor, endothelin B receptor or 5-hydroxytryptamine receptor 1D, to stimulate NO release and induce vasodilation (58). Our *in vitro* experiments showed that knockdown of Gαi-2 or Gαi-3, but not Gαi-1, significantly reduced E2-induced cGMP signaling and H_2S production. To the best of our knowledge, we for the first time reveal a molecular link between Gαi and H_2S, which adds a new explanation to the mechanisms of Gαi activation and vasorelaxation.

Mutagenesis and the addition of blocking peptide revealed that amino acids 251-260 of ERα contains Gαi binding domain that mediates Src and ERK1/2 activation (32). Furthermore, point mutation of $ERα^{D258A}$ prevented the interaction of ERα with Gαi and blocked non-genomic activation of its downstream signalings (33). In line with this, our data demonstrated that $ERα^{D258A}$ mutant prevented E2-induced ERα-Gαi interaction, non-genomic activation of Akt and ERK1/2, CSE phosphorylation and H_2S production. We also found that AAV-mediated silencing of pGC-A and CSE significantly reduced E2-induced vasodilation. These results indicated that the described non-genomic mechanism of E2-induced H_2S release is essential for physiological effect of E2 on vasorelaxation. The rapid, non-genomic ER signaling contributes to long-term vascular protective actions, such as anti-atherogenesis and inhibition of neointima formation (59). Whether E2-induced rapid H_2S release plays an important role in its long-term vascular protective actions remains to be investigated in our future study.

We found that E2 rapidly stimulated endothelial H_2S release and it is insensitive to transcription or protein inhibitor. In addition, the cell-impermeant E2-BSA compound mimicked E2's effect. These data suggest that E2- induced endothelial H_2S release through its non-genomic action, which does not require transcription or translation of E2's target genes. Instead, E2 binds to its membrane receptors and rapidly

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exerts the generation of intracellular second messengers, and various signal-transduction within minutes such as phosphorylation of CSE, thereby mediating transient H_2S release. Classically, three isoforms of membrane estrogen receptor, including mERα, mERβ, as well as GPR30 (also known as G Protein-Coupled Estrogen Receptor 1, GPER1), exist in different tissues (60). We revealed that ERα interacted with Gαi and the point mutation of ERα blocked E2-elicited downstream signaling and H_2S release. This is consistent with our previous studies showing that mERα is the critical receptor for estrogen's non-genomic effects in different type of cells (14,61). GPR30 has no effect on H_2S release. However, GPR30 antagonist G15 itself enhanced H_2S release. This may be due to the low-affinity cross-reactivity of the G15 to ERα (62). Hence, our data provides a new example to the concept that the modulation of different membrane estrogen receptor isoform can differ markedly in their stimulatory and/or inhibitory effects. The selective activation of these membrane ERs, therefore, is of great importance in the prevention and treatment of E2-related physiological functions and diseases.

In conclusion, we showed that E2 stimulated endothelial H_2S release in a non-genomic manner. E2 enhanced the interaction of mERα and Gαi-2/3, which transactivated pGC-A to produce cGMP and subsequently activate PKG-Iβ to interact with CSE protein, leading to its phosphorylation and rapid H_2S release (Figure 10). Our study provides a novel mechanistic insights into E2's non-genomic effects and also identifies the mERα signaling as a promising target for the development of novel therapeutic strategies in cardiovascular system.

Experimental procedures

Ethical statement

Human umbilical cords for Human umbilical vein endothelial cells (HUVECs) isolation

Human umbilical cords for isolation of HUVECs were collected from Guangzhou First Municipal People's Hospital. All participants gave informed consent. This study was approved by The Research Ethics Committee of Guangzhou First Municipal People's Hospital and Guangzhou Medical University, and complied with the Declaration of Helsinki.

Mice

All animal procedures were reviewed and approved by Institutional Animal Care and Use Committee of Guangzhou Medical University. Four-week-old female mice in C57BL/6 background were obtained from Guangdong animal experiment center (Guangdong, China), raised in the Experimental Animal Center of Guangzhou Medical University. The mice were kept under specific pathogen-free and temperature-controlled conditions on a 12 h light/dark cycle and maintained feedings and drinking water free.

Reagents and antibodies

17β-estradiol (Catalog No. #E2758), β-Estradiol 6-(O-carboxy¬methyl)oxime: BSA fluorescein iso¬thio¬cyanate conjugate (E2-BSA) (Catalog No. #E6507), Cycloheximide (Catalog No. #C7698), Actinomycin D (Catalog No. #A9415), Sodium nitroferricyanide(III) dehydrate (Catalog No. #228710), Nω-Nitro-L-arginine (Catalog No. #N5501), PET-cGMP (Catalog No. # P0622-1VL), and Acetylcholine chloride

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(Catalog No. #A6625) were purchased from Sigma-Aldrich (St. Louis, MO, USA). ICI 182,780 (Catalog No. #ab120131), KT5823 (Catalog No. #ab120423) were obtained from Abcam (Cambridge, MA, USA). G1 (Catalog No. #G6798) and G15 (Catalog No. #G6548) were supplied by Santa Cruz (St. Louis, MO, USA). NS2028 (Catalog No. #4517) was obtained from Tocris Bioscience (Minneapolis, MN, USA). Endothelial Cell Medium (Catalog No. #SC-1001-prf) was purchased from sciencell (Carlsbad, CA, USA). Opti-MEM (Catalog No. #31985) and fetal bovine serum (Catalog No. #12484) were obtained from Invitrogen (Carlsbad, CA, USA). Phenylephrine (Catalog No. #S1723) was obtained from Selleck (Houston, TX, USA). γ-cystathionine (CSE) polyclonal (Catalog No. 12217-1-AP, Lot No. 00050744) antibody and NPR1 (pGC-A) (Catalog No. 55116-1-AP, Lot No. 00048172) polyclonal antibody were purchased from Proteintech (Rosemont, PA, USA), anti-Phosphotyrosine (Catalog No. #ab190824, Lot No. GR260170-1) antibody and anti-Phospho-(Ser/Thr)Phe (Catalog No. #ab17464, Lot No. GR321281-1) antibody, anti-ERβ antibody (Catalog No. #ab3576, Lot No. GR3192192-3) were obtained from Abcam. Anti-ERα (Catalog No. #8644s, Lot No. 4) antibody, anti-PKG-Iα (Catalog No. #13511s, Lot No. 1) antibody, anti-phospho-VASP^{ser239} (Catalog No. #3114S, Lot No. 5) antibody, anti-VASP antibody (Catalog No. #3112S, Lot No. 2) anti-Gαi antibody (Catalog No. #5290, Lot No. 3), anti-VCAM1 (Catalog No. #12367, Lot No.1), HA-Tag Magnetic Bead Conjugate (Catalog No. #11846, Lot No. 6) were obtained from Cell Signaling Technology (Danvers, MA, USA), anti-PKG-Iβ antibody (Catalog No. #PA527325, Lot No. PA5-27325) was purchased from Thermo fisher (Waltham, MA, USA). Anti-Gαi-1 (Catalog No. #sc-13533, Lot No. not available), Anti-Gαi-2 (Catalog No. #sc-7276, Lot No. not available), Anti-Gαi-3 (Catalog No. #sc-262, Lot No. not available) antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). All other chemicals were of analytical grade and purchased from Guangzhou Chemical Reagents (Guangzhou, China).

