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The effect of 17 β -estradiol on intracellular calcium homeostasis in human endothelial cells

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Abstract

The cardiovascular effects of estrogen are mediated in part by augmenting the function of endothelial nitric oxide synthase. Endothelial nitric oxide synthase activity is dependent on many cofactors including Ca^{2+} . Hence, we investigated the effect of chronic 17 β -estradiol treatment on the intracellular Ca^{2+} concentration and endothelial nitric oxide synthase protein expression in the human endothelial cell line, EA.hy926, using spectrofluorometry and Western blot, respectively. Inhibiting the sarco(endo)plasmic reticulum Ca^{2+} ATPase with thapsigargin caused an increase in the intracellular Ca^{2+} concentration, which was higher in chronically 17 β -estradiol-treated (1 μM , 24 h) cells loaded with Fura-2-acetoxymethyl ester compared to vehicle-treated cells, suggesting a higher endoplasmic reticulum Ca^{2+} content in 17 β -estradiol-treated cells. An enhanced Ca^{2+} influx pathway in chronically 17 β -estradiol-treated cells was also observed. In addition, 17 β -estradiol-treated cells expressed higher levels of endothelial nitric oxide synthase protein in comparison to vehicle-treated cells. The chronic effect of 17 β -estradiol on Ca^{2+} homeostasis and endothelial nitric oxide synthase expression was attenuated with the nonselective estrogen receptor inhibitor, ICI 162,780 (10 μM , 13-methyl-7-[9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl]-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthrene-3,17-diol). Furthermore, analysis of the thapsigargin-evoked Ca^{2+} response in chronically 17 β -estradiol-treated estrogen receptor α -knockdown cells showed no significant difference in Ca^{2+} response compared to vehicle-treated estrogen receptor α -knockdown cells, indicating that the regulation of Ca^{2+} homeostasis by 17 β -estradiol is mediated through an estrogen receptor α -dependent pathway. These data revealed an estrogen receptor α -dependent modulation of Ca^{2+} homeostasis accompanying the enhancement of endothelial nitric oxide synthase expression in 17 β -estradiol-treated human endothelial cells.

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Keywords

estrogen; estrogen receptor alpha; endothelial nitric oxide synthase; calcium

1. Introduction

During their reproductive years, women have a lower incidence of coronary artery disease compared to men of similar age (Barrett-Connor and Bush, 1991). However, women experience a dramatic increase in the incidence of coronary artery disease with the onset of menopause. Loss of endogenous estrogen associated with menopause contributes to the increased cardiovascular risk as evidenced by the fact that estrogen replacement therapy in postmenopausal women reduces mortality due to cardiovascular diseases (Barrett-Connor and Bush, 1991). However, despite supportive epidemiological data (Grady et al., 1992), clinical trials (Herrington et al., 2000; Hulley et al., 1998; Simon et al., 2001) have called into question whether hormone replacement therapy regimens have cardioprotective effects. A better understanding of the mechanism underlying the beneficial effects of estrogen on the vasculature is clearly needed to optimize possible therapeutic applications.

Many of estrogen's beneficial vascular effects may be mediated by its direct action on vascular reactivity, especially by modifying the functional state of endothelial nitric oxide production (Hayashi et al., 1992; Rahimian et al., 1998). Nitric oxide is produced in vascular endothelial cells by the enzyme endothelial nitric oxide synthase. We previously reported that administering 17 β -estradiol to ovariectomized rats improved vascular function through its effects on nitric oxide (Rahimian et al., 2002; Rahimian et al., 1997a; Rahimian et al., 1997b). These findings have been demonstrated by a number of studies in both human and animals (Hayashi et al., 1992; Reis et al., 1994). The effects of estrogen on nitric oxide may stem from increasing endothelial nitric oxide synthase expression or activity (Hayashi et al., 1995; Stirone et al., 2003).

An endothelial nitric oxide synthase cofactor, Ca^{2+} , plays a key role in regulating endothelial nitric oxide synthesis (Pollock et al., 1991). An increase in intracellular Ca^{2+} concentration is an early step in the signaling process leading to vasodilation (Bryan et al., 2005; Busse et al., 2002). However, the effects of estrogen on Ca^{2+} homeostasis are unclear. It has been shown that acute treatment of human arterial endothelial cells with 17 β -estradiol (10 nM, 5 min) induces nitric oxide synthesis in a Ca^{2+} -dependent mechanism (Stefano et al., 2000).

