**ORIGINAL RESEARCH**

I7β-estradiol induces vasorelaxation by stimulating endothelial hydrogen sulfide release

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**ABSTRACT:** Estrogen exerts vascular protective effects, but the underlying mechanisms remain to be understood fully. In recent years, hydrogen sulfide (H2S) has increasingly been recognized as an important signaling molecule in the cardiovascular system. Vascular H2S is produced from L-cysteine, catalyzed by cystathionine γ-lyase (CSE). In our study, apolipoprotein E (ApoE)-deficient mice were ovariectomized and implanted with placebo (OVX mice) or 17β-estradiol (E2) pellets (OVX + E2 mice). Compared with OVX mice, OVX + E2 mice showed increased plasma H2S levels (P = 0.012) and decreased aortic lesion area (P = 0.028). These effects were largely reversed when supplementing with the irreversible CSE inhibitor DL-propargylglycine (PPG) in the OVX + E2 + PPG mice. Meanwhile, the nitric oxide and prostacyclin-resistant responses to cumulative application of acetylcholine (ACH) were studied among all the three groups of femoral arteries. Compared with the arteries in the OVX group, the vasodilator sensitivity of arteries to ACH was increased in the OVX + E2 group and attenuated in the OVX + E2 + PPG group. E2 and estrogen receptor (ER) α agonist 4′,4′,4′′-(-propyl-[1H]-pyrazole-1,3,5-triyli)trisphenol rapidly increased H2S release in human endothelial cells, but not partially selective ERα agonist 2,3-bis-(4-hydroxyphenyl)-propionitrile. These effects were inhibited by ER antagonist ICI 182780 or by protein kinase G (PKG) inhibitor KT5823. Furthermore, endothelial PKG activity was increased by E2 (P = 0.003) and E2-induced vasodilation was inhibited by KT5823 (P = 0.009). In conclusion, the endothelial CSE/H2S pathway is activated by E2 through PKG, which leads to vasodilation. These actions may be relevant to estrogen’s anti-atherogenic effect.

**Key words:** 17β-estradiol / vasorelaxation / atherosclerosis / hydrogen sulfide / vascular endothelial cells

**Introduction**

Estrogen retards the progression of atherosclerosis (Hodis et al., 2001; Torri et al., 2003; Billon-Gales et al., 2009). In recent years, there have been numerous attempts to understand estrogen’s cardiovascular actions, in particular its endothelial mechanisms (Miller and Duckles, 2008; Xing et al., 2009). For example, 17β-estradiol (E2) has been shown to augment endothelium-dependent vasodilation (Simoncini et al., 2000; Sobrino et al., 2010), promote endothelial integrity (Simoncini et al., 2006; Filipe et al., 2008) and to attenuate the inflammatory response in endothelium (Chakrabarti et al., 2010). Notwithstanding, the endothelial mechanisms of estrogen action are far from understood.

It has been well documented that E2 induces vasorelaxation through activation of endothelial nitric oxide synthase (eNOS), leading to NO release from the endothelium (Williams et al., 1992; Haynes et al., 2000; Russell et al., 2000; Simoncini et al., 2000; Kubickiene et al., 2008). In addition, endothelial prostacyclin (PGI2) also contributes to the E2-induced vasodilatory effect (Sobrino et al., 2010). These findings are consistent with the current concept that endothelial NO and PGI2 are the principal molecules that act on the adjacent smooth muscle cells to induce relaxation (Villar et al., 2006). However, it was reported that NO and PGI2 are not the predominant endothelium-derived relaxing factors in female mice (Scotland et al., 2005). In parallel, E2 potentiates NO- and PGI2-resistant vasodilation in response to acetylcholine (ACH) (Burger et al., 2009). These data

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suggested that apart from NO and PGI2, endothelium-derived hyperpolarizing factor (EDHF) also plays a crucial role in E2-provoked vasorelaxation.

The exact nature of EDHF remains undefined. Recent studies raised the candidacy of hydrogen sulfide (H2S) as a new EDHF (Wang, 2009). Endogenous H2S is now considered the third member of the gaseo-transmitter family, along with NO and carbon monoxide (Szabo, 2007). In the cardiovascular system, the production of H2S is catalyzed by cystathionine γ-lyase (CSE), the enzyme predominantly expressed in the vasculature and heart (Yang et al., 2008). H2S potently induces vasorelaxation and decreases blood pressure (Yang et al., 2008). Stimulates endothelial cell-related angiogenic properties (Papapetropoulos et al., 2009; Yang et al., 2006) and diminishes atherosclerotic lesions (Wang et al., 2009). However, there is no evidence indicating a functional relationship between estrogen and H2S in the cardiovascular system.