Cell cultures and treatments

HUVECs were isolated and cultured as previously described (13). Human aortic endothelial cells (HAECs) were obtained from Sciencell (Catalog #6100). Before treatments, HUVECs and HAECs were cultured in ECM (Catalog No. #1001-prf) containing 5% steroid-deprived FBS. Cell cultures were maintained at 37°C in a humidified incubator in a 5% CO_2 plus 95% O_2 atmosphere. All plasmid transfections were performed by using lipofectamine 3000 reagent (Catalog No. #L3000015, Invitrogen) according to manufactures' instructions. All siRNA at 20nM working concentration were transfected using Lipofectamine RNAiMAX Reagent followed by the manufactures' instructions (Catalog No. #13778, Invitrogen). Cells (60% confluent in dishes) were serum-starved for 6-8 hour followed by incubation with siRNA or plasmid for another 6 hours in serum-free media. Target protein silencing or overexpression was assessed by western blot after 48 hours of transfection.

Plasmids and siRNA and adeno-associated virus (AAV) -shRNA

ESR1 (Catalog No. #H8846), EGFP-ESR1-D258A (Catalog No. #H5536), EGFP-HA-Empty vector (Catalog No. #H7638), EGFP-HA-CSE-WT (Catalog No.

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#H7673), EGFP-HA-CSE- S377A (Catalog No. #H316) and EGFP-HA-CSE- S377D (Catalog No. # H8157) expression plasmid were generated in Obio Technology Corp., Ltd (ShangHai, China). The PKG-Iα and PKG-Iβ expression plasmid were bought from Addgene (Watertown, MA, USA). The PKG-Iα (Catalog No. #5626772), PKG-Iβ (Catalog No. #5872111) and pGC A (Catalog No. #5625849) siRNA were purchased from Invitrogen (Carlsbad, CA, USA), Gαi2 and Gαi3 siRNA were ordered from Guangzhou RiboBio Co., Ltd (Guangzhou, China). The mouse AAV-Control-shRNA, AAV-CSE-shRNA and AAV-pGC-A-shRNA were ordered from Vigene Biosciences Inc (ShanDong, China).

Real-time PCR

Total RNA was isolated using RNeasy mini kit (Qiangen, Germany) and reverse transcribed PrimeScript™ RT Master Mix (Takara, RR036A). Real-time PCR was performed using SYBR Green qPCR kit (Takara, RR820A) in the QuantStudio 5 real-time system (Life technologies). Data was expressed as relative fold changes using the $2^{-\Delta\Delta}$CT method. qPCR primers are summarized as below:

PFKFB3,
F: 5′-TGGCAGATGACCAGCACA-3′;
R: 5′-CTTCAGAGAGAGGAAGCCGA-3′;
ICAM-1,
F: 5′-TCTTCCTCGGCCTTCCCATA-3′,
R:5′-AGGTACCATGGCCCCAAATG-3′;
β-Actin,
F: 5′-CATGTACGTTGCTATCCAGGC-3′;
R: 5′-CTCCTTAATGTCACGCACGAT-3′.

Immunoblottings

After treatments, HUVECs were washed thrice with pre-cooling PBS before addition of the lysis buffer (100mM Tris-HCl, pH=6.8, 4% SDS, 20% glycerol, 1mM sodium orthovanadate, 1mM NaF and 1mM phenylmethylsulfonylfluoride, 3mM PMSF) to cell-culture dish on ice. Then cell lysates were scraped carefully, boiled for 18min before centrifuged for 7min at 13500 rpm. Cell lysates were separated by SDS-PAGE. The antibodies were used at the following concentrations: CSE (1:2000), pGC-A (1:1000), Phosphotyrosine (1:2000), Phospho-(Ser/Thr)Phe (1:2000), ERβ (1:2000), ERα (1:4000), PKG-Iα (1:1000), phospho-VASP (1;4000), VASP (1:2000), Gαi (1:2000), PKG-Iβ (1:2000). Semi-quantitative analysis of western blot data was performed using Image J software (National Institutes of Health). The relative protein levels normalized by that of intracellular β-actin were then expressed as the percentage compared with respective controls. All semi-quantitative data are presented in Figure S3, S4.

Immunoprecipitation assay

Immunoprecipitation was performed according to the manufactures' instructions (Catalog No. #10007D, Dynabeads Protein G, Invitrogen, USA). In brief, equal amount (200 μg) of cell lysate were immunoprecipitated with IP antibody-conjugated Dynabeads magnetic beads for 10 min at 4°C under gentle agitation. The magnetic bead-Ab-Ag complex were washed with washing Buffer for three times. 20 μl Elution Buffer consisting of 0.2M glycine (pH 2.0) was used to remove immunoprecipitates from the beads. The immunoprecipitates were analyzed by western blotting.

Mass spectrometry analysis

HEK293 cells were transfected with 2μg wild type CSE plasmid for 24 h, and immunoprecipitation was performed with anti-Phospho-(Ser/Thr)Phe as describe

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above. The anti-Phospho-(Ser/Thr)Phe immunoprecipitates were subjected to trypsin digestion, and the phosphopeptides were enriched using TiO2 beads (GL Sciences) according to manufacturer's instructions. The enriched phosphopeptides separated from nanoHPLC were subjected into the tandem mass spectrometry Q-EXACTIVE (Thermo Fisher Scientific, San Jose, CA) by BGI Mass Spectrometry Center (Shenzhen, China). The parameters for MS analysis are listed as following: electrospray voltage: 1.6kV; precursor scan range: 350-1600m/z at a resolution of 70,000 in Orbitrap; MS/MS fragment scan range: >100m/z at a resolution of 17,500 in HCD mode; The number of MS/MS scans following one MS scan: 20 most abundant precursor ions above a threshold ion count of 20000.

Measurement of cGMP levels

HUVECs were treated with 10nM E2 at the indicated time points. Cells were lysed in 0.1M HCl/1%Triton. cGMP was then measured by using a cGMP ELISA kit (Catalog No. #80101, NewEast Biosciences, King of Prussia, PA, USA). Each time the calibration of cGMP standards are assayed at the same time as the samples. The standard wells, testing sample wells and blank wells were set. Briefly, the testing sample and cGMP standards were added to testing sample wells and standard wells respectively, the blank wells contained lysis buffer. HRP-conjugate reagent, chromogen solution and stop solution were added to each well. The optical density at 450 nm was determined using a microtiter plate reader. The concentration of cGMP in the samples was then determined by comparing the O.D. of the samples to the standard curve.

Measurement of H_2S levels

HUVECs were treated and then the medium was collected for measuring the H_2S levels as we previously reported (24). Amperometric H_2S sensors (ISO-H_2S-100, World Precision Instruments, Sarasota, FL, USA) were used for the measurement of dissolved H_2S concentration in the medium. In brief, the amperometric H_2S sensor was calibrated with $Na_2S.9H_2O$ stock solution before each experiment and a calibration curve was made. The tip of the H_2S sensor was immersed into the medium for 10-15 mm and the current output was recorded. The concentration of dissolved H_2S in the solution was calculated based on the calibration curve.

Animals

At 8 weeks of age, the mice were randomly divided into three groups and accepted one tail vein injection of AAV-Control-shRNA ($5.0*10^{11}$ v.g per mouse), AAV-pGC-A-shRNA ($5.0*10^{11}$ v.g per mouse) or AAV-CSE-shRNA ($7.5*10^{11}$ v.g per mouse), respectively. After 4 weeks, the aortae were dissected out and the AAV-mediated silencing of target protein was evaluated through immunofluorescence staining and western blot.