We previously reported that long-term (chronic) treatment of EA.hy926, human endothelial cells, with 17 β -estradiol (1 μM , 24 h) increased the level of endothelial nitric oxide synthase mRNA expression, which correlated with higher nitric oxide production (Rahimian et al., 2004). Considering that increases in intracellular Ca^{2+} concentration in endothelial cells plays a key role in nitric oxide production, we hypothesized that treatment with 17 β -estradiol modulates endothelial nitric oxide synthase protein expression as well as Ca^{2+} homeostasis, leading to an increase in endothelial Ca^{2+} response to pharmacological agent, such as thapsigargin. Since the effects of estrogen on nitric oxide synthesis are mediated via estrogen receptors (Hall et al., 2001), we also explored the role of estrogen receptor in 17 β -estradiol-mediated changes to Ca^{2+} homeostasis using RNA inhibition to decrease the expression of estrogen receptor in EA.hy926.

2. Materials & Methods

2.1. Cell culture

The permanently established EA.hy926 endothelial cell line (Edgell et al., 1983) was the kind gift of CJS Edgell (University of North Carolina). Cells were cultured in low glucose (1000 mg/L) Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C with 95% air and 5% CO₂, until approximately 70% confluent. Cells were sequentially cultured in phenol red-free, serum-free Dulbecco's Modified Eagle's Medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine for 18 h to induce quiescence. Cells were treated with either vehicle (0.09% ethanol) or 17 β-estradiol (1 µM, Sigma, St. Louis, MO) for 24 h. The role of estrogen receptors in 17 β-estradiol-mediated effects were analyzed with the nonselective estrogen receptor inhibitor, ICI 182,780 (10 µM, 13-methyl-7-[9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl]-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthrene-3,17-diol, Tocris, Ellisville, MO), added 1 h prior to treatment with 17 β-estradiol. During treatments, cells were cultured in phenol red-free Dulbecco's Modified Eagle's Medium supplemented with 10% charcoal/dextran-treated fetal bovine serum (Hyclone, Logan, UT) and 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

2.2. Western Blot

Cells were grown on 60 mm Petri dishes and treated as previously mentioned. Cells were then harvested by scraping in Hank's balanced salt solution without phenol red. Cells were pelleted by centrifuging at 500 × g for 4 min. Cell pellets were lysed with RIPA Buffer (Sigma-Aldrich, St. Louis, MO) supplemented with Halt Protease Inhibitor Cocktail and 5 mM EDTA (ethylenediaminetetraacetic acid, Pierce Biotechnology, Rockford, IL). Protein concentrations were determined using Pierce® BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) according to manufacturer suggestions. Twenty µg of total protein were separated on 8.0% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, transferred to nitrocellulose membrane, and then probed with an endothelial nitric oxide synthase monoclonal antibody (1:1000, BD Biosciences, San Jose, CA) or estrogen receptor α polyclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), for respective studies. Detection was accomplished using a goat anti-rabbit secondary horseradish peroxidase conjugated antibody (1:5000, Santa Cruz Biotechnology) and a chemiluminescent substrate for detecting horseradish peroxidase (Pierce Biotechnology, Rockford, IL). Protein concentration was quantified using densitometric analysis with UN-SCAN-IT gel (Silk Scientific Corporation, Orem, UT).

2.3. Real time RT-PCR

RT-PCR was used to analyze estrogen receptor expression in EA.hy926 and comparisons were made to wild type estrogen receptor α and estrogen receptor β positive plasmids, generously supplied by Dr. Pierre Chambon (Université Louis Pasteur, Strasbourg, France). Cells grown on 60 mm Petri dishes were harvested by scraping. Total RNA was collected using the MasterPure™ RNA Purification Kit as recommended by the manufacturer (Epicenter, Madison, WI). The Omniscript RT Kit (Qiagen, Valencia, CA) was used to synthesize the cDNA from 2 µg of total extracted RNA. One µl of total cDNA was used for specific mRNA expression quantified with real time RT-PCR (MyIQ, ICycler, BioRad, Hercules, CA) using iQ™ SYBR Green Supermix (BioRad, Hercules, CA). Relative mRNA expression levels were normalized to 18s ribosomal RNA and analyzed as previously reported by Muller et al (2002). Primers for PCR were designed by the web based program, Oligo Perfect™ Designer (Invitrogen). The human estrogen receptor α primers used were forward 5'-AGCAGCAGGTCAATAGAGGGCA-3', and reverse 5'-