In this study, we investigated the role of H2S in vasodilatory response to ACh with E2 treatment and in E2-induced anti-atherogenic effects in apolipoprotein-E knockout (apoE−/−) mice. Moreover, we observed the rapid regulation on H2S release by E2 in human endothelial cells.

Materials and Methods

Materials

E2, DL-propargylglycine (PPG) and KT5823 were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4′,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triy) triphenol (PPT), 2,3-bis-(4-hydroxyphenyl)-propionitrile (DPN) and ICI 182780 were purchased from Toscrs Cookson (Bristol, UK). Dulbecco’s modified Eagle’s medium (DMEM), Opti-MEM and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA, USA). All other chemicals were of analytical grade and purchased from Guangzhou Chemical Reagents (Guangzhou, China).

Animals

Six-week-old homozygous ApoE−/− female mice in C57BL/6 background were obtained from Jackson Laboratory (Maine, NE, USA) and raised in the Experimental Animal Center of Zhongshan School of Medicine, Sun Yat-sen University. The mice were kept under specific pathogen-free and temperature-controlled conditions on a 12-h light/dark cycle and fed ad libitum an atherogenic diet containing 1.25% (w/w) cholesterol (Guangdong Medical Animal Experimental Center, Guangzhou, China) and drinking water. At 8 weeks of age, the female mice were anesthetized and bilaterally ovariectomized (OVX) through a 1-cm dorsal incision. After surgery the mice were allowed to recover for 1 week, at which point pellets containing E2 (0.25 mg, 60-day release, Innovative Research of America) were implanted into the mice. Untreated mice were injected daily with saline. The doses of E2, PPG were used in this study as described previously (Rayner et al., 2009; Wang et al., 2009). All procedures were performed in accordance with the guidelines for animal welfare of Sun Yat-sen University.

Assessment of aortic atherosclerotic lesions

Aortic atherosclerotic lesions were assessed as described (Wang et al., 2009). In brief, after 8 weeks of treatment, blood was collected from the retro-orbital venous plexus. The heart, arterial tree and uterus were dissected and collected carefully. The heart and the arterial tree were incubated with phosphate-buffered saline (PBS, 0.01 mol/l, pH 7.4) and fixed in 4% formalin. Tissues were embedded in optimum cutting temperature compound and serially sectioned at 6 μm using a Leica cryostat (Leica Microsystems, Wetzlar, Germany). To quantify the dimensions of atherosclerotic lesions, 10–12 sections per animal in each group with tricuspid valves were stained with oil-red O and counterstained with hematoxylin. Lesion areas were captured and quantified with the Leica Qwin imaging software (Leica Microsystems).

Artery preparation and wall tension measurement

In this study, we obtained femoral arteries from ApoE−/− mice to test the vasodilatory response to ACh, on the basis of the fact that the atherosclerotic lesions were less frequently formed in the femoral artery compared with aorta, right and left external carotid artery. Therefore, the vasodilatory response to ACh is more efficient (Seo et al., 1997). In vitro tension measurement was carried out as we previously reported (Xiang et al., 2010). In brief, after 8 weeks of treatment, small femoral arteries were isolated and cut into four 2-mm-wide ring segments in ice-cold physiological salt solution [with the following composition (mmol/l): NaCl 119, KCl 4.7, CaCl2 2.5, MgSO4 1.17, NaHCO3 25, KH2PO4 1.18, EDTA 0.026 and glucose 5.5]. The artery rings were mounted on a two-chamber Danish Myotechnology M610 wire myograph under a stereomicroscope. The organ bath was filled with oxygenated Krebs solution and maintained at 37°C. 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, 100 mmol/l), and the functional integrity (over 90% relaxation) of the endothelial layer was determined by adding ACh (100 μmol/l). 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 10–15 min and then its response to ACh was recorded.