Artery preparation and wall tension measurement

After Tail vein injection of AAV for four weeks, aortic blood vessels were taken to detect vascular tension. *In vitro* tension measurement was carried out as we previously reported (24). In brief, aortic were isolated and cut into four segments (2mm per segment) in ice-cold physiological salt solution. The artery rings were mounted on a needle holder of DMT620 multi-channel isolated vascular tone measurement system (World Precision

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Instruments, Sarasota, FL, USA) under a stereomicroscope. The organ bath was filled with oxygenated tissue buffer (PSS) and maintained at 37.8C. Isometric contractions were recorded by a force transducer connected to an analog-to-digital converter system. Thirty minutes after mounting in the organ bath, all the rings were contracted using phenylephrine (Phe, 1μM), and the functional integrity (over 90% relaxation) of the endothelial layer was determined by adding acetylcholine (ACh, 10μM). The rings were then allowed to equilibrate for an additional 60 min and the rings were contracted for a second time by the addition of Phe for 30 min and then its response to ACh or E2 was recorded.

Statistical Analysis

Data are presented as means ± SD. All experiments were repeated at least 3 times. Statistical comparisons were made using one way-ANOVA followed by the post-hoc LSD test or Mann-Whitney test where the data is not normally distributed with SPSS software, the difference was considered significant if $p< 0.05$.

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Author Contributions: X.D. F. conceived and supervised the study. X.D. F., D.X. Z. and J.X. L. wrote the manuscript. S.T. revised the manuscript. X.Y. X., Q. Y., P. L., X.Y. L., X.S. L., Y.W. C., J.X. L. performed the experiments. All authors analyzed the data and revised the manuscript.

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FOOTNOTES

The abbreviations used are: Ach: Acetylcholine chloride; Act D: Actinomycin D; ANP: Artial natriuretic peptide; BAY: BAY41-2272; cGMP: Guanosine 3',5'-cyclic monophosphate; CHX: Cycloheximide; CSE: Cystathionine γ-lyase; CVD: Cardiovascular disease; DPN: 2,3-bis-(4--hydroxyphenyl)-propionitrile; E2: 17β-estradiol; E2-BSA: β-Estradiol 6-(O-carboxy¬methyl)oxime: BSA fluorescein iso¬thio¬cyanate conjugate; ECM: Endothelial Cell Medium; ER: Estrogen receptor; ERα: Estrogen receptor α; ERT: Estrogen Replacement Therapy; FBS: Fetal bovine serum; GCs: Guanylyl cyclases; GPR30: G-protein-coupled ER; GTP: Guanosine triphosphate; H_2S: Hydrogen sulfide; ICI: ICI182780; IRAG: IP3RI-associated cGMP kinase substrate; L-NNA: NG-Nitro-L-arginine; NPR-A: Natriuretic peptide receptor A; pGC: Particulate GC; pGC-A: Particulate guanylate cyclase-A; Phe: Phenylephrine; PKG: Protein kinase G; PKG-I: Protein kinase G type I; PPT: 4′,4″,4′″-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol. sGC: Soluble GC; SNP: Sodium nitroprusside dehydrate; VASP: Vasodilator-stimulated phosphoprotein;

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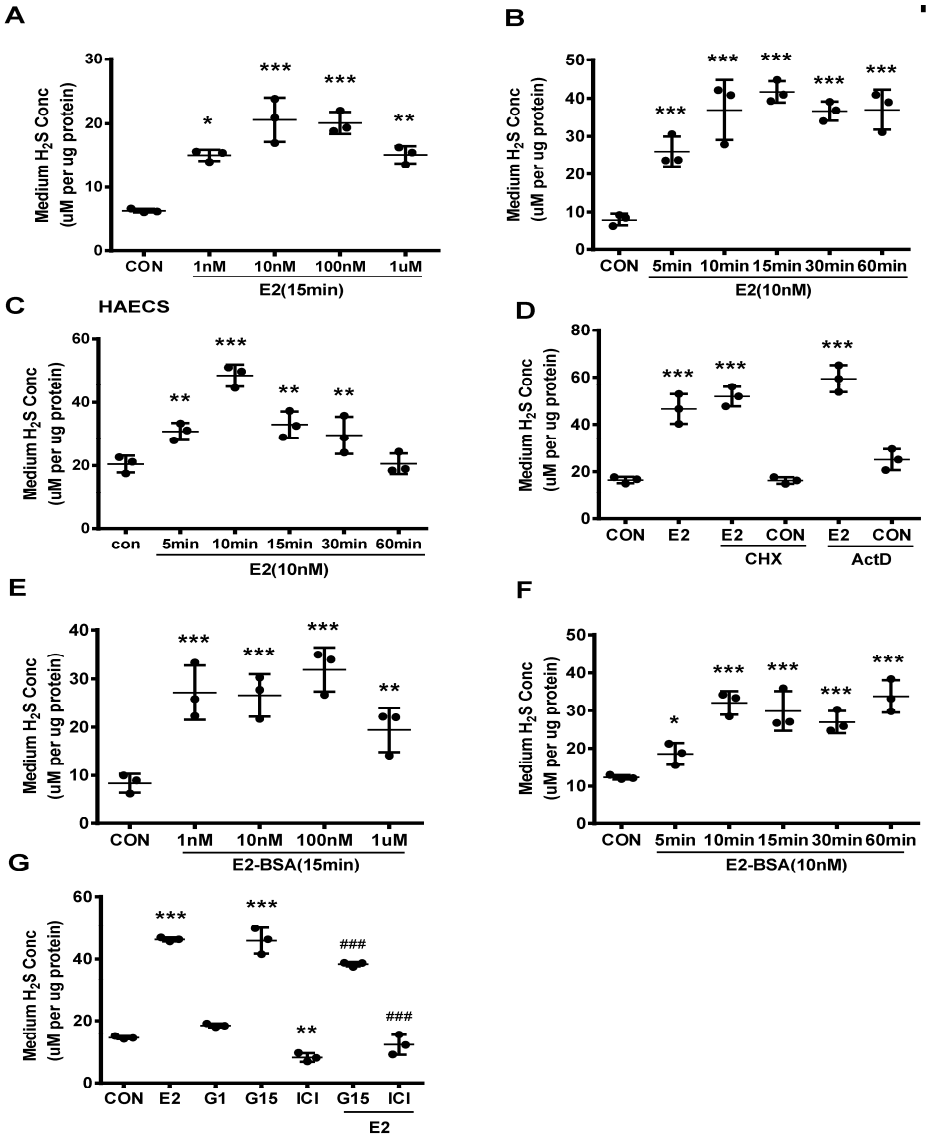

Figure 1. E2 promotes vascular endothelial H_2S release through its non-genomic effect. A. HUVECs were exposed to different concentrations of E2 for 15 min, and the medium H_2S concentrations were measured (mean ± SD, n=3 independent experiments, *P<0.05, **P<0.01, ***P<0.001 vs CON, one way-ANOVA followed by the post-hoc LSD test). B. HUVECs were treated with E2 (10nM) at different time points, and the medium H_2S concentrations were measured (mean ± SD, n=3 independent experiments, ***P<0.001 vs CON, one way-ANOVA followed by the post-hoc LSD test). C. HAECs were treated with E2 (10nM) at different time points, and the medium H2S concentrations were measured (mean ± SD, n=3 independent experiments, **P<0.01, ***P<0.001 vs CON, one way-ANOVA followed by the post-hoc LSD test). D. HUVECs were treated with E2 in the presence or absence of the protein synthesis inhibitor (CHX, 200μM), and the transcription inhibitor Actinomycin D (Act D, 10μM). The medium H_2S concentrations were measured (mean ± SD, n=3 independent experiments, ***P<0.001 vs CON, one way-ANOVA followed by the post-hoc LSD test). E-F. HUVECs were treated with E2-BSA in different concentrations or at different time points, and the medium H_2S concentrations were measured (mean ± SD, n=3 independent experiments, *P<0.05, **P<0.01, ***P<0.001 vs CON, one way-ANOVA

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followed by the post-hoc LSD test). G. HUVECs were treated with E2 in the presence or absence of ER antagonist ICI182,780 (ICI, 10μM), GPR30 antagonist (G15, 10μM), GPR30 agonist G1 (G1, 10μM), and the medium H_2S concentrations were measured (mean ± SD, n=3 independent experiments, **P<0.01, ***P<0.001 vs CON, $^{\#\#\#}$P<0.001 vs E2, one way-ANOVA followed by the post-hoc LSD test).

