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Interaction among estrogen, IGF-1, and H₂S on smooth muscle cell proliferation

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Abstract

Both estrogen and hydrogen sulfide (H₂S) inhibit the proliferation of vascular smooth muscle cells (SMCs) and development of atherosclerosis. In the absence of endogenous H₂S as occurred in CSE-knockout (KO) mouse, however, estrogen stimulates the proliferation of vascular SMCs. The underlying mechanisms for this seemingly controversial vascular effect of estrogen are unclear. In the present study, we demonstrated that the stimulatory effect of estrogen on the proliferation of CSE-KO SMCs was suppressed by the inhibitor of insulin-like growth factor-1 receptor (IGF-1R) or knockdown of IGF-1R protein expression. Estrogen downregulated the expression of insulin-like growth factor-1 (IGF-1) and IGF-1R in aortic tissues or aortic SMCs isolated from WT and CSE-KO mice. Furthermore, endogenous H₂S downregulated IGF-1R, but upregulated estrogen receptor (ER)- α , in aortic tissues or SMCs. ER- α and IGF-1R were co-located in SMCs and co-immunoprecipitated, which was decreased by H₂S. Finally, both endogenous and exogenous H₂S induced the S-sulfhydration of IGF-1R, but not ER- α , in WT-SMCs and CSE-KO SMCs, which underlies the decreased formation of IGF-1R/ER- α hybrid in the presence of H₂S. Thus, the absence of H₂S favors the interaction of estrogen with IGF-1R/ER- α hybrid to stimulate SMCs proliferation. The appreciation of a critical role of H₂S in preventing estrogen-induced SMCs proliferation will help better understand the regulation of complex vascular effects of estrogen and sex-related cardiovascular diseases.

Key Words

- ▶ estrogen
- ▶ hydrogen sulfide
- ▶ insulin like growth factor-1 receptor
- ▶ smooth muscle cells

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Introduction

Cardiovascular beneficiary effects of estrogen have been widely reported in human studies, such as the reduced incidence of cardiovascular diseases in premenopausal women (Reslan & Khalil 2012, Yang *et al.* 2013). Studies carried out *in vitro* have shown that estrogen attenuates

the formation of atheroma and neointima. Estrogen plays an anti-atherosclerotic effect *in vivo* through different subtypes of estrogen receptors (ER) by engaging an array of signaling cascades such as cGMP, cAMP, mitogen-activated protein kinase (MAPK), phosphatidyl inositol

3-kinase (PI3K)/Akt and protein phosphatase pathways (Katzenellenbogen 1996, Nilsson *et al.* 2001, Harrington *et al.* 2003, Chakrabarti *et al.* 2014, Matthews & Gustafsson 2003). Animal studies have shown the inhibitory effect of estrogen on smooth muscle cell (SMC) proliferation via the activation of ER- α , not ER- β or G protein-coupled estrogen receptor 1 (Li *et al.* 2012).

The adverse effects of estrogen hormone replacement therapy (HRT) on certain populations of post-menopausal women have also been documented (Wells & Herrington 1999). For example, for women who started HRT 'more than 10 or 20 years from menopause onset or are aged 60 years or older, the benefit-risk ratio appears less favorable because of the greater absolute risks of coronary heart disease, stroke, venous thromboembolism, and dementia' (North American Menopause Society 2017). Li *et al.* reported that estrogen stimulated, rather than inhibited, SMC proliferation in the absence of endogenous hydrogen sulfide (H₂S) (Li *et al.* 2012). The protective effect of estrogen/ER- α against atherosclerosis also relies on a functional cystathionine gamma-lyase (CSE)/H₂S system (Li *et al.* 2017). It appears that endogenous H₂S level holds key for determining whether estrogen protects or disturbs cardiovascular homeostasis. This hypothesis has never been tested before.

H₂S is one of gasotransmitters that are produced in mammalian cells and perform important physiological functions (Wang 2003, 2011, 2014). H₂S is synthesized endogenously with cystathionine beta-synthase (CBS), CSE, and 3-mercaptopyruvate sulfurtransferase (3-MST) as the catalyzing enzymes and L-cysteine or homocysteine as the substrates (Wang 2003, 2011, Zhao *et al.* 2001). H₂S regulates the processes of anti-atherosclerosis, anti-inflammation, anti-oxidant, vasodilatation, and protection of ischemia injury (Yang *et al.* 2008). We have demonstrated previously that H₂S, either endogenously produced by CSE in vascular tissues or exogenously applied, induces apoptosis of human aortic SMCs (Yang *et al.* 2004).

The present study specifically investigated the inhibitory effect of H₂S on estrogen-induced stimulation of SMC proliferation as well as the underlying mechanisms.

Materials and methods

Materials

IGF-1R inhibitor (picropodophyllin, PPP) was from Santa Cruz Biotechnology. Anti-IGF-1R antibody and anti-p-IGF-1R was obtained from Cell Signaling Technology.

Anti-IGF-1 antibody was purchased from Novus Biologicals (Littleton, CO). Anti-ER- α antibody and okadaic acid were from Abcam Biotechnology company. Estradiol EIA kit was from Cayman Chemical Company. The optimal cutting temperature compound (OCT) was from Ager Scientific (Stansted, UK). The goat anti-mouse IgG Alexa Fluor Plus 488 and the donkey anti-rabbit IgG (H+L) Alexa Fluor 594 were from Fisher Scientific (Hampton, NH). The plasmids of control single-guide RNA (sgRNA), IGF-1R sgRNA, and Cas9 were obtained from GeneCopoeia (Rockville, MD). All other chemicals were from Sigma or New England Biolabs (Camarillo, CA).