CACATGATCAACTGGGCGAAGAG-3'. The human endothelial nitric oxide synthase primers were forward 5'-AGCAGCAGGTCAATAGAGGGGCA-3', and reverse 5'-CACATGATCAACTGGGCGAAGAG-3'. The human transient receptor potential 1 primers were forward 5'-TCAACATCATTCCCTCACCA-3', and reverse 5'-AACTGTTTTGCCGTTTGACC-3' and human sarco(endo)plasmic reticulum Ca^{2+} -ATPase primers were 5'-ACCCACATTCGAGTTGGAAG-3', and reverse 5'-CAGTGGGTTGTCATGAGTGG-3'. The human 18s ribosomal RNA primers were supplied by Applied Biosystems/Ambion (Austin, TX).

2.4. Intracellular Ca^{2+} measurements

Ca^{2+} homeostasis in EA.hy926 cells was studied using the pharmacological tool thapsigargin (1 μM). Intracellular Ca^{2+} concentration was monitored from cell populations loaded with Fura-2 acetoxymethyl ester (Molecular Probes, Carlsbad, CA) using a fluorescence spectrophotometer equipped with a xenon lamp (Photon Technology International, Lawrenceville, NJ). Cells were grown on poly-L-lysine coated glass coverslips and treated with vehicle (0.09% ethanol), 17 β -estradiol (1 μM), or ICI 182,780 (10 μM) plus 17 β -estradiol (1 μM) for 24 h. After treatments, cells were washed with phenol red-free Hank's balanced salt solution and loaded with 1.5 μM Fura-2 acetoxymethyl ester dissolved in Hank's balanced salt solution from a stock solution of 1.5 mM in DMSO containing 20% pluronic acid at room temperature for 30 min in the dark. Cells were then washed with Hank's balanced salt solution to remove extracellular dye and incubated in Hank's balanced salt solution for an additional 5 min. Average fluorescence ratio was measured using a rapid alternating excitation (340 nm and 380 nm) with emission at 510 nm. Fluorescence ratios were taken 3 times per second (Felix Fluorescent Analysis Software, Photon Technology International, Lawrenceville, NJ).

2.5. Short inhibiting RNA-mediated estrogen receptor α knockdown

Short inhibiting RNA (siRNA) was delivered to cells using 10 μl of LipofectamineTM 2000 (Invitrogen, Carlsbad, CA) to knockdown estrogen receptor α expression in EA.hy926. Cells were grown to approximately 80% confluence in 60 mm Petri dish. Cells were then washed with Opti-MEM (Invitrogen, Carlsbad, CA) and transfected with 4 ml of Opti-MEM containing 90 pmol of estrogen receptor α specific short inhibiting RNA or nonspecific short inhibiting RNA in 10 μl of LipofectamineTM 2000 (Invitrogen). After 5 h incubation, transfection medium was replaced with phenol red-free, antibiotic-free Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (24 h). Cells then underwent one passage and were treated with vehicle (0.09% ethanol) or 17 β -estradiol (1 μM) for 24 h. Western blot and real time RT-PCR were used to analyze estrogen receptor α and endothelial nitric oxide synthesis expression after the RNA inhibition procedure.

2.6. Statistical analysis

All Ca^{2+} traces were normalized to the average baseline taken from the first 75 seconds of each respective Ca^{2+} trace for all analyses. The area under the curve of the fluorescent ratio was calculated using the trapezoid method, $\Sigma[(\text{ratio}_2 + \text{ratio}_1)/2 * (\text{time}_2 - \text{time}_1)]$ (Padar et al., 2004). Depending on the comparison being made, the area under the curve integration bounds contained either 300, 425, or 525 seconds, but the length of integration was the same for each matched up experimental comparison.

Values were expressed as mean $\pm$ standard deviation. Single comparisons were made with a t-test and multiple comparisons were made using one-way ANOVA. Individual differences from the one-way ANOVA were analyzed using Bonferroni post hoc test, after demonstrating significant intergroup differences by ANOVA. For all analysis, $n=3$ or 5 and $P<0.05$ was considered significant.