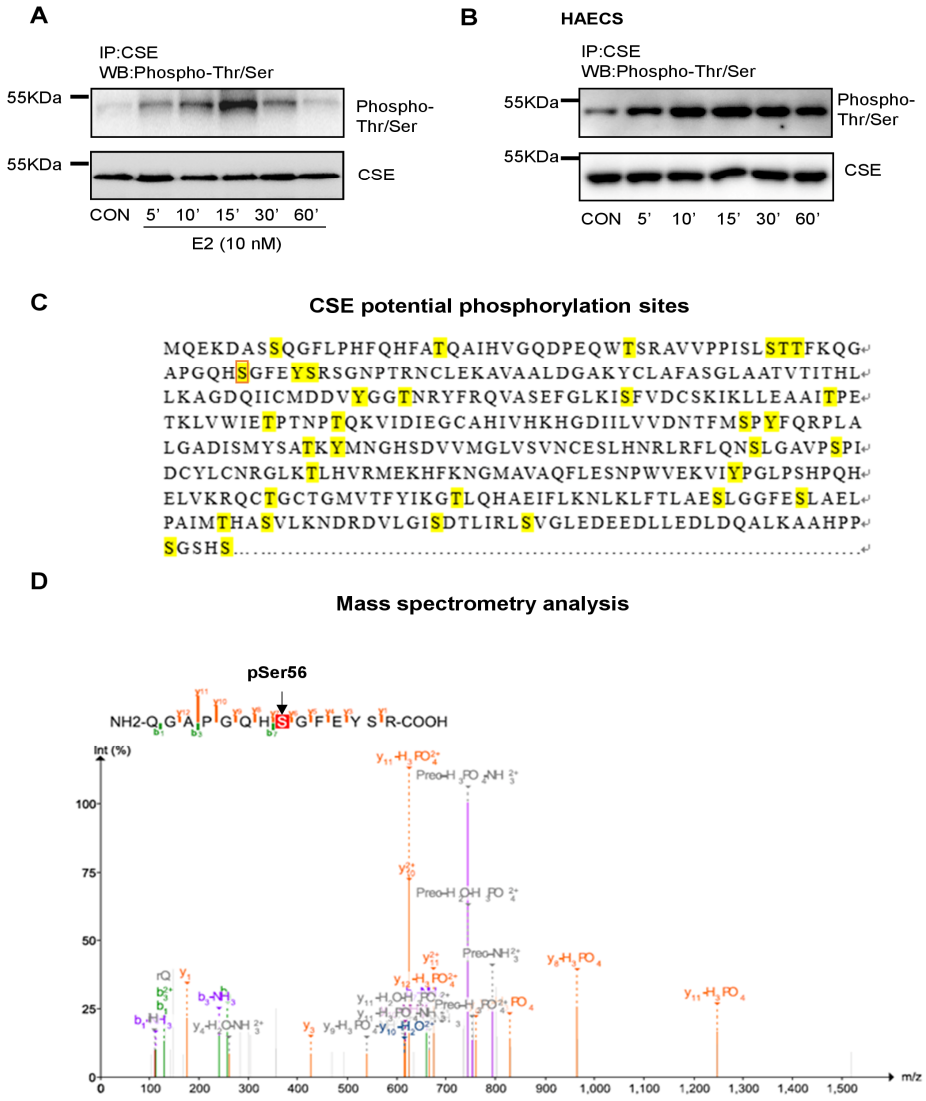

Figure 2. E2 regulates endothelial H_2S release through phosphorylation of CSE. A-B, HUVECs and HAECs were treated with E2 at different time points, and Thr/Ser phosphorylation of CSE and total CSE level were measured by immunoprecipitation and western blot. C. Potential phosphorylation sites marked with yellow were predicted by NetPhos 3.1 Server. D. Mass Spectrometry analysis of immunoprecipitated samples anti-Phospho-(Ser/Thr)Phe from HEK293 cells.

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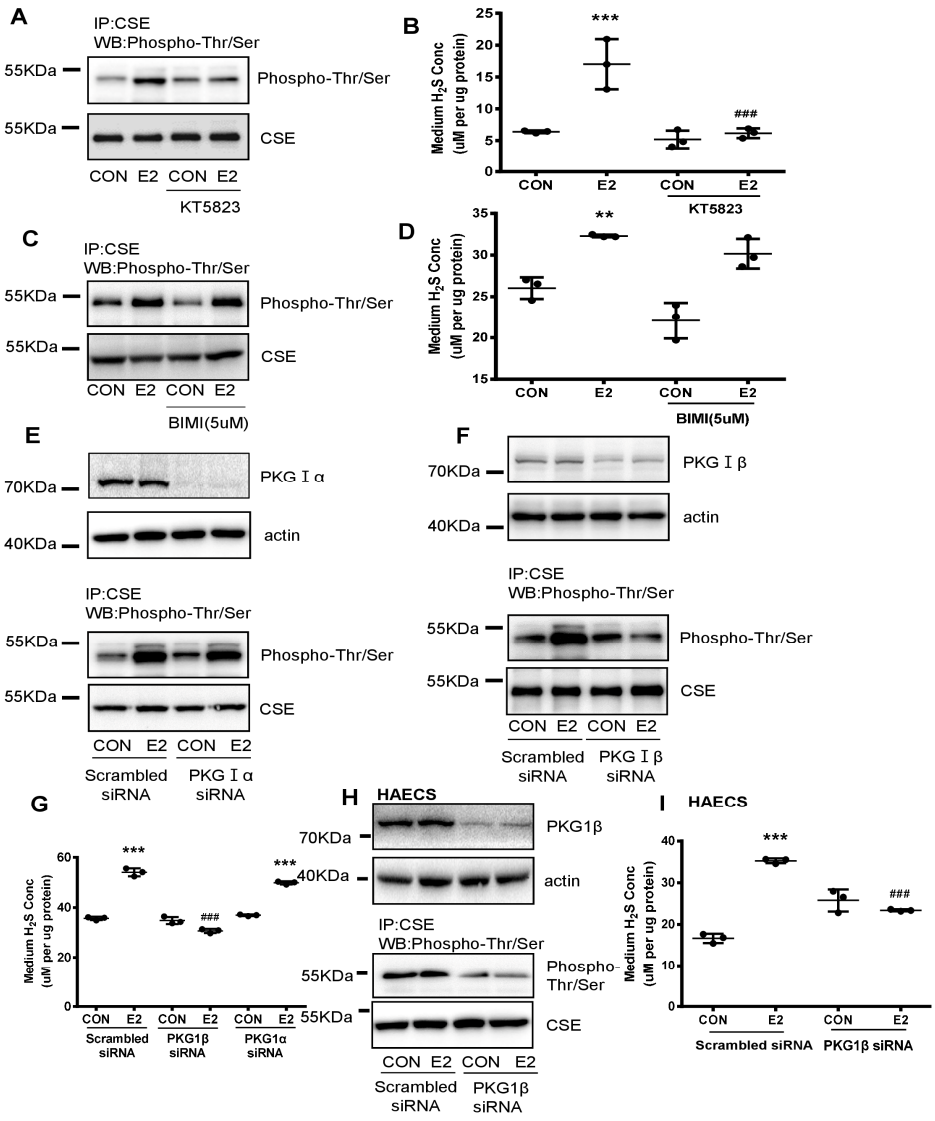