Animal experiment

WT mice were maintained on C57BL/6J $\times$ 129SvEv background. In-house-bred homozygous CSE-KO mice were generated after backcross of at least ten generations of heterozygous of CSE-knockout mice with WT mice with the same genetic background (Yang *et al.* 2008). All WT and CSE-KO mice were verified by genotyping and used in this study. Each group of WT and their matching CSE-KO mice were from the same generations. Aortic tissues were isolated from 12-weeks-old male and female WT and age-matched CSE-KO mice. For H₂S supplement experiments, the mice received either PBS as the control or NaHS (39 μ mol/kg body weight, i.p.). The animal use protocol was in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Animal Care Committee of Laurentian University, Canada.

Isolation and culture of SMCs

The euthanasia of mice was conducted by CO₂ inhalation, and the aortae were isolated for subsequent experiments. SMCs were isolated from collected mouse descending aortae and cultured as previously described (Berk 2001, Li *et al.* 2017). These SMCs were cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. SMC identity was confirmed by staining with SMC-specific α -actin MAB (Sigma). The morphology of freshly isolated SMCs is spindle shaped and when cultured to confluence these cells exhibited the typical 'hill-and-valley' pattern (Berk 2001, Li *et al.* 2012). The cell culture medium was changed every 2 days. All cells used in the experiments were from passages 6–8.

Cell proliferation assay

The amount of total living cells was assessed by MTT assay as described previously (Li *et al.* 2012, Shuang *et al.* 2018). Equal numbers (2.0×10^3) of SMCs were seeded onto each well of 96-well plates for 24 h. Then the serum-free medium was given for another 12 h. After different treatments, MTT (5 mg/mL) was added to each well, and the cells were incubated for additional 4 h at 37°C. After removing the culture medium, 200 μ L dimethyl sulfoxide (DMSO) was added for 20 min to dissolve insoluble purple formazan which can only be produced by living cells. The FLUOstar OPTIMA microplate spectrophotometer (BMG LABTECH, Offenburg, Germany) was used to measure the absorbance of solubilized formazan products at 570 nm. The amounts of living cells in the absence of any special treatment were considered as control level (100%).

Bromodeoxyuridine (BrdU) incorporation was also used to measure proliferated cells as described previously (Li *et al.* 2012, Shuang *et al.* 2018). SMCs were plated at equal number (2.0×10^3 per well) in 96-well plates and incubated for 24 h. The serum-free medium was applied for another 12 h. Afterwards, 10% FBS was added together with NaHS (100 μ M), PPP (0.05 μ M) or 17 β -estradiol (E2) (1–300 nM) for 48 h incubation. BrdU label was added to each well for additional 24 h. After the fixative/denaturing solutions were added and incubated for 1 h to separate DNA into single strands so that the primary antibody has access to the incorporated BrdU. Then anti-BrdU antibody and peroxidase goat anti-mouse IgG HRP conjugate were pipetted to each well and incubated for 24 h. With the supplement of the substrate solution, incorporated BrdU was detected as instructed by the manufacturer (Calbiochem). FLUOstar OPTIMA microplate spectrophotometer (BMG LABTECH, Offenburg, Germany) was used to measure the absorbance of newly generated DNA at 595 nm. Each measurement was composed of six replications and all experiments were performed at least four times.

E2 measurement

The level of 17 β -estradiol (E2) in aorta was detected by Estradiol EIA kit from Cayman Chemical Company. Aortic tissue samples (50 μ L) from each collection was added to 96-well plate. Each sample was assayed in duplicate. FLUOstar OPTIMA microplate spectrophotometer (BMG LABTECH, Offenburg, Germany) was used to measure the absorbance at 420 nm. All experiments were performed at least four times.

Gene silencing with single guide RNA (sgRNA)

IGF-1R knock down was conducted using clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (Cas9) system as described previously (Bourque *et al.* 2018, Shuang *et al.* 2018). IGF-1R sgRNA contains a 20-base sequence specifically complemented to the 5' non-variable scaffold sequence of IGF-1R DNA. A non-targeting RNA sequences that do not recognize any sequence in genome and provide a non-edited control of edited cells was applied as the control sgRNA. The plasmids of control sgRNA and IGF-1R sgRNA were constructed by Genecopoeia (Rockville, MD). SMCs were plated and grown to 80% confluence for plasmid transfection following the protocol of Invitrogen. SMCs were seeded in 10 cm² dishes with the medium containing 10% FBS and transfected for 24 h with both CRISPR/Cas9 plasmid and IGF-1R-specific sgRNA plasmid together with lipofectamine 2000 for IGF-1R gene knockdown. The cells transfected with Cas9/control sgRNA plasmids were used as control.

Protein detection

Western blotting was performed on aortic tissues and cultured SMCs as described before (Li *et al.* 2012, Shuang *et al.* 2018). The dilutions of primary antibodies were 1:1000 for ER- α , 1:1000 for IGF-1R, and 1:5000 for β -actin.

For ER- α and IGF-1R double immunofluorescence staining, freshly isolated aortae were embedded in OCT compound (Ager Scientific) and cut to cryostat sections (5–10 μ m). For staining of cultured SMCs, the cells were plated on glass coverslips in 6-well plates and incubated at 37°C for 48 h. The coverslips were rinsed with PBS and fixed for 10 min with 3.7% formaldehyde and 0.1% Triton X-100 in PBS. Fixed aorta sections or SMCs were incubated in blocking buffer (1% BSA in Tween/Tris-buffered saline) for 30 min at room temperature. Aorta sections or SMCs were then incubated with anti-IGF-1R antibody (rabbit) and anti-ER- α antibody (mouse) in blocking buffer for 1 h at room temperature. After washing, the coverslips were incubated with rhodamine conjugate anti-mouse and Fluorescein isothiocyanate (FITC) conjugate anti-rabbit secondary antibodies for 1 h. Visualization was done by IX71 Inverted Microscope (Olympus) and X-Cite 120Q fluorescence illumination system (Excelitas Technologies). All images were processed by DP2-BSW Version 2.1q.