For aorta preparation, female Sprague–Dawley rats were obtained from the Center of Experimental Animals at Sun Yat-Sen University (Guangzhou, China). The rats were sacrificed by cervical dislocation. Thoracic aortas were collected from a region 1.5 cm away from the aortic arch and placed in a dissecting dish filled with an ice-cold oxygenated Krebs solution. The procedure to measure wall tension is similar to the aforementioned process. For all the functional experiments, firstly the rings were incubated for 30 min with inhibitors.

Measurement of H2S levels

Amperometric H2S sensors (ISO-H2S-100, World Precision Instruments, Sarasota, FL, USA) were used for the real-time measurement of dissolved H2S concentration in the medium or in the plasma (Suzuki et al., 2011). In brief, the amperometric H2S sensor was calibrated before each experiment with freshly prepared Na2S·9H2O stock solution (range of final concentrations: 0, 2, 4, 8 and 10 μM) and a calibration curve was made. The tip of the H2S sensor was immersed ~10–15 mm into the medium or plasma solution and the current output on the display was recorded after it reached a plateau. The concentration of dissolved H2S in the solution was calculated by a linear regression analysis based on the calibration curve. After that, the protein concentration in each well was measured by the bicinchoninic acid (BCA) method and the protein concentration of the control well was defined as 1. Then the protein concentrations in other
wells were adjusted to the control. The final concentrations of H2S were adjusted to these protein concentration values. For measuring H2S release from endothelial cells, every 3 wells of cultured cells were treated with E2 for each time points (0–60 min) and then the medium was collected.

**Cell cultures and treatments**

Human umbilical vein endothelial cells (HUVECs) were cultured as we previously described (Bae et al., 2009). Before treatments, HUVECs were kept for 24 h in DMEM containing steroid-stripped FBS. Whenever an inhibitor was used, the compound was added 30 min before starting the treatments. The control cells received the same amount of ethanol (solvent for E2, 0.01% final concentration).

**Western blotting**

After treatments, endothelial cells were rinsed once with ice-cold PBS before addition of the lysis buffer (100 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 1 mM sodium orthovanadate, 1 mM NaF and 1 mM phenylmethylsulfonylfluoride). The cell lysates were scraped, boiled and centrifuged for 2 min at 13,000 rpm. Cell lysates were separated by SDS–PAGE. Antibodies used were: CTH (CSE) (Sigma-Aldrich, St. Louis, MO, USA) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Primary and secondary antibodies were incubated with the membranes using the standard technique. Immunodetection was accomplished using enhanced chemiluminescence (Chemidoc XRS+ System, Bio-Rad, Hercules, CA, USA).

**Protein kinase G activity**

Protein kinase G (PKG) activity was measured according to the manufacturer’s instructions (Cyclex, MBL International, Woburn, MA). In brief, cells were harvested and pelleted by centrifugation and were resuspended with extraction buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulphonyl fluoride, 1 μg/ml pepstatin, 0.5 μg/ml leupeptin, 2 mM NaF, 0.2 mM Na3VO4, 5 mM beta-mercaptoethanol). Then the cells were lysed by three cycles of freezing and thawing. Protein concentration was determined by the BCA kit (Pierce), and 30 μg of lyase from each sample was used. The lysates were diluted in Kinase Buffer to 100 μl and added to the wells together with 90 μl of cGMP plus Kinase Reaction Buffer. After incubation at 30°C for 30 min, the wells were washed five times with Wash Buffer and 100 μl of horse-radish peroxidase-conjugated Detection Antibody 10H11 was pipetted into each well. After incubation at room temperature for 60 min, the wells were washed five times and 100 μl of substrate reagent was added to each well for incubation at room temperature for 10 min. Finally 100 μl of stop solution was added to each well within 30 min and spectrophotometric absorbance was measured at 450 nm.

**Statistical analysis**

Data are presented as means ± SD, representing at least three independent experiments. Statistical comparisons were made using Student’s t test or one-way analysis of variance followed by a post hoc analysis (Tukey test) where applicable, and the significance level was set at P < 0.05.

**Results**

**Quantification of the dimensions of atherosclerotic lesions**

In oil-red O staining atherosclerotic plaques were extensively found in the OVX group of ApoE−/− mice and the size was 0.42 ± 0.05 mm² (Fig. 1A and B). In contrast, the plaque was significantly diminished in the OVX + E2 mice (0.28 ± 0.04 mm²) (n = 7, P = 0.028), but it was largely reversed in the OVX + E2 + PPG mice (0.36 ± 0.04 mm²) (n = 7, P = 0.036) compared with the OVX + E2 mice (Fig. 1A and B). However, PPG did not completely abolish E2 action, since there was still a significant difference between the OVX and the OVX + E2 + PPG mice (n = 7, P = 0.042).