Figure 3. E2 induced endothelial H_2S release and phosphorylation of CSE is dependent on PKG-Iβ. A. HUVECs were exposed to E2 for 15min in the presence or absence of the Protein kinase G inhibitor (KT5823, 10μM), and CSE phosphorylation level and total CSE was detected by immunoprecipitation and western blot. B. HUVECs were exposed to E2 for 15min, in the presence or absence of the Protein kinase G inhibitor (KT5823, 10μM), the medium H_2S concentrations were measured (mean ± SD, n=3 independent experiments, ***P<0.001 vs CON, $^{###}$P<0.001 vs E2, Mann-Whitney test). C-D, HUVECs were exposed to E2 for 15min in the presence or absence of the Protein Kinase C inhibitor (BIMI, 5μM), CSE phosphorylation level and total CSE was detected by immunoprecipitation and western blot, and the medium H_2S concentrations were measured (mean ± SD, n=3 independent experiments, **P<0.01, vs CON, one way-ANOVA followed by the post-hoc LSD test). E-F. HUVECs were transfected with PKG-Iα (20nM) or PKG-Iβ siRNA(20nM) for 36h and treated with E2 for 15 min, the cell content of PKG-Iα and PKG-Iβ were detected by western blot, CSE phosphorylation level and total CSE was detected by immunoprecipitation and western blot. G, HUVECs were transfected with PKG-Iα (20nM) or PKG-Iβ siRNA(20nM) for 36h and treated with E2 for 15 min, the medium H_2S concentrations were measured (mean

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± SD, n=3 independent experiments, ***P<0.001, vs Scrambled siRNA CON, ###P<0.001, vs Scrambled siRNA E2, one way-ANOVA followed by the post-hoc LSD test). H-I, HAECs were transfected with PKG-Iβ siRNA(20nM) for 36h and treated with E2 for 15 min, PKG-Iβ, CSE phosphorylation level and total CSE was detected by immunoprecipitation and western blot, and the medium H_2S concentrations were measured.

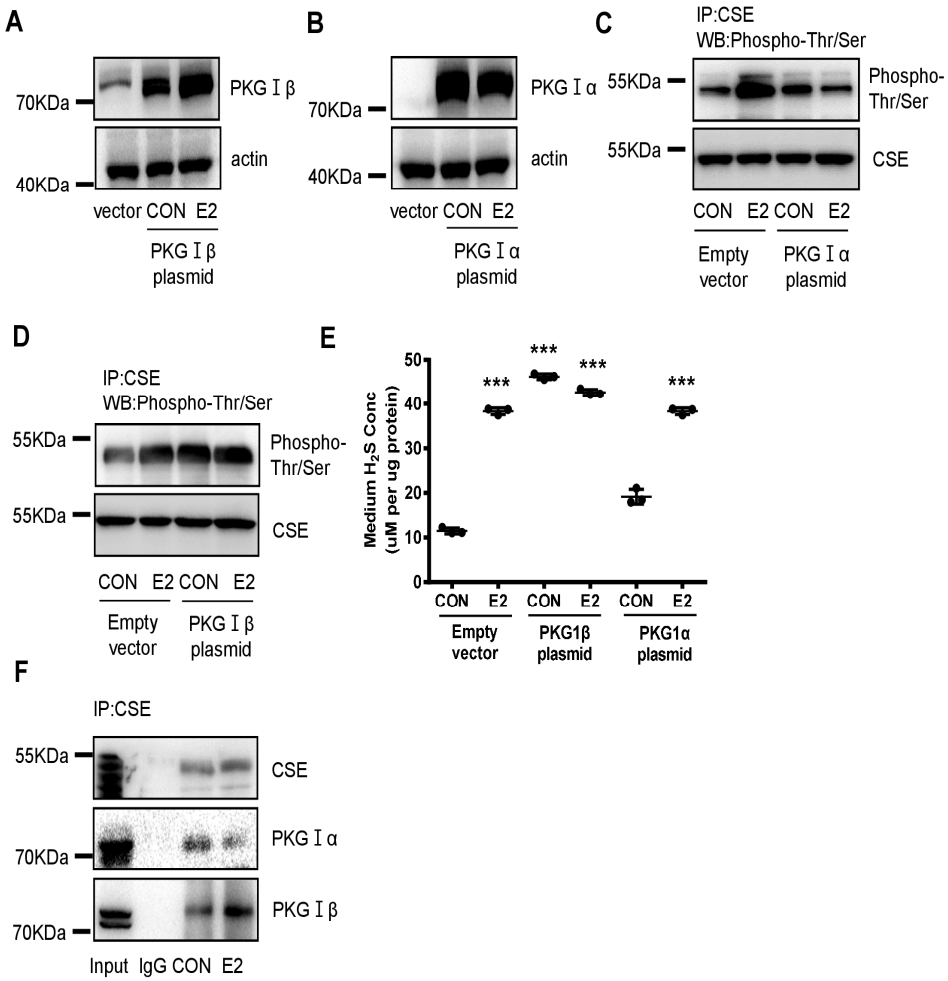

Figure 4. PKG-Iβ directly interacts with CSE and mediates E2-induced phosphorylation of CSE and endothelial H_2S release. A-B. HUVECs were transfected with PKG-Iα (2μg) or PKG-Iβ (2μg) plasmid for 48h, and treated with E2 for 15 min. The efficiency of overexpression was confirmed by western blot. C-D. CSE phosphorylation level and total CSE was detected by immunoprecipitation and western blot. E. HUVECs were transfected with PKG-Iα (2μg) or PKG-Iβ (2μg) plasmid for 48h, and treated with E2 for 15 min, the medium H_2S concentrations were measured (mean ± SD, n=3 independent experiments, **P<0.01, vs Empty vector CON, one way-ANOVA followed by the post-hoc LSD test). F. The interaction between CSE protein and PKG-Iβ or PKG-Iα protein was detected by immunoprecipitation and western blot. Input refers to whole cell lysates, and IgG refers to normal rabbit IgG.