Protein phosphatase 2A (PP2A) and IGF-1R activity

PP2A and IGF-1R activities in whole cell lysates were determined by serine/threonine phosphatase assay

according to manufacturer's protocol (EMD Millipore). Briefly, total proteins (200 µg) from homogenized SMC lysates were incubated with 2 µg of anti-PP2Ac antibody or anti-pIGF-1R antibody at 4°C for 2 h, respectively, supplemented with 25 µL protein A agarose. The protein-bead complexes were precipitated by centrifugation at 600 g for 1 min, washed at least three times with PBS and the last time with Ser/Thr assay buffer. Then the beads were incubated with diluted phosphopeptide for 10 min at 30°C. After centrifugation, supernatant was incubated with Malachite Green Phosphate Detection Solution. Accumulation of dephosphorylated substrate was measured in the supernatant using FLUOstar OPTIMA microplate spectrophotometer (BMG LABTECH, Offenbourg, Germany) at 650 nm wavelength.

Measurement of H₂S production

H₂S level in SMCs was measured by lead sulfur method as described previously (Hine *et al.* 2015, Li *et al.* 2017). Cell homogenate of 100 mg was mixed with PBS buffer supplemented with 10 mM cysteine. A lead acetate H₂S detection paper (Sigma) was placed above 96-well plate containing the mixture and incubated at 37°C for 2 h, and then lead sulfur paper was scanned with Canon scanner (CanoScan LiDE 700F).

Co-immunoprecipitation

Protein co-immunoprecipitation was detected as described previously (Shuang *et al.* 2018). Total proteins (300 µg) from each collection (homogenized aortic tissues or cultured SMC lysates) were incubated for 12 h at 4°C with 3 µg anti-ER-α (rabbit polyclonal) antibody. Same amount of rabbit IgG was used as a negative control of co-immunoprecipitation to determine whether non-specific proteins were pulled down. Then 40 µL G-Sepharose beads were added into each protein sample. Beads were washed several times with PBS before being used. The proteins were pulled down by anti-ER-α antibody and then precipitated by protein G-Sepharose beads at 4°C for 3 h under gentle rotation. The protein-bead complexes were precipitated by centrifugation at 600 g for 1 min, washed at least five times with PBS and mixed with 2 × SDS-PAGE loading buffer. After boiled for 5 min, immunoprecipitated samples were resolved on SDS-PAGE and subjected to Western blot analysis with anti-IGF-1R antibody.

Proximity ligation assay

The mouse/rabbit red Duolink® kit (Sigma) was used for this experiment. SMCs cultured on slides were fixed and permeabilized as aforementioned for immunofluorescence studies. The slides were incubated with blocking buffer (provided in the kit) for 1 h in room temperature followed by further incubation with primary antibodies overnight at 4°C. After three times of buffer (supplied with the kit) washing (15 min for each), ligation reaction was carried out at 37°C for 1 h in a humid chamber followed by buffer washing again. At last, the cells were incubated with amplification mix for 100 min at 37°C in a darkened humidified chamber. After washing, the slides were examined under IX71 Inverted Microscope (Olympus) with an X-Cite 120Q fluorescence illumination system (Excelitas Technologies). All images were processed by DP2-BSW Version 2.1q.

S-sulphydration assay (biotin switch assay)

Protein S-sulphydration was detected as described previously (Untereiner *et al.* 2011, 2016, Altaany *et al.* 2014, Shuang *et al.* 2018). Briefly, aortic tissues or cultured SMCs were homogenized in HEN buffer (250 mM Hepes-NaOH (pH 7.7), 1 mM EDTA and 0.1 mM neocuproine) supplemented with 100 µM deferoxamine and 1% proteinase inhibitor cocktail, then centrifuged at 14,000 g for 15 min at 4°C. All samples were blocked with HEN buffer adjusted to 2.5% SDS and 20 mM S-methyl methanethiosulfonate (MMTS) at 50°C for 20 min with frequent and gentle vortexing to avoid foaming. After addition of four volumes of acetone, proteins were precipitated at -20°C for 1 h. Next, acetone was removed after 10 min centrifugation (2000 g) and protein pellets were re-suspended with HENS buffer (HEN buffer supplemented with 1% SDS and 30 mM biotin-HPDP) for 3 h incubation at room temperature. Biotinylated proteins were then precipitated by streptavidin-agarose beads and washed with HEN buffer and PBS. Biotinylated proteins were eluted by SDS-PAGE gel and subjected to Western blot analysis using anti-IGF-1R and anti-ER-α antibodies, respectively.

Statistical analysis

All data were expressed as mean ± s.e. and represented at least four independent experiments. All statistical analyses were performed with SPSS, version 25.0 (IBM Corp) for Windows. Significance level was set at $P < 0.05$.

Results

Effects of estrogen on the proliferation of SMCs

E2 is the major form of estrogens in mammals and responsible for the most physiological effects of estrogens (Nilsson *et al.* 2001). As reported in our previous study (Li *et al.* 2012), E2 treatment of cultured WT-SMCs and CSE-KO SMCs yielded opposite effects on cell proliferation. E2 treatment significantly decreased total number of WT-SMCs but increased that of CSE-KO SMCs from both male and female mice (Fig. 1A and B). A PP2A specific inhibitor, okadaic acid (2 nM), blocked the inhibitory effect of E2 on WT-SMCs but did not affect the stimulatory effect on CSE-KO SMCs (Fig. 1C and D). PPP (0.05 μ M) is a specific inhibitor of IGF-1R

tyrosine phosphorylation. PPP completely abolished the stimulatory effect of E2 on CSE-KO SMCs (Fig. 1D) but had no effect on the inhibitory effect of E2 on WT-SMCs (Fig. 1C). Another IGF-1 analog, JB1, had also no effect on WT-SMCs but inhibited the stimulatory effect of E2 on KO-SMCs. After NaHS treatment (100–300 μ M) for 2 h, the stimulatory effect of E2 on the proliferation of CSE-KO SMCs was significantly abolished (Fig. 1E) ($P < 0.05$). Our previous study had shown that NaHS treatment potentiated the inhibitory effect of E2 on the growth of WT-SMCs (Li *et al.* 2012).