**Changes in plasma concentration of H2S**

Compared with that in the OVX mice, plasma H2S concentration was significantly elevated in the OVX + E2 mice (n = 7, P = 0.012). The increase in plasma H2S concentration induced by E2 was largely inhibited by the addition of PPG (n = 7, P = 0.009 when OVX + E2 versus OVX + E2 + PPG) (Fig. 1C).

**Estrogen replacement enhances vasodilatory response to ACh**

To preclude the effects of NO and PGI2, the arteries were incubated with NG-nitro-L-arginine (L-NNA; NOS inhibitor) and indomethacin (Indo; COX inhibitor) for 30 min to inhibit the production of NO and prostacyclin, respectively. Then the NO- and prostacyclineresistant responses of all the three groups of femoral arteries to cumulative application of ACh were studied. Figure 2A shows representative ACh-induced changes in the tensins of Phe-preconstricted arteries obtained from the OVX, OVX + E2 as well as the OVX + E2 + PPG mice. In the presence of L-NNA (200 μM) and Indo (10 μM), ACh effectively dilated vessels from the three groups of mice (Fig. 2A—D). However, compared with the arteries from the OVX mice, the ACh concentration–response curve was shifted to the left in arteries of the OVX + E2 mice (n = 4, P = 0.037 when OVX + E2 versus OVX mice to 0.1 μM ACh; n = 4, P = 0.007 when OVX + E2 versus OVX mice to 1 μM ACh), while it was partially reversed to the right in arteries of the OVX + E2 + PPG mice (n = 4, P = 0.030 when OVX + E2 versus OVX + E2 + PPG mice to 0.1 μM ACh; n = 4, P = 0.022 when OVX + E2 versus OVX + E2 + PPG mice to 1 μM ACh) (Fig. 2A—D), indicating a significant enhancement of H2S-mediated vasodilation. Although the vasodilatory response to ACh in the OVX + E2 + PPG group was not reduced to the same level as in the OVX group, there was no significant difference between them (n = 4, P > 0.05).

**E2 rapidly stimulates endothelial H2S release**

Treatment with E2 (10 nM) resulted in a rapid increase in H2S release on HUVECs (n = 3, P = 0.006 when E2 versus control at 5 min; n = 3, P = 0.024 when E2 versus control at 10 min) (Fig. 3A). The increase went up to maximal level around 5–10 min and returned to baseline ~15 min after E2 treatment (Fig. 3A). CSE expression did not change during this time frame (Fig. 3B). Meanwhile, increased H2S release was found throughout a range of E2 concentrations that fall within the physiological range (n = 3, P < 0.01) (Fig. 3C).

When HUVECs were alternatively exposed to E2 (10 nM) or to selective ERα agonist PPT (10 nM) or to partially selective ERβ agonist DPN (10 nM), enhanced H2S release was found only with E2 (n = 3, P = 0.006) or with PPT (n = 3, P = 0.009), but not with DPN treatment (n = 3, P > 0.05), implying that ERβ is not implicated in the regulation of endothelial H2S (Fig. 3D). Those effects elicited by E2 or PPT were inhibited by ER pure antagonist ICI 182780 (ICI—100 nM) (n = 3,
$P = 0.016$ when $E_2$ versus $E_2 + ICI; n = 3, P = 0.018$ when $PPT$ versus $PPT + ICI$) (Fig. 3D). In addition, $ICI$ use alone had no effect on $H_2S$ production compared with the control ($n = 3, P > 0.05$).

**E2 regulates endothelial H2S through PKG**

PKG activation was reported to be associated with increased $H_2S$ generation (Salloum et al., 2009). Therefore, we investigated the role of PKG in $E_2$-stimulated endothelial $H_2S$ release. As shown in Fig. 4A, PKG inhibitor KT5823 (KT, 10 $\mu$M) largely inhibited $E_2$-enhanced endothelial $H_2S$ release ($n = 3, P = 0.009$ when $E_2$ versus $E_2 + KT$). In addition, KT alone also reduced $H_2S$ release without significant difference compared with the control ($n = 3, P > 0.05$) (Fig. 4A). Moreover, treatment of $E_2$ increased endothelial PKG activity ($n = 3, P = 0.003$ when $E_2$ versus CON), which was abrogated by the addition of ICI ($n = 3, P = 0.005$ when $E_2$ versus $E_2 + ICI$) (Fig. 4B). ICI use alone had no effect on PKG activity when compared with control ($n = 3, P > 0.05$) (Fig. 4B).