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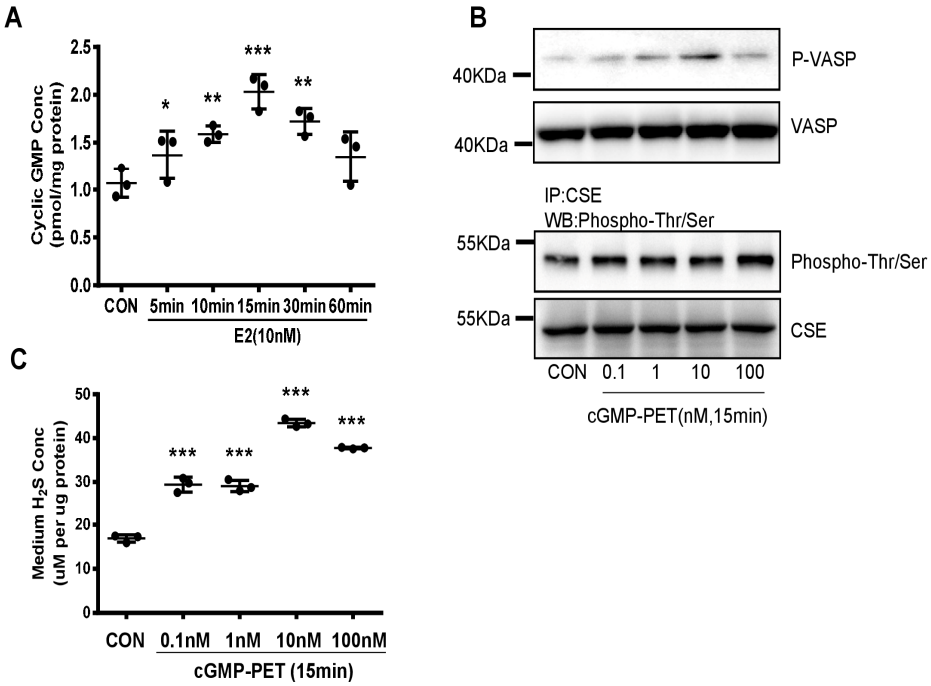

Figure 5. E2 induced endothelial H_2S release through cGMP. A. HUVECs were treated with E2 at different time points, the level of cGMP was measured by Elisa (mean ± SD, n=3 independent experiments, *P<0.05, **P<0.01, ***P<0.001, vs CON, one way-ANOVA followed by the post-hoc LSD test). B. HUVECs were treated with different concentrations of cGMP-PET for 15 min, the cell content of p-VSAP was detected by western blot, the cell content of CSE phosphorylation level and total CSE was detected by immunoprecipitation and western blot. C. HUVECs were treated with different concentrations of cGMP-PET for 15 min, the medium H_2S concentrations were measured (mean ± SD, n=3 independent experiments, ***P<0.001, vs CON, one way-ANOVA followed by the post-hoc LSD test).

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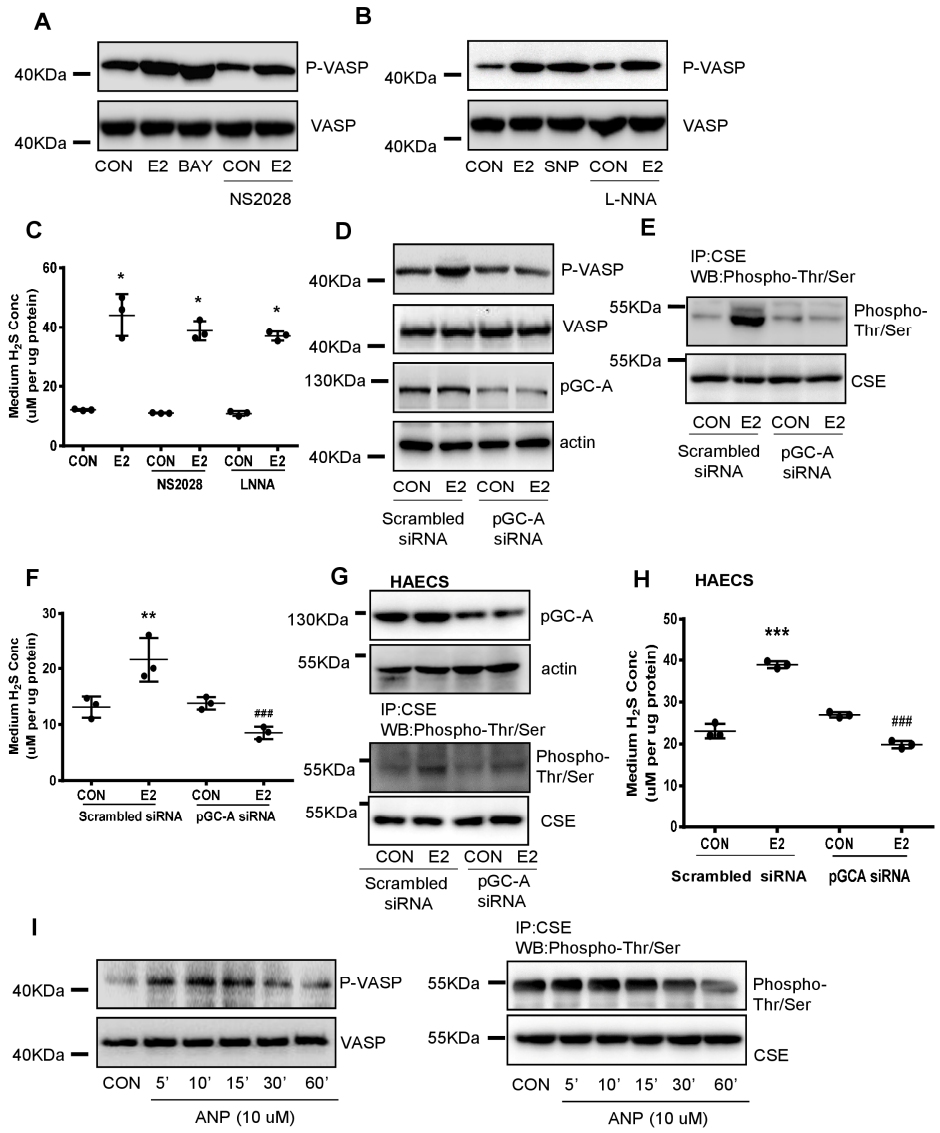

Figure 6. pGC-A is critical in E2-induced cGMP/PKG/CSE signaling pathway and endothelial H_2S production. A-C. HUVECs were exposed to E2 for 15 min in the presence or absence of the sGC activator (BAY41-2272, $3*10^{-6}$M), sGC inhibitor (NS2028, 10^{-5}M), donor of NO (SNP, 10^{-4}M), inhibitor of NO (LNNA, 10^{-3}M). The cell content of p-VSAP was detected by western blot, and the medium H_2S concentrations were measured (mean ± SD, n=3 independent experiments, *P<0.05, vs CON, one way-ANOVA followed by the post-hoc LSD test). D. HUVECs were transfected with pGC-A siRNA (20nM) for 36h and treated with E2 for 15 min, pGC-A, p-VASP and VASP was detected by western blot. E. HUVECs were transfected with pGC-A siRNA (20nM) for 36h, and treated with E2 for 15 min, CSE phosphorylation level and total CSE was detected by immunoprecipitation and western blot. F. HUVECs were transfected with pGC-A siRNA (20nM) for 36h, and treated with E2 for 15 min, the medium H_2S concentrations were measured (mean ± SD, n=3 independent

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experiments, **P<0.01, vs Scrambled siRNA CON, one way-ANOVA followed by the post-hoc LSD test). G-H. HAECs were transfected with pGC-A siRNA (20nM) for 36h, and treated with E2 for 15 min, pGC-A, CSE phosphorylation level, total CSE, and the medium H_2S concentrations were measured (mean ± SD, n=3 independent experiments, ***P<0.01, vs Scrambled siRNA CON, one way-ANOVA followed by the post-hoc LSD test). I. HUVECs were exposed to ANP (pGC-A ligand) (10^{-6}M) for different time, the cell content of p-VASP level was detected by western blot, the cell content of CSE phosphorylation level and total CSE was detected by immunoprecipitation and western blot.