E2 concentration in aortic tissues was significantly higher in WT female mice (1.98 ± 0.34 pg/mg) than in WT male mice (1.00 ± 0.25 pg/mg) (Fig. 1F). Aortic tissues from male CSE-KO mice had the lowest E2 level

Figure 1

Stimulatory and inhibitory effects of E2 on the survival and proliferation of SMCs. SMCs isolated from aortae of WT and CSE-KO mice were treated with different concentrations of E2 for 72 h. (A) MTT assay of total numbers of viable CSE-KO SMCs and WT-SMCs. (B) BrdU assay of proliferated CSE-KO SMCs and WT-SMCs. (C) Changes in total numbers of viable WT-SMCs and CSE-KO SMCs under different conditions. (D) Changes in BrdU incorporation in WT-SMCs and CSE-KO-SMCs under different conditions. SMCs from male mice in (C and D) were treated with 0.05 μ M PPP, 2 nM okadaic acid, 0.25 μ g/mL JB1, and 100 nM E2 for 72 h. The numbers of viable cells without any treatment were considered as baseline control of 100%. $n = 4$ samples for each group. Comparison was made with Mann-Whitney test. (E) NaHS abolished the stimulatory effect of E2 on CSE-KO SMCs. $n = 4$ samples for each group. Comparison was made with Mann-Whitney test. (F) E2 levels in WT and CSE-KO aortic tissues. $n = 6$ samples. Comparison was made with Student's *t*-test. For (A and B), * $P < 0.05$ vs WT Ctrl; # $P < 0.05$ vs CSE-KO Ctrl. For (C, D and E), * $P < 0.05$ vs Ctrl; # $P < 0.05$ vs estrogen treatment only group. For (F), * $P < 0.05$ vs WT male group; # $P < 0.05$ vs WT female group. Ctrl, control group; E2, 17 β -estradiol; KO, CSE-knock out; N.S., not significant ($P > 0.05$); OA, okadaic acid; PPP, picropodophyllin; SMCs, smooth muscle cell. Each sample is a collection from six mice in (A, B, C, D, E and F).

(0.27 ± 0.05 pg/mg). In all subsequent experiments to test the effects of exogenous E2, only WT-SMCs and CSE-KO SMCs from male animals were used in order to minimize the impact of pre-existing endogenous E2.

Effects of estrogen on ER- α and PP2A in aortae and SMCs

ER- α protein level in aortic tissues was significantly lower in male and female CSE-KO than in WT mice but not different between male WT and female WT mice (Fig. 2A). PP2A protein levels were the same in all groups (Fig. 2A). Treatment of WT-SMCs and CSE-KO SMCs with 100 nM E2 for 72 h did not change ER- α and PP2A protein levels in

both WT-SMCs and CSE-KO SMCs (Fig. 2B). E2 treatment, however, increased the activity of PP2A both in WT-SMCs and CSE-KO SMCs (Fig. 2C). This stimulatory effect was significantly higher in WT-SMCs than in CSE-KO SMCs (Fig. 2C).

Effects of estrogen and H₂S on IGF-1R and IGF-1 in aortae and SMCs

IGF-1R protein level in aortic tissues was significantly lower in male WT than in male CSE-KO mice but not different between female WT and female CSE-KO mice (Fig. 2A). IGF-1R protein expression was also lower in female mice aortae than male littermates in both WT and

Figure 2

Estrogen-regulated expressions of IGF-1R and IGF-1 proteins in mouse aortae and aortic SMCs. (A) Comparison of the expression levels of selective proteins between aortic tissues from WT and CSE-KO mice. Each sample was composed of aortic tissues from at least five different mice. (B) Comparison of the expression levels of selective proteins between WT-SMCs and CSE-KO SMCs. SMCs were treated with/without E2 (100 nM) for 24 h. (C) Comparison of PP2A activities between WT-SMCs and CSE-KO SMCs after E2 treatment. (D) Comparison of pIGF-1R activities between WT-SMCs and CSE-KO SMCs after E2 treatment. * $P < 0.05$ vs WT male group in (A) and WT Ctrl in (B) or the groups without E2 treatment in (C and D). # $P < 0.05$ vs KO male group in (A) and KO Ctrl in (B) or all other groups in (C and D). & $P < 0.05$ vs WT female group in (A) and WT with estrogen treatment group in (B, C and D). Ctrl, control group. KO, CSE-KO mice. $n = 4$ samples and each sample was a collection from four to six mice in (A), four dishes of SMCs in (B) or in four wells of SMCs in (C and D). All data were compared using the Mann-Whitney test.

CSE-KO groups (Fig. 2A). These results indicate that H₂S and estrogen downregulate IGF-1R protein expression. The downregulatory effect of E2 on IGF-1R is independent of endogenous H₂S levels, but there is no direct proof that endogenous H₂S works independently to diminish IGF-1R expression due to the potential presence of endogenous E2 in these cells (Fig. 2B). IGF-1 protein level was lower in female than in male WT and CSE-KO mice (Fig. 2A), but there was no difference between WT and CSE-KO mice with the same sex.

The protein levels of IGF-1R, but not IGF-1, were significantly higher in CSE-KO SMCs than WT-SMCs (Fig. 2B). E2 treatment of WT-SMCs and CSE-KO SMCs for

72 h significantly downregulated the expression of IGF-1R as well as IGF-1 proteins in both WT-SMCs and CSE-KO SMCs (Fig. 2B).

The activity of pIGF-1R of WT-SMCs under basal conditions, considered as 100% level, was not significantly different from that of CSE-KO SMCs. After the treatment with E2 (100 nM) for 2 h, the activity of pIGF-1R was significantly increased to $412.8 \pm 52.2\%$ in CSE-KO SMCs ($n = 4$, $P < 0.05$) and $198.2 \pm 32.7\%$ in WT-SMCs ($n = 4$, $P < 0.05$) (Fig. 2D).