3. Results

3.1. Effect of 17 β -estradiol on endothelial nitric oxide synthase expression

Endothelial nitric oxide synthase protein expression was analyzed in 1) vehicle-treated, 2) ICI 182,780-treated, 3) 17 β -estradiol-treated, 4) or ICI 182,780 plus 17 β -estradiol-treated cells using Western blot. The endothelial nitric oxide synthase monoclonal antibody detected a protein band at 150 kD, which is the reported molecular weight of endothelial nitric oxide synthase. As shown in Fig. 1, 17 β -estradiol-treated (1 μ M, 24 h) cells showed a 3.1 ± 0.3 fold increase in endothelial nitric oxide synthase protein expression as compared to vehicle-treated cells ($P < 0.01$, $n = 3$). 17 β -estradiol-mediated effects on endothelial nitric oxide synthase protein expression were attenuated with ICI 182,780 pretreatment, while ICI 182,780 alone did not affect endothelial nitric oxide synthase expression.

3.2. Effect of 17 β -estradiol on Ca^{2+} homeostasis

Using bioassay, we previously showed that 24 h treatment with 17 β -estradiol (1 μ M) induced higher endothelium derived relaxing factor from EA.hy926 than 24 hr vehicle treatments (Rahimian et al., 2004). Here, we investigated the effects of chronic 17 β -estradiol treatment (1 μ M, 24 h) on Ca^{2+} homeostasis. Intracellular Ca^{2+} concentration of 17 β -estradiol-treated or vehicle-treated cells were monitored in Hank's balanced salt solution containing 1.2 mM Ca^{2+} or Ca^{2+} -free solution. In solution containing 1.2 mM Ca^{2+} , thapsigargin inhibition of the sarco(endo)plasmic reticulum Ca^{2+} -ATPase induced a biphasic Ca^{2+} response characterized by a rapid increase in intracellular Ca^{2+} concentration followed by a plateau phase, in which a high intracellular Ca^{2+} concentration was maintained in both vehicle- and 17 β -estradiol-treated cells (Fig. 2A). The plateau phase returned to baseline upon treating the cell population with Ni^{2+} (5 mM), a nonselective cation channel inhibitor. The extent of thapsigargin-evoked intracellular Ca^{2+} concentration increase was higher in 17 β -estradiol-treated cells than vehicle-treated cells, as indicated by the area under the curve (Fig. 2B). Furthermore, the effects of 17 β -estradiol on the thapsigargin-evoked Ca^{2+} response were reduced with ICI 182,780 (10 μ M) as observed in the ICI 182,780 plus 17 β -estradiol-treated cells.

In Ca^{2+} -free Hank's balanced salt solution, thapsigargin treatment resulted in a single Ca^{2+} transient in both vehicle- and 17 β -estradiol-treated cells (Fig. 3A). Subsequently raising the extracellular Ca^{2+} concentration to 1.2 mM led to a robust increase in intracellular Ca^{2+} concentration, which was completely blocked by Ni^{2+} . The extent of the thapsigargin-evoked Ca^{2+} response from 17 β -estradiol-treated cells was also higher than the vehicle-treated cells as indicated by the area under the curve (Fig. 3B and 3C). Inhibition of estrogen receptor with ICI 182,780 attenuated the effect of 17 β -estradiol on the Ca^{2+} response as observed in the ICI 182,780 plus 17 β -estradiol-treated cells (Fig. 3A–3C).

3.3. Analysis of transient receptor potential 1 and sarco(endo)plasmic reticulum Ca^{2+} ATPase expression

To determine whether the enhanced thapsigargin-induced Ca^{2+} signals in 17 β -estradiol-treated cells are due to altered store operated channel and sarco(endo)plasmic reticulum Ca^{2+} ATPase expression, we analyzed transient receptor potential 1 and sarco(endo)plasmic reticulum Ca^{2+} ATPase mRNA expression using real-time RT-PCR. There were no significant differences in the mRNA expression levels of transient receptor potential 1 and sarco(endo)plasmic reticulum Ca^{2+} ATPase between vehicle-treated and 17 β -estradiol-treated cells (data not shown).

3.4. Reduction of estrogen receptor expression in EA.hy926

We used RT-PCR to determine that EA.hy926 cells express estrogen receptor α but not estrogen receptor β (data not shown). Since EA.hy926 showed no detectable estrogen receptor β expression under the current experimental conditions, we chose to inhibit estrogen receptor α expression in EA.hy926 with estrogen receptor α -specific short inhibiting RNA to study the role of estrogen receptor on 17 β -estradiol-mediated effects in the thapsigargin-evoked Ca^{2+} response.