To further confirm the role of PKG in $E_2$-induced $H_2S$ release and vasodilation, we obtained aorta from SD rats and measured the wall tension. In our settings, a contraction response to Phe was stable within 1–2 min and would last for more than 1 h (Xiang et al., 2010). $E_2$ (10 nM) relaxed the aorta with Phe at a magnitude of $(72.6 \pm 5.48)\%$ in 15 min (Fig. 4C). In the presence of L-NNA and Indo, $E_2$ (10 nM) still relaxed the aorta at a magnitude of $(33.6 \pm 4.75)\%$ ($n = 4, P = 0.009$ when $E_2$ versus $E_2 + L-NNA + Indo$) (Fig. 4C). This effect was attenuated by the pretreatment of PPG ($n = 4, P = 0.007$ when $E_2 + L-NNA + Indo$ versus $E_2 + L-NNA + Indo + PPG$) or KT ($n = 4, P = 0.009$ when $E_2 + L-NNA + Indo$ versus $E_2 + L-NNA + Indo + KT$) (Fig. 4C).

**Discussion**

The major finding of our present work is the identification of endothelial $H_2S$ as a new target molecule regulated by estrogen. Estrogen rapidly stimulates endothelial $H_2S$ release and results in vasodilation, which may be beneficial to the cardiovascular system, including anti-atherogenesis.

Estrogen prevents the development of atherosclerosis in human and in animal models (Hodis et al., 2001; Torii et al., 2003; Billon-Gales et al., 2009). In agreement, this study indicated that $17\beta$-estradiol ($E_2$) was efficient in reducing atherosclerotic lesions. This could be partially ascribed to the ability of $E_2$ to favorably alter the lipoprotein profile (Bourassa et al., 1996; Rayner et al., 2009). However, the crucial determinants are the direct effects of $E_2$ on vasculature, not the changes in lipids (Mendelsohn and Karas, 2005). For example, our previous work indicated that $E_2$ maintains endothelial morphology and preserves endothelial functions (Kublickiene et al., 2008).
These actions are implemented by a number of molecules (Xing et al., 2009), among which NO is the critical one (Chambliss and Shaul, 2002). Notwithstanding, there was some evidence that E2 was still potent to lower blood pressure and diminish atherosclerotic lesion in ApoE–/– mice, which are deficient in endothelial eNOS (Hodgin et al., 2002). Our work shows that E2 replacement in ApoE–/– mice increases plasma H2S levels and PPG, a potent irreversible inhibitor of CSE (Thompson et al., 1982), largely reduces H2S levels and reverses the inhibitory effect of E2 on atherosclerotic lesion size, indicating that apart from NO, H2S also plays an important role in estrogen’s anti-atherogenesis effect.

Endothelial dysfunction is the early step of atherosclerosis (Vanhoutte et al., 2009). In post-menopausal women, vascular endothelial cells become dysfunctional (Kubiliene et al., 2008), which is characterized by the reduction in the levels of relaxing factors and/or increase in the levels of vasoconstrictors (Vanhoutte et al., 2009). Acute administration of E2 improves endothelium-dependent vasodilation in post-menopausal women (Lima et al., 2005), which is attributed mainly to NO release from the endothelium (Simoncini et al., 2000; Kubiliene et al., 2008). In addition, PGI2 also contributes to E2-induced vasodilatory effect (Sobrino et al., 2010). However, in the presence of L-NNA and Indo, ACh effectively dilated vessels from rats in a concentration-dependent manner and E2 replacement potentiated vasodilatory response to ACh (Burger et al., 2009). These studies concluded that the third mediator, EDHF, was responsible for estrogen-induced vasorelaxation. The present study further showed that the CSE inhibitor PPG largely inhibited E2-induced NO- and PGI2-resistant vasodilation, which provides new evidence to support the premise that H2S is the EDHF that plays an important role in E2-induced vasodilation.