IGF-1R-specific sgRNA significantly knocked down IGF-1R protein expression in CSE-KO SMCs and WT-SMCs by $61.7 \pm 1.04\%$ and $62.7 \pm 2.58\%$, respectively (Fig. 3A).

Figure 3

Knocking down of IGF-1R expression reversed the proliferative effect of estrogen on CSE-KO SMCs. (A) Comparison of IGF-1R and IGF-1 protein levels between WT-SMCs and CSE-KO SMCs after Cas9/sgRNA transfection. $n = 4$ samples for each group and each sample was a collection from four dishes of SMCs. The comparison was made using Mann-Whitney test. (B) E2 levels in WT-SMCs and CSE-KO SMCs after Cas9/sgRNA transfection. $n = 6$ samples for each group and each sample was a collection from four dishes of SMCs. The comparison was made using Student's t-test. (C) Comparison of PP2A activities between WT-SMCs and CSE-KO SMCs after Cas9/sgRNA transfection with/without E2 treatment (100 nM). $n = 4$ samples for each group and each sample was a collection from four dishes of SMCs. The comparison was made using Mann-Whitney test. (D) Comparison of pIGF-1R activities between WT-SMCs and CSE-KO SMCs after Cas9/sgRNA transfection with/without E2 treatment (100 nM). $n = 4$ samples for each group and each sample was a collection from four dishes of SMCs. The comparison was made using Mann-Whitney test. (E) MTT assay of total numbers of viable CSE-KO SMCs and WT-SMCs. $n = 4$ samples for each group and each sample was a collection from six different wells of SMCs. The comparison was made using Mann-Whitney test. (F) BrdU incorporation assay of proliferated CSE-KO SMCs and WT-SMCs. $n = 4$ samples for each group and each sample was a collection from six different wells of SMCs. The comparison was made using Mann-Whitney test. For (A and B), * $P < 0.05$ vs control sgRNA group. For (C and D), * $P < 0.05$ vs all other groups of control sgRNA; # $P < 0.05$ vs all other groups of IGF-1R sgRNA; & $P < 0.05$ vs control sgRNA + E2 group. For (E and F), * $P < 0.05$ between two groups at the same E2 concentrations. N.S., not significant ($P > 0.05$).

This CRISPR knockdown efficiency is similar to the reported 79% knockdown of BRCA1 proteins in adipose stem cells (Zhao *et al.* 2019) or 70% knockdown of progranulin mRNA (Tian *et al.* 2019). IGF-1R knockout had no effects on IGF-1 protein expression (Fig. 3A), E2 levels (Fig. 3B) or PP2A activity (Fig. 3C). E2 (100 nM)-induced increase in pIGF-1R activities was abolished in WT-SMCs and significantly decreased in CSE-KO-SMCs after partially knock-down of IGF-1R protein expression (Fig. 3D). The stimulatory effects of E2 on the survival (Fig. 3E) and proliferation (Fig. 3F) of CSE-KO SMCs were reversed after partially knocking down IGF-1R, suggesting that the stimulatory effects of E2 on cell proliferation were largely mediated by IGF-1R. On the other hand, IGF-1R knockdown did not impact on the inhibitory effect of E2 on the survival (Fig. 3E) and proliferation (Fig. 3F) of WT-SMCs.

Co-localization and interaction of IGF-1R with ER- α

Using double immunofluorescence staining with anti-IGF-1R and anti-ER- α antibodies, we explored co-location

of IGF-1R and ER- α in cultured SMCs and aortic tissues. Since we did not count the numbers of positively stained cells in each cell population and immunofluorescence detection is not a quantitative technique, the fluorescence intensities were not quantitatively compared between cell populations. Both WT-SMCs and CSE-KO-SMCs were labeled with immunofluorescence of IGF-1R (red) and ER- α (green) (Fig. 4A). The images of IGF-1R and ER- α were merged or co-localized well in both WT-SMCs and KO-SMCs. Similarly, aortic tissues from WT and CSE-KO mice were both stained with IGF-1R and ER- α immunofluorescence (Fig. 4B). The merged images of IGF-1R and ER- α as well as the nuclear marker DAPI appeared to localize in all layers of aortic tissues from WT and KO mice, including smooth muscles, endothelium and adventitia. Proximity ligation of IGF-1R and ER- α , mostly in cell nuclei, was stronger in CSE-KO SMCs than in WT-SMCs (Fig. 4C). We further examined physical interaction between IGF-1R and ER- α . Co-immunoprecipitation results showed that IGF-1R proteins were pulled down by anti-ER- α antibody (Fig. 5A, B, D and E) and ER- α proteins were pulled

Figure 4

Co-localization of IGF-1R with ER- α . (A) Double immunofluorescence staining was conducted on WT-SMCs and CSE-KO SMCs. IGF-1R was detected by rhodamine red fluorescence and ER- α by FITC green fluorescence. These two images were overlapped (merged) to show the relative location of IGF-1R and ER- α . (B) Double immunofluorescence staining was conducted on aortic tissues from male WT and CSE-KO mice. IGF-1R was detected by rhodamine red fluorescence and ER- α by FITC green fluorescence. The nuclei were detected by DAPI. These images were overlapped (merged) to show the relative location of IGF-1R and ER- α . (C) Immunofluorescence staining was conducted on WT-SMCs and CSE-KO SMCs with proximity ligation assay. The nuclei were marked by DAPI. These images were overlapped (merged) to show the ligation of IGF-1R and ER- α . The white scale bars are 20 μ m in length.