Both real time RT-PCR and Western blot were used to quantify the expression of estrogen receptor α mRNA (Fig. 4A) and protein (Fig. 4B), in the LipofectamineTM 2000-transfected cells, nonspecific short inhibiting RNA-transfected cells, and estrogen receptor α -specific short inhibiting RNA-transfected cells (estrogen receptor α -knockdown cells). Estrogen receptor α polyclonal antibody detected estrogen receptor α protein in Lipofectamine-transfected, nonspecific siRNA-transfected, and estrogen receptor α -specific short inhibiting RNA-transfected cells. Compared to the transfection with Lipofectamine alone, the nonspecific-short inhibiting RNA reduced estrogen receptor α by $35.1 \pm 12.5\%$ (mRNA) and $63.1 \pm 16.0\%$ (protein), while the estrogen receptor α -specific short inhibiting RNA reduced estrogen receptor α expression by $86.7 \pm 19.3\%$ (mRNA) and $85.3 \pm 2.7\%$ (protein) ($P < 0.05$, $n = 3$). To determine whether the short inhibiting RNA was nonspecifically decreasing other gene expression in the cells, we measure the effect of short inhibiting RNA on endothelial nitric oxide synthase mRNA expression. Endothelial nitric oxide synthase mRNA expression was not significantly altered in nonspecific short inhibiting RNA transfected or estrogen receptor α -specific short inhibiting RNA transfected cells (Fig. 4C).

3.5. Effect of estrogen receptor α knockdown on 17 β -estradiol-mediated changes in intracellular Ca^{2+}

Ca^{2+} homeostasis in estrogen receptor α -knockdown cells was characterized in Ca^{2+} -free Hank's balanced salt solution using thapsigargin. The thapsigargin-evoked Ca^{2+} response from cells undergoing the knockdown process was similar to the Ca^{2+} response described in section 3.2 (Fig. 5A–5C). Transfections with Lipofectamine alone did not alter 17 β -estradiol-mediated effects on Ca^{2+} homeostasis, since 17 β -estradiol treatment showed a higher thapsigargin-evoked Ca^{2+} increase than vehicle treatments in the Lipofectamine-transfected cells (Fig. 5A). The thapsigargin-evoked Ca^{2+} response from 17 β -estradiol-treated nonspecific short inhibiting RNA-transfected cells was higher than the vehicle-treated nonspecific short inhibiting RNA-transfected cells (Fig. 5B). However, 17 β -estradiol-treated estrogen receptor α -knockdown cells displayed a similar thapsigargin-induced Ca^{2+} response to vehicle-treated estrogen receptor α -knockdown cells (Fig. 5C). Comparison of differences between individual cell phenotypes and treatments were based on the area under the curve of thapsigargin-evoked Ca^{2+} signal in Ca^{2+} free buffer and the elevation of intracellular Ca^{2+} concentration in response to added extracellular Ca^{2+} (1.2 mM) (Fig. 5D and 5E).

3.6. Acute effects of 17 β -estradiol on Ca^{2+} homeostasis

Since 17 β -estradiol has both short-term and long-term effects, we examined the thapsigargin-induced Ca^{2+} response in the absence of extracellular Ca^{2+} from cells treated with 17 β -estradiol (1 μM) for 5 min. Acute 17 β -estradiol treatment did not alter the thapsigargin-evoked Ca^{2+} profile compared to vehicle treatment (Fig. 6A), as indicated by no differences in the area under the curve (Fig. 6B and 6C). Cells undergoing a 30 min 17 β -estradiol treatment also showed no difference in the thapsigargin-evoked Ca^{2+} profile compared to vehicle treatment (data not shown). We also examined the direct effect of 17 β -estradiol on Ca^{2+} homeostasis. Direct stimulation of cells with 17 β -estradiol (1 μM) by itself did not increase intracellular Ca^{2+} concentration in EA.hy926 (data not shown).

4. Discussion

Despite extensive studies on the role of Ca^{2+} in endothelial nitric oxide synthase activity and the role of endothelial nitric oxide synthase in the cardiovascular effect of estrogen, the mechanisms linking altered Ca^{2+} homeostasis to estrogen have yet to be fully investigated. Hence, we investigated the effects of chronic 17 β -estradiol treatment on intracellular Ca^{2+} homeostasis in human endothelial cells, EA.hy926. We showed that the extent of thapsigargin-evoked intracellular Ca^{2+} concentration increase was higher in 17 β -estradiol-treated cells than vehicle-treated cells, which was dependent on estrogen receptor α .