Estrogen exerts biological effects through the interaction with intracellular receptors (ERs, including ERα and ERβ) or membrane estrogen receptors (mERs). The rapid nature of the response of E2-induced H2S release suggests that it is likely to be mediated by extra-nuclear pathways (Burley et al., 2007). Indeed, the existence of mERs in different tissues is well documented (Levin, 2009). Here we found that the selective ERα agonist PPT mimicked estrogen’s effects, while not selective ERβ agonist DPN. It demonstrated that membrane-localized ERα was responsible for E2-regulated H2S release, not ERβ. This is consistent with our previous studies showing that mERα is the critical receptor for estrogen’s non-genomic effects in different types of cells (Simoncini et al., 2006; Zheng et al., 2011).

H2S is produced from L-cysteine, catalyzed by CSE in vasculature (Yang et al., 2008). The enhanced CSE protein expression or CSE activity leads to the increase in H2S release, which is involved in a variety of biological processes, including vasodilation, angiogenesis, and neuroprotection.
of pathophysiological processes of cardiovascular disease, such as atherosclerosis, myocardial ischemia, hypertension and so on (Pan et al., 2012). In this study, CSE protein expression did not alter during the time-course of E2 treatment, indicating that enhanced CSE activity is responsible for elevated H2S levels.

The mechanisms of estrogen’s non-genomic actions depend on the recruitment of kinase cascades via the interaction of mERs with specific domains of kinases, such as c-Src or PI3K (Fu and Simoncini, 2008). In this study, we found that PKG was the key intermediate, based on the fact that E2 activated PKG in short time and PKG inhibitor KT largely inhibited E2-induced H2S release and vasodilation. It should be noted that PKG inhibitor KT may also inhibit other kinases and lead to non-specific effects. To avoid this, small interference RNA against PKG should be used to further confirm the role of PKG in E2 action. PKG is cGMP-dependent protein kinase and there are two types of PKG present in eukaryotic cells: type I and type II (Burley et al., 2007). Activation of PKG phosphorylates many downstream proteins and regulates important cardiovascular functions such as angiogenesis and vasorelaxation (Coletta et al., 2012). Indeed, it was reported that PKG was activated by E2 through non-genomic mechanisms in vascular endothelial cells and smooth muscle cells (White et al., 1995; Keung et al., 2011). However, how PKG activation is associated with increased H2S generation by E2 is not clear from the present study. Although continuing efforts are put into the regulatory mechanisms of CSE protein expression (for example, the role of specificity protein 1 has been defined in this process; Yang et al., 2011), the mechanisms for rapid CSE activation are far from understood. From this point of view, further efforts to analyze and locate the active sites of CSE enzyme are needed.

In conclusion, our findings indicate that endothelial H2S is one target of estrogen and it plays a crucial role in estrogen’s anti-atherogenesis effect. Estrogen rapidly stimulates endothelial H2S release and induces vasodilation through the activation of PKG. Our findings provide original mechanistic insights into estrogen’s cardiovascular effects and hint that the H2S deficiency may be relevant to the pathogenesis progress of cardiovascular diseases in post-menopausal women.

Figure 3 E2 rapidly increases endothelial H2S release. (A) HUVECs were exposed to E2 for different times as indicated and the medium H2S concentrations were measured. Post hoc Tukey’s tests were used for these statistical comparisons. n = 3, ***P < 0.01 versus CON, *P < 0.05 versus CON. (B) HUVECs were exposed to E2 for 0–60 min and cell content of CSE or β-actin was shown by western blot. (C) HUVECs were exposed to different concentrations of E2 for 5 min and the medium H2S concentrations were measured. Post hoc Tukey’s tests were used for these statistical comparisons n = 3, ***P < 0.01 versus CON. (D) HUVECs were exposed to E2 (10 nM), PPT (10 nM) or DPN (10 nM) for 5 min, respectively, in the presence or absence of pure ER antagonist ICI 182780 (ICI—100 nM). Tukey’s tests were used for these statistical comparisons. a is significantly different from b (n = 3, P < 0.05).
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Authors’ roles

All the authors meet the qualification of Molecular Human Reproduction. K.W.Z. and Q.G. designed and carried out the experiments, analyzed the data and drafted and revised the manuscript; S.H.Z., S.N.P., P.L. and K.S. carried out some experiments; S.T. and T.H.W. discussed the project and the results and reviewed the manuscript; X.D.F. raised funds for the project, designed the experiments, analyzed the data and drafted and revised the manuscript.

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Conflict of interest

None declared.

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