Figure 5

H₂S inhibits the formation of IGF-1R/ER- α hybrid. (A) Co-immunoprecipitation of IGF-1R and ER- α with anti-ER- α to pull down the lysate. * $P < 0.05$ vs WT male mice, # $P < 0.05$ vs CSE-KO male mice. (B) Co-immunoprecipitation of IGF-1R and ER- α was significantly decreased in CSE-KO aortae after the intraperitoneal injection of NaHS (39 μ mol/kg). PBS injection at the same volume was used as control. # $P < 0.05$. (C) Co-immunoprecipitation of IGF-1R and ER- α with anti-IGF-1R to pull down the lysate. CSE-KO SMCs were transfected with Cas9 and IGF-1R specific sgRNA or Cas9 and control sgRNA for 24 h. # $P < 0.05$. In both WT-SMCs (D) and CSE-KO SMCs (E), co-immunoprecipitation of IGF-1R and ER- α was decreased after 2 h NaHS treatments. * $P < 0.05$ vs control group (0 NaHS). Rabbit IgG was included as negative control to co-immunoprecipitation. $n = 4$ samples and each sample was a collection of aortic tissues from three to six mice in (A and B) or four plates of SMCs (C, D and E). All data were compared using Mann-Whitney test.

down by anti-IGF-1R antibody (Fig. 5C). The levels of co-immunoprecipitated IGF-1R proteins were significantly lower in aortic tissues from WT mice than from CSE-KO mice in both male and female mice, suggesting that endogenous H₂S inhibited the formation of IGF-1R/ER- α hybrid (Fig. 5A). After intraperitoneal injection of NaHS in CSE-KO mice, the levels of co-immunoprecipitated IGF-1R proteins in aorta were significantly decreased (Fig. 5B). In the absence of endogenous H₂S in CSE-KO mice, significant levels of IGF-1R/ER- α hybrid were detected after intraperitoneal injection of PBS as control. After intraperitoneal injection of NaHS in these CSE-KO mice, the levels of co-immunoprecipitated IGF-1R proteins in aorta were significantly decreased (Fig. 5B), indicating that added exogenous H₂S inhibited the formation of IGF-1R/ER- α hybrid. Knocking down IGF-1R expression

with IGF-1R sgRNA also lowered the level of IGF-1R/ER- α hybrid (Fig. 5C), confirming that the availability of IGF-1R protein affects the formation of IGF-1R/ER- α hybrid. On the other hand, NaHS at 100 μ M and 300 μ M significantly inhibited IGF-1R/ER- α hybrid formation in both WT-SMCs (Fig. 5D) and CSE-KO SMCs (Fig. 5E).

S-sulphydration of IGF-1R and its impact on the formation of IGF-1R/ER- α hybrid

The C domain of human ER- α protein, which mediates DNA binding, is rich of cysteine. Human IGF-1R also has a cysteine-rich domain between residues 223 and 274 in its ligand binding site (Kavran *et al.* 2014, Cai *et al.* 2017). Basal level of S-sulphydrated IGF-1R was significantly higher in aortic tissues from WT mice than from CSE-KO

mice, indicating that endogenous H₂S generated by CSE induced the S-sulphydration of IGF-1R (Fig. 6A). Since there was no difference in S-sulphydrated IGF-1R levels between male and female mice (Fig. 6A), estrogen seems not playing a role in mediating IGF-1R S-Sulphydration. NaHS at 100 and 300 μ M increased the S-sulphydration of IGF-1R in CSE-KO SMCs (Fig. 6B) as well as in WT-SMCs (Fig. 6C). On the other hand, ER- α was not S-sulphydrated in both WT-SMCs and CSE-KO SMCs (Fig. 6D).

Discussion

Increased incidence of atherosclerosis in postmenopausal women can be related to declined estrogen levels (North American Menopause Society 2017). Previous studies have shown that anti-atherosclerotic effect of

estrogen is mediated by CSE-generated H₂S *in vitro* and *in vivo* (Li *et al.* 2012, 2017). E2 treatment (80 μ g/kg/day) attenuated hypercholesterolemia, oxidative stress, ICAM-1 expression, and NF- κ B in atherogenic diet-fed WT mice but not in atherogenic diet-fed CSE-KO mice (Li *et al.* 2017). There was more SMC proliferation in aortic atherosclerotic lesions in CSE-KO female mice than in WT female mice (Li *et al.* 2017). E2 treatment significantly inhibited the proliferation of WT-SMCs, but stimulated that of CSE-KO SMCs (Li *et al.* 2012). It appears that, in the absence of H₂S, the inhibitory effect of estrogen on SMC proliferation was reversed to a stimulatory action (Li *et al.* 2012). H₂S mediated estrogen-inhibited proliferation of SMCs through selective activation of ER- α , but not ER- β , and locked cell cycle in G1 phase by downregulating cyclin D1 pathways (Li *et al.* 2014). On the other hand, the underlying mechanism for estrogen-stimulated SMC proliferation in the absence of H₂S has been unclear. This understanding is crucial for devising strategies to minimize adverse cardiovascular effects of estrogen as shown with estrogen HRT but maximize beneficiary effects of estrogen (Yang *et al.* 2013).

In the present research, we found that E2 treatment for 72 h significantly decreased total numbers and proliferations of WT-SMCs from both male and female mice (Fig. 1A and B). This same E2 treatment at different concentrations, however, increased total numbers and the proliferation of CSE-KO SMCs by 10–40% over that of non-treated SMCs from male and female CSE-KO mice (Fig. 1A and B). Estrogen activates two distinct signaling pathways. It binds to its cognate receptors and subsequently translocates to the nucleus and transcriptionally regulates the relevant gene expression (Mendelsohn & Karas 2005). Estrogen also directly enhances PP2A activity (Ueda *et al.* 2013). Although protein levels of PP2A were not different between WT-SMCs and CSE-KO SMCs (Fig. 2A and B), E2 treatment for 2 h increased PP2A activity in both WT-SMCs and CSE-KO SMCs. This stimulatory effect, however, was significantly lower in CSE-KO SMCs than in WT-SMCs (Fig. 2C). Okadaic acid, a specific PP2A inhibitor, suppressed the inhibitory effect of E2 on the proliferation of WT-SMCs but not its pro-proliferative effect on CSE-KO SMCs (Fig. 1C and D). These results demonstrated that the stimulatory effect of estrogen on the proliferation of CSE-KO SMCs is not mediated by PP2A.