Nitric oxide production plays an important role in mediating the effects of 17 β -estradiol on the vasculature. Increased nitric oxide production in endothelial cells may result from increased endothelial nitric oxide synthase expression (Rahimian et al., 2004) as well as enhanced association of cofactors to endothelial nitric oxide synthase, such as Ca^{2+} (Goetz et al., 1999). We previously reported that there was a gender difference in the basal intracellular Ca^{2+} concentration in rat valvular endothelial cells (Rahimian et al., 1998). We also showed that chronic treatment of EA.hy926 cells with 17 β -estradiol (1 μM , 24 h) enhances nitric oxide production and endothelial nitric oxide synthase mRNA expression (Rahimian et al., 2004). In this study, we examined whether the same treatment of EA.hy926 cells with 17 β -estradiol would alter the endothelial nitric oxide synthase protein expression and Ca^{2+} homeostasis. We showed that treatment of cells with 17 β -estradiol (1 μM , 24 h) increases endothelial nitric oxide synthase protein expression. 17 β -estradiol-mediated increases in endothelial nitric oxide synthase activity from ovine fetal pulmonary artery endothelial cells (Lantin-Hermoso et al., 1997) correlate with an increase in intracellular Ca^{2+} concentration. Ca^{2+} -bound calmodulin partakes in endothelial nitric oxide synthase activation (Pollock et al., 1991) to increase nitric oxide synthesis in endothelial cells. However, such activities are mainly considered as characteristics of acute 17 β -estradiol treatment and not much is known about the long-term effects of 17 β -estradiol on Ca^{2+} homeostasis.

We used thapsigargin to analyze the long-term effects of 17 β -estradiol on Ca^{2+} homeostasis. Thapsigargin-evoked increased intracellular Ca^{2+} concentration via inhibition of the sarco (endo)plasmic reticulum Ca^{2+} ATPase (Thastrup et al., 1990). In the presence of extracellular Ca^{2+} , thapsigargin induced a biphasic Ca^{2+} response. The magnitude of initial rise in intracellular Ca^{2+} concentration in buffer containing Ca^{2+} was similar to the response in Ca^{2+} -free buffer, and the plateau phase only appeared in the presence of extracellular Ca^{2+} . Therefore, we concluded that the first phase was due to Ca^{2+} release from the endoplasmic reticulum, and the second phase was due to Ca^{2+} influx. Furthermore, the thapsigargin-evoked Ca^{2+} response was significantly higher in 17 β -estradiol-treated cells. Ca^{2+} release from the endoplasmic reticulum results in a high concentration of cytoplasmic Ca^{2+} , which accompanies a high level of intracellular Ca^{2+} concentration due to Ca^{2+} influx through store operated channels. Ca^{2+} influx serves to refill the Ca^{2+} stores in a process referred to as capacitative Ca^{2+} entry (Putney, 1986). As expected, nonselective inhibition of store operated channels on the plasma membrane with Ni^{2+} caused intracellular Ca^{2+} concentration to return to baseline in both vehicle- and 17 β -estradiol-treated cells. Although the functional consequences of an elevated thapsigargin-induced Ca^{2+} response are unclear, our previous data suggests that enhanced nitric oxide production in these cells (Rahimian et al., 2004) may be partly due to an increase in intracellular Ca^{2+} concentration. Our working hypothesis is consistent with recent data demonstrating that 17 β -estradiol replacement in ovariectomized rats enhanced acetylcholine-induced relaxation in precontracted uterine arteries, which was associated with an increased acetylcholine-stimulated intracellular Ca^{2+} concentration in endothelial cell (Burger et al., 2009).

The enhanced thapsigargin-induced Ca^{2+} signals in 17 β -estradiol-treated cells may at least partially result from either an elevated level of store operated channel or sarco(endo)plasmic reticulum Ca^{2+} ATPase expression. Transient receptor potential 1 is a candidate for store operated channels (reviewed in Salido et al., 2009). However, our analysis of transient receptor potential 1 and sarco(endo)plasmic reticulum Ca^{2+} ATPase mRNA expression in vehicle- and 17 β -estradiol-treated cells showed no significant differences. In agreement with our findings, Gifford et al. showed that enhanced agonist-induced rise in intracellular Ca^{2+} from uterine artery endothelial cells of pregnant ewes compared to non-pregnant ewes did not accompany Ca^{2+} channel expression changes, but may relate to the enhanced interaction between transient receptor potential channel and inositol trisphosphate receptor (2006a, 2006b).