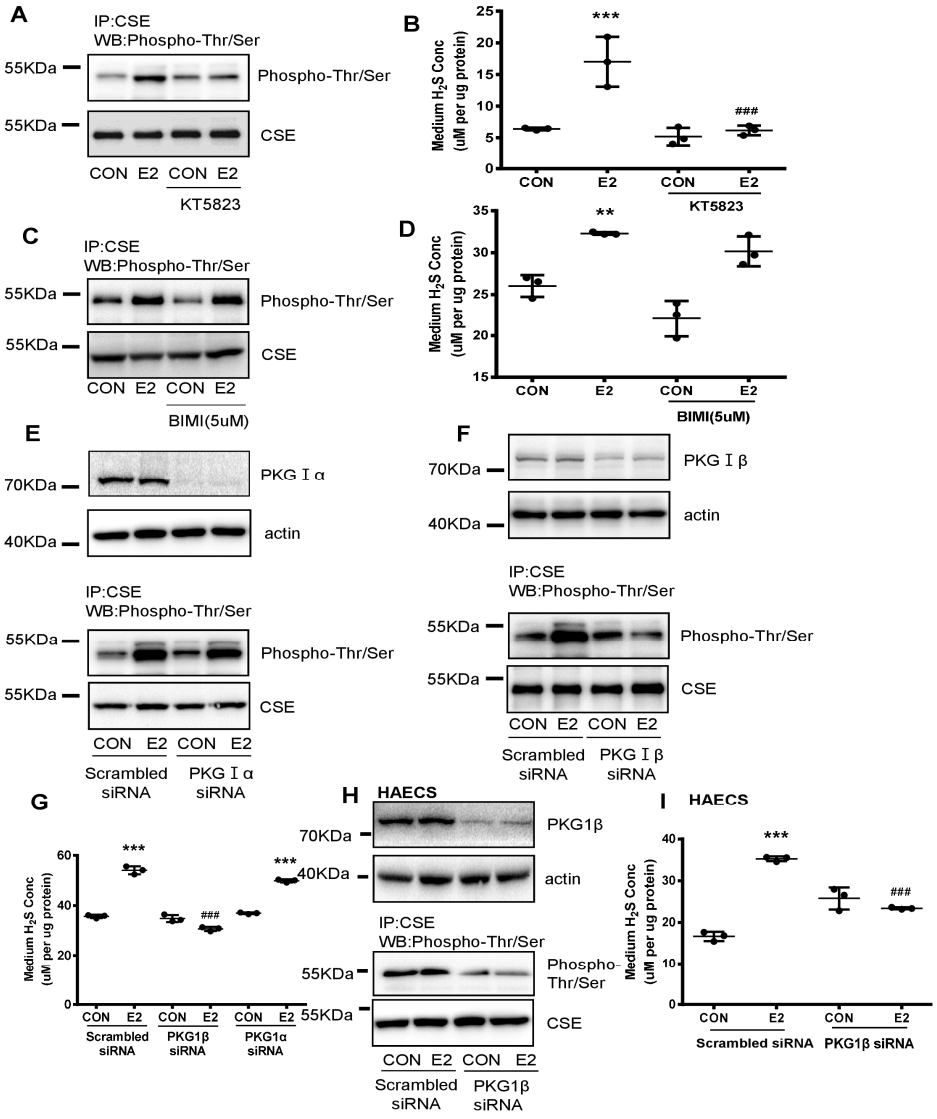

Figure 7. E2 activated cGMP/PKG pathway and endothelial H_2S release through the enhanced interaction of ERα with Gαi2/3 and pGC-A. A. HUVECs were exposed to E2 for 15 min, the interaction between Gαi1/2/3, pGC-A and mERα was detected by immunoprecipitation and western blot. B-C. HUVECs were transfected with scrambled siRNA or Gαi2/3 siRNA for 48h, and treated with E2 for 15 min, the cell content of Gαi2/3 and p-VASP was detected by western blot. D-E. HUVECs were transfected with scrambled

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siRNA or Gαi2/3 siRNA for 48h, and treated with E2 for 15 min, CSE phosphorylation level and total CSE was detected by immunoprecipitation and western blot. E2. F-G. HUVECs were transfected with scrambled siRNA or Gαi2/3 siRNA for 48h, and treated with E2 for 15min, the medium H_2S concentrations were measured (mean ± SD, n=3 independent experiments, **P<0.01, ***P<0.001, vs Scrambled siRNA CON, $^{###}$P<0.001, vs Scrambled siRNA E2, one way-ANOVA followed by the post-hoc LSD test).

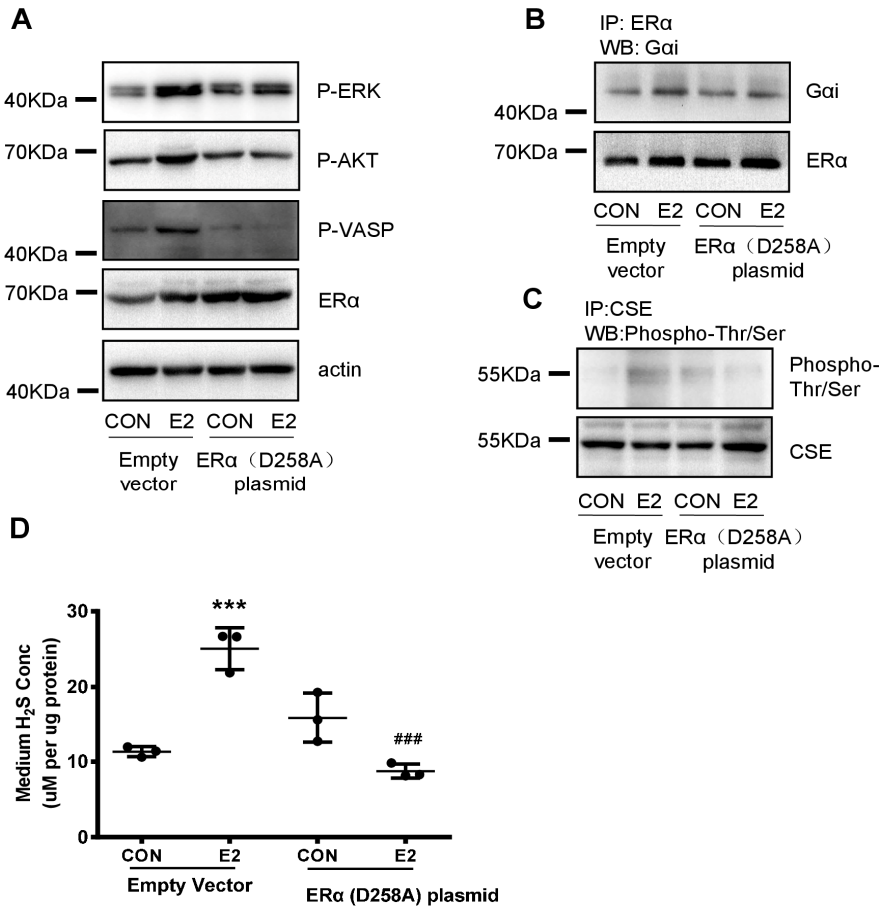

Figure 8. ERα^{D258A} prevents the non-genomic effect of E2 on phosphorylation of CSE and endothelial H_2S release. A. HUVECs were transfected with empty vector (2μg) or mutant ERα (D258A, 2μg) plasmid for 48h and treated with E2 for 15min, p-VSAP, VASP, p-ERK, p-AKT and ERα were detected by western blot. B. HUVECs were transfected with empty vector (2μg) or mutant ERα (D258A, 2μg) plasmid for 48h and treated with E2 for 15min, the interaction between ERα and Gαi was measured by immunoprecipitation and western blot. C-D. HUVECs were transfected with empty vector (2μg) or mutant ERα (D258A, 2μg) plasmid for 48h and treated with E2 for 15min, CSE phosphorylation level and total CSE was detected by immunoprecipitation and western blot, and the medium H_2S concentrations were measured (mean ± SD, n=3 independent experiments, ***P<0.001, vs Empty vector CON, $^{###}$P<0.001, vs Empty vector E2, one way-ANOVA followed by the post-hoc LSD test).