The involvement of IGF-1R in E2-induced stimulation of SMC proliferation was examined. IGF-1 binds to the ectodomain of IGF-1R, inducing tyrosine phosphorylation of the activation loop and juxta-membrane region and thus phosphorylating IRS-1 and AKT (Delafontaine *et al.* 2004,

Figure 6

S-sulphydration of IGF-1R, but not ER- α , in aortic tissues and SMCs. (A) IGF-1R was S-sulphydrated in both WT and CSE-KO aortae with a greater extent in the former. (B and C) IGF-1R was S-sulphydrated by NaHS in cultured CSE-KO SMCs and WT SMCs. (D) ER- α was not S-sulphydrated in WT-SMCs and CSE-KO SMCs. Aortic tissue (2 mg) or SMC lysates (5 μ g/ μ L) were used with Biotin Switch assay. The S-sulphydrated IGF-1R protein level was normalized by the load quantity of total IGF-1R proteins. * $P < 0.05$ vs WT groups; # $P < 0.05$ vs control group (0 NaHS). $n = 4$ samples for each data groups and each sample is a collection from four to six mice in A and from four to five plates of SMCs in (B, C and D). All data were compared using Mann-Whitney test.

Kavran *et al.* 2014). Different from estrogen, IGF-1 stimulates proliferation and differentiation and protects from apoptosis of murine SMCs (Rubin & Baserga 1995, Shuang *et al.* 2018). Estrogen downregulates IGF-1 and IGF-1R mRNA and protein expression in rat vascular SMCs (Delafontaine *et al.* 2004) but upregulates the expression of IGF-1 and IGF-1R in ovarian granulosa cells and breast cancer cells (Stewart *et al.* 1990, Umayahara *et al.* 1994). We demonstrated previously that endogenous H₂S downregulated the expression of IGF-1R proteins but not IGF-1, in murine SMCs (Shuang *et al.* 2018). In the present study, we found that the levels of IGF-1 and IGF-1R proteins in aortae were higher in male mice than their female counterparts (Fig. 2A). IGF-1R protein level, but not that of IGF-1, was lower in WT than in CSE-KO littermates of the same sex.

PPP inhibits the autophosphorylation of IGF-1R (Razuvaev *et al.* 2007). As shown in Fig. 1C and D, PPP (0.05 μ M) alone did not change the viability and proliferation of WT-SMCs and CSE-KO SMCs. The inhibitory effects of E2 on the viability and proliferation of WT-SMCs were not altered by PPP treatment, indicating that the inhibitory effect of E2 does not involve IGF-1R activation, but likely to be mediated entirely by ER- α (Fig. 1C and D). However, PPP blocked the stimulatory effect of E2 on the viability and proliferation of CSE-KO SMCs (Fig. 1C and D). JB1, an IGF-1 peptide analog that potently inhibits the autophosphorylation of IGF-1R, also attenuated E2-increased viability and proliferation of CSE-KO SMCs but did not alter the effects of E2 on WT-SMCs (Fig. 1C and D). It is thus reasoned that the pro-proliferative effect of E2 on CSE-KO SMCs is mediated by the activation of IGF-1R and this effect is inhibited by endogenous H₂S.

Cellular biological processes are regulated by crosstalk among multiple signaling pathways. IGF-1, acting mainly on IGF-1R, induces activation and phosphorylation of ER in uterine cells (Aronica & Katzenellenbogen 1993). The interaction between IGF-1R and ER results in synergistic growth stimulation of HepG2 cells and BG-1 cells transfected with the vitA2-sv40-ERE reporter construct (Umayahara *et al.* 1994, Smith 1998). Co-administration of E2 and IGF-1 increases target gene expression to an extent greater than either agent alone (Ignar-Trowbridge *et al.* 1996). We previously demonstrated that E2 inhibited the proliferation of WT-SMCs, which was entirely mediated by ER- α (Li *et al.* 2012). Knocking down of ER- α has also been reported to abolish the pro-proliferative effect of IGF-1 on prostatic SMCs (Zhou *et al.* 2011). E2-stimulated proliferation of cultured bovine satellite cells was inhibited by JB1 (Kamanga-Sollo 2008). In the absence of IGF-1,

IGF-1R was reported to be activated by E2 (Quesada *et al.* 2007). These previous reports indicate the co-presence of ER- α and IGF-1R in different cell types and that their interaction determines whether cell proliferation would be increased or inhibited.

Endogenous H₂S regulates the proliferation of vascular SMCs. We previously showed that over-produced H₂S derived from CSE overexpression decreased cell growth and CSE deficiency significantly increased the growth of cultured mouse SMCs (Yang *et al.* 2010). We also reported a pro-apoptotic effect of endogenous H₂S on human aortic SMCs (Yang *et al.* 2006). In the present study, we found that the total number of living CSE-KO SMCs was increased after 72 h treatment with E2 and this stimulatory effect of E2 was reversed after knocking down of IGF-1R expression by CRISPR/Cas9-sgRNA (Fig. 3E). Knocking-down IGF-1R in SMCs did not change E2 concentration in SMCs (Fig. 3B) and PP2A activity (Fig. 3C) but decreased E2-increased pIGF-1R activity significantly (Fig. 3D). Increased number of viable cells could result from decreased cell apoptosis or increased cell proliferation or both. Our BrdU assay showed that E2 treatments increased the proliferation of CSE-KO SMCs and this increase, too, was reversed after knocking down IGF-1R expression (Fig. 3F). Moreover, knocking down IGF-1R expression in WT-SMCs did not affect the effects of E2 on the viability or the proliferation of these cells (Fig. 3E and F). The comparison of the results of MTT assay and BrdU assay (Fig. 3E and F) suggests that IGF-1R-mediated stimulatory effect of E2 on the total number of viable CSE-KO SMCs is largely attributed to increased SMC proliferation.