To address the role of estrogen receptors in 17 β -estradiol-mediated altered Ca^{2+} homeostasis and increased endothelial nitric oxide synthase expression, EA.hy926 were treated with an excess of ICI 182,780 prior to treatments with 17 β -estradiol. The ICI 182,780 plus 17 β -estradiol-treated cells showed a reduced 17 β -estradiol-mediated effect on intracellular Ca^{2+} concentration, as well as a reduced level of endothelial nitric oxide synthase protein, suggesting a possible role for estrogen receptors in 17 β -estradiol-mediated effects. Analysis of wild type estrogen receptor α and estrogen receptor β mRNA in EA.hy926 using RT-PCR suggests that EA.hy926 expresses estrogen receptor α but not estrogen receptor β . The presence of wild type estrogen receptor α but not estrogen receptor β in EA.hy926 is consistent with previous reports documenting the expression of estrogen receptor α in EA.hy926 (Figtree et al., 2003) and human umbilical vein endothelial cells (Venkov et al., 1996). To our knowledge, the expression of estrogen receptor β has not been documented in this cell line but has been reported in human umbilical vein endothelial cells (Toth et al., 2008).

To evaluate the role of estrogen receptor α in 17 β -estradiol-mediated effects, we inhibited estrogen receptor α expression in EA.hy926 using short inhibiting RNA. Both estrogen receptor α -specific short inhibiting RNA and a nonspecific short inhibiting RNA significantly reduced estrogen receptor α protein and mRNA expression, although the extent of reduced estrogen receptor α expression was significantly greater in cells transfected with the estrogen receptor α -specific short inhibiting RNA. To assure that the reduction in estrogen receptor α was not reflective of total RNA reduction in the cells, we compared the expression of endothelial nitric oxide synthase among the three differently transfected cell populations. The expression of endothelial nitric oxide synthase was not significantly different between the Lipofectaminetransfected, nonspecific short inhibiting RNA-transfected, and estrogen receptor α -specific short inhibiting RNA, suggesting that the nonspecific short inhibiting RNA had some silencing effects for estrogen receptor α . However, it is important to note that the reduced estrogen receptor α expression due to the nonspecific short inhibiting RNA did not alter the 17 β -estradiol-mediated effects on Ca^{2+} homeostasis. Furthermore, in the estrogen receptor α -specific short inhibiting RNA-transfected cells (estrogen receptor α -knockdown), 17 β -estradiol treatment showed a similar thapsigargin-evoked Ca^{2+} response to vehicle treatment. The similarity in the thapsigargin-evoked Ca^{2+} response between 17 β -estradiol and vehicle treatment in the estrogen receptor α -knockdown cells suggests that the observed 17 β -estradiol-mediated effect on Ca^{2+} homeostasis was indeed dependent on estrogen receptor α . Further studies are needed to disclose the relative minimal expression of estrogen receptor α required for 17 β -estradiol-mediated change to Ca^{2+} homeostasis in EA.hy926.

Direct treatment with 17 β -estradiol (10 nM) has been shown to induce intracellular Ca^{2+} mobilization in ovine fetal pulmonary artery endothelial cells (Lantin-Hermoso et al., 1997). In the current study, however, 17 β -estradiol (1 μM) by itself did not mobilize intracellular Ca^{2+} in EA.hy926. We also investigated the acute effects of 17 β -estradiol (1 μM , 5 min) on thapsigargin-evoked Ca^{2+} increase. The thapsigargin-evoked Ca^{2+} release and influx from 5 min 17 β -estradiol-treated cells showed no significant difference from vehicle-treated cells,

suggesting that acute 17 β -estradiol treatment did not influence Ca^{2+} homeostasis. In contrast, Goetz et al (1999) reported that a 5 min treatment of bovine aortic endothelial cells with 17 β -estradiol (10 nM) induced translocation of endothelial nitric oxide synthase in a Ca^{2+} -dependent manner. The underlining mechanisms for the long-term effects of 17 β -estradiol on Ca^{2+} homeostasis observed in this study are unknown. Perhaps increases in the activity of Ca^{2+} regulatory channels and pumps, such as transient receptor potentials and sarco(endo)plasmic reticulum Ca^{2+} ATPase, may be involved, but these factors remain to be investigated.