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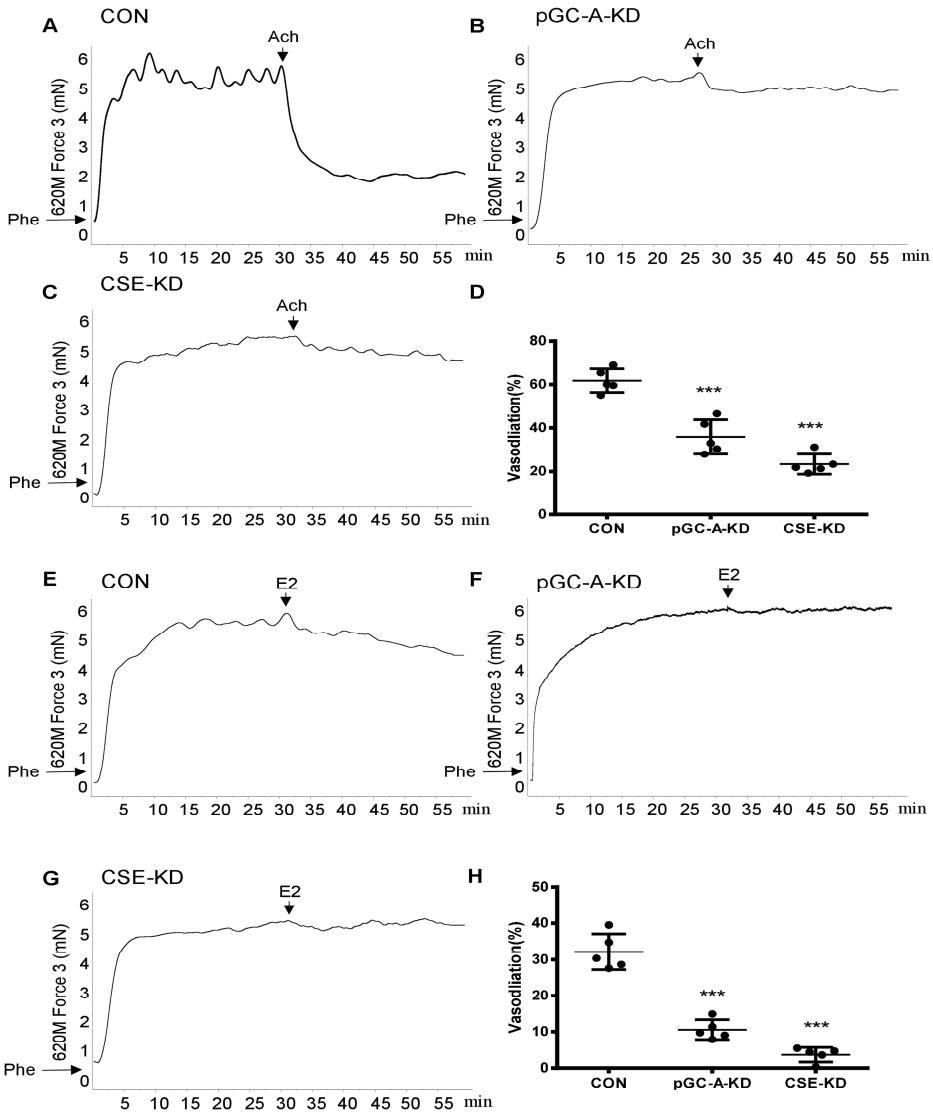

Figure 9. AAV-mediated silencing of pGC-A or CSE significantly attenuated E2-induced vasodilation. Mice at 8 weeks of age were injected from the tail vein with AAV-control-shRNA ($5.0*10^{11}$ v.g), AAV-pGC-A-shRNA ($5.0*10^{11}$ v.g), AAV-CSE-shRNA ($7.5*10^{11}$ v.g). A-D. After 4 weeks, the aortae were dissected out and pretreated for 30 min with 200mM L-NNA (NO inhibitor) and 10mM Indo (prostaglandin synthesis inhibitors) and pre-constricted with phenylephrine (Phe, 10^{-6}M) before testing with acetylcholine (Ach, 10^{-5}M) (mean ± SD, n=5 independent experiments, ***P<0.001, vs CON, one way-ANOVA followed by the post-hoc LSD test). E-H. Knockdown of pGC-A or CSE expression significantly attenuated E2-induced vasodilation (mean ± SD, n=5 independent experiments, ***P<0.001, vs CON, one way-ANOVA followed by the post-hoc LSD test).

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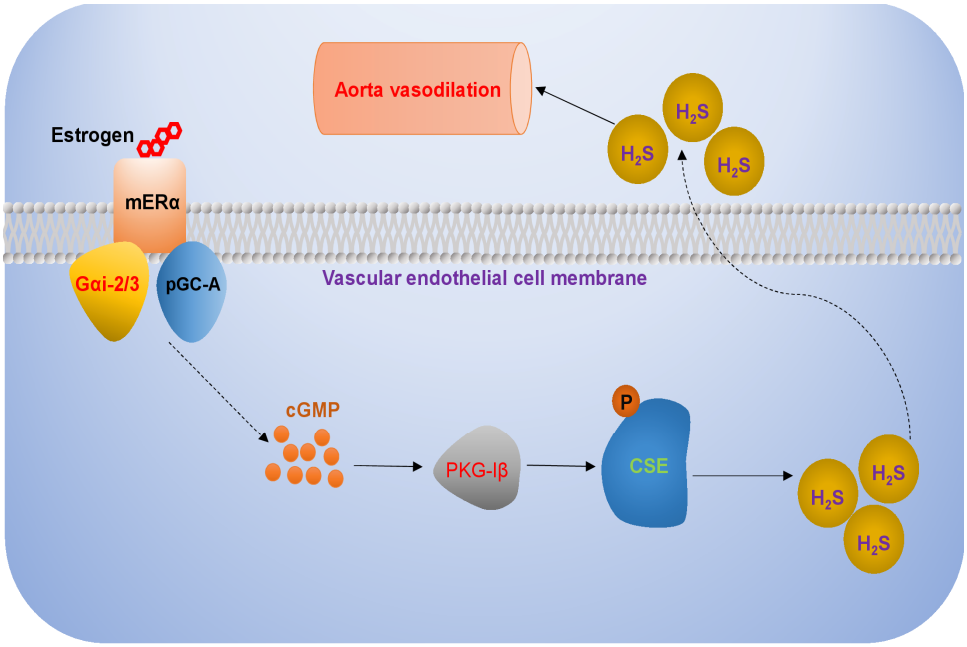

Figure 10. Schematic representation of non-genomic action of E2 on vascular endothelial H_2S release. E2 enhances the interaction of membrane estrogen receptor α (ERα) and G alpha subunit Gαi-2/3, which subsequently transactivates particulate guanylate cyclase-A (pGC-A) to produce cGMP and activates protein kinase G type I β (PKG-Iβ). The isoform of active PKG-Iβ interacts with CSE protein, leading to its phosphorylation and rapid endothelial H_2S release.

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17β-Estradiol non-genomically induces vascular endothelial H2S release by promoting phosphorylation of cystathionine γ-lyase

Xingyan Xu, Qing Yan, Xiaoyun Liu, Ping Li, Xiaosa Li, Yiwen Chen, Tommaso Simoncini, Junxiu Liu, Dongxing Zhu and Xiaodong Fu

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