IGF-1R can form a hybrid with diverse proteins. Insulin receptor/IGF-1R hybrid receptors in human or rat SMCs are activated by both insulin and IGF-1 (Johansson & Arnqvist 2006, Cai *et al.* 2017). The interaction of ER with IGF-1R has also been reported on neuroprotection, blood brain barrier integrity, and angiogenesis (Sohrabji 2015, Quesada *et al.* 2007). Quesada *et al.* reported that IGF-1R and ERs (ER- α and ER- β) were co-expressed in many neurons and glial cells in the CNS (Quesada *et al.* 2007). Interestingly, Mendez reported that estrogen-activated ER- α can phosphorylate IGF-1R, leading to dimerization of ER- α and IGF-1R (Mendez 2003). This IGF-1R/ER- α hybrid receptor serves as a common substrate for synergistic interactions between E2 and IGF-1 (Rubin & Baserga 1995). In our study, using double immunofluorescence staining and proximity ligation assay we detected co-localization of IGF-1R and ER- α in SMCs, mostly in the nucleus (Fig. 4).

Our co-immunoprecipitation study also showed that IGF-1R was pulled down by anti-ER- α antibody and vice versa, more in CSE-KO SMCs than in WT SMCs (Fig. 5). These results indicate that IGF-1R and ER- α form a hybrid in SMCs and its formation is favored in the absence of endogenous H₂S as occurred in CSE-KO SMCs. Taken together, we conclude that endogenous H₂S inhibits the formation of IGF-1R/ER- α hybrid and by doing so inhibits the pro-proliferation effect of estrogen.

The formation of a hybrid receptor depends on the expressions and activities of both constituting components. Knocking down of IGF-1R expression downregulated the formation of IGF-1R/ER- α hybrid receptor (Fig. 5C). Interestingly, our co-immunoprecipitation study (Fig. 5D and E) showed that the binding ability of ER- α to IGF-1R was inhibited both in WT-SMCs and CSE-KO SMCs after 2 h NaHS treatment. This short period of NaHS treatment should not affect the expression of IGF-1R protein or CSE protein (Shuang *et al.* 2018) but may cause post-translational modification of the protein. S-sulfhydration is a post-translational modification in which H₂S converts thiol (SH) groups in selective cysteines to hydropersulfide (SSH) (Mustafa *et al.* 2009, Altaany *et al.* 2014, Módis *et al.* 2016). S-sulfhydration of selective proteins has been shown to inhibit protein synthesis, decrease DNA damage, cause vasorelaxation, and attenuate cell apoptosis (Yang 2014). Whereas ER- α was not S-sulfhydrated by H₂S (Fig. 6D), IGF-1R was S-sulfhydrated in the aortae from WT male and female mice, which was significantly decreased in their CSE-KO counterparts (Fig. 6A). In addition to CSE-generated endogenous H₂S, exogenous H₂S treatment for 2 h increased the S-sulfhydration of IGF-1R in CSE-KO SMCs and WT SMCs (Fig. 6B and C). It is reasoned that the S-sulfhydration of IGF-1R changes IGF-1R conformation so that its binding affinity to ER- α is decreased (Fig. 5E).

In summary, endogenous production of H₂S in mouse aortic SMCs is mostly, if not entirely, catalized by CSE. While the expression of CSE proteins in vascular SMCs has been confirmed by numerous studies, the expression of CBS and/or 3-MST in the same type of SMCs has not been generally acknowledged, if any. In the current study, we tried, but failed, to detect CBS and 3-MST proteins in mouse aortic SMCs (Supplementary Fig. 1A, B and C, see section on [supplementary materials](#) given at the end of this article). Using the same antibodies, we were able to detect the expression of CBS and 3-MST proteins in mouse liver (Jin *et al.* 2020) and airways tissues

(Wang *et al.* 2017), respectively. Low endogenous level of H₂S in CSE-KO SMCs due to CSE deficiency promotes the formation of IGF-1R/ER- α hybrid, which is activated by E2 and leads to the stimulation of SMCs proliferation. Both endogenous and exogenous H₂S upregulate ER- α expression, downregulate IGF-1R expression, and induce IGF-1R S-sulfhydration, thus inhibiting the formation of IGF-1R/ER- α hybrid. The net outcome is the blockage of IGF-1R/ER- α hybrid signaling pathway. Consequently, E2 activates ER- α , rather than IGF-1R/ER- α , and inhibits SMCs proliferation (Fig. 7). Our study provides novel insight on estrogen-stimulated SMCs proliferation and elucidates the critical role of H₂S in reversing the stimulatory effect of estrogen on SMCs proliferation to an inhibitory effect. Not only this study will help understand mechanisms for the seemingly contradictory effects of estrogen on the cardiovascular system but also suggest cardiovascular protective benefits for a co-administration of H₂S and estrogen treatment regime.

Figure 7

Proposed mechanisms for H₂S-mediated estrogen effects on SMC proliferation. Arrowed lines denote stimulatory effects. Bar capped lines indicate inhibitory effects. Estrogen can activate both ER- α and IGF-1R/ER- α hybrid. Activation of the former inhibits SMC proliferation whereas activation of the latter stimulates SMC proliferation. H₂S *per se* upregulates ER- α expression, downregulates IGF-1R expression, and induces IGF-1R S-sulfhydration. In the presence of H₂S, estrogen activates ER- α and H₂S inhibits IGF-1R as well as the formation of IGF-1R/ER- α hybrid. The net outcome is the inhibition of SMC proliferation. In the absence of H₂S, ER- α is downregulated; IGF-1R upregulated; and the formation of the IGF-1R/ER- α hybrid increased. Consequently, estrogen activates IGF-1R/ER- α hybrid to stimulate SMC proliferation.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-20-0190>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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