The relevance of the present data to understanding how estrogens function *in vivo* should be considered with regard to the experimental model (a cultured endothelial cell line derived from human umbilical vein endothelial cells) and with regard to the concentration of 17 β -estradiol that were used in the current study. We are fully aware that there may be a functional difference between the EA.hy926 and primary endothelial cell lines. Furthermore, the concentration of 17 β -estradiol used in this study may not be considered as physiologically relevant, since free estrogens may not reach micromolar levels in the plasma. However, as White (2002) reported, the “physiological” concentration of steroids is questionable and the concentration at the cellular level is not known. In many cases, maximal vascular effects of estrogen as well as other steroids (Deenadayalu et al., 2001) *in vitro* require a 10–1000 times higher concentration of free hormone than concentrations typically present in circulating plasma. However, there is reason to believe that free plasma levels of estrogen may far underestimate actual effective concentrations within targeted cells (White, 2002).

5. Conclusion

The present work revealed an estrogen receptor α -mediated modulation of Ca^{2+} homeostasis accompanying the enhancement of endothelial nitric oxide synthase expression in long-term 17 β -estradiol-treated human endothelial cell line EA.hy926. This presents a possible and novel mechanism by which long-term 17 β -estradiol treatments may maintain a sustained Ca^{2+} -dependent nitric oxide synthesis in endothelial cells, although further studies are required to provide the underlying mechanism behind 17 β -estradiol effects on Ca^{2+} homeostasis and nitric oxide synthesis. Our data support the hypothesis that steroid sex hormone can influence endothelial function, which may contribute to gender differences observed in the pathogenesis of cardiovascular disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Estrogen treatment enhances endothelial nitric oxide synthase protein expression

Western blot analysis of endothelial nitric oxide synthase (eNOS) expression in cells undergoing long-term treatments with vehicle, ICI 182,780 (ICI), 17 β -estradiol (E₂), and ICI 182,780 plus 17 β -estradiol (ICI + E₂). Significant difference from the control is indicated (*, $P < 0.05$, $n = 3$), analyzed using one-way ANOVA, followed by a Bonferroni post hoc test.

Figure 2. Thapsigargin-evoked Ca^{2+} response in the presence of 1.2 mM extracellular Ca^{2+}
 A) Representative Ca^{2+} traces from EA.hy926 in response to thapsigargin (1 μM) in the presence of 1.2 mM extracellular Ca^{2+} , followed by Ni^{2+} (5 mM) treatment in vehicle-treated, 17 β -estradiol-treated (E_2), and ICI 182,780 plus E_2 -treated (ICI+ E_2) cells. B) The mean $\pm$ standard deviation of the area under the curve from the thapsigargin response in the presence of extracellular Ca^{2+} . Significant differences among individual treatment groups are indicated ($\bullet$ — $\bullet$, $P < 0.05$, $n = 5$), analyzed using a one-way ANOVA, followed by a Bonferroni post hoc test.

Figure 3. Thapsigargin-evoked Ca^{2+} response in the absence of extracellular Ca^{2+}

A) Representative Ca^{2+} traces from EA.hy926 in response to thapsigargin ($1 \mu\text{M}$) in Ca^{2+} -free Hank's balanced salt solution, followed by the addition of extracellular Ca^{2+} (1.2 mM) and Ni^{2+} (5 mM) in vehicle-treated, 17β -estradiol-treated (E_2), and ICI 182,780 plus 17β -estradiol-treated (ICI+ E_2) cells. B) The mean $\pm$ standard deviation of the area under the curve from the thapsigargin-transient response in Ca^{2+} -free Hank's balanced salt solution. C) The mean $\pm$ standard deviation of the area under the curve from the thapsigargin response upon the addition of extracellular Ca^{2+} . Significant differences among individual treatment groups are indicated ($\bullet$ — $\bullet$, $P < 0.05$, $n=5$), as analyzed using a one-way ANOVA, followed by a Bonferroni post hoc test.

Figure 4. Analysis of estrogen receptor α and endothelial nitric oxide synthase expression in Lipofectamine-transfected cells, nonspecific short inhibiting RNA-transfected cells, and estrogen receptor α -knockdown cells

Analysis of estrogen receptor α mRNA expression with real time RT-PCR (A) and estrogen receptor α protein expression with Western blot (B) show that estrogen receptor α specific short inhibiting RNA can effectively knockdown estrogen receptor α mRNA and protein expression, respectively. In B, the protein bands represented by a, b, and c are from Lipofectamine-transfected cells, nonspecific short inhibiting RNA-transfected cells, and estrogen receptor α -knockdown cells, respectively. C) Transfections with siRNA, specific or nonspecific, did not affect endothelial nitric oxide synthase (eNOS) mRNA expression. Values are expressed as mean $\pm$ standard deviation, significance among groups are indicated ($\bullet$ — $\bullet$, $P < 0.05$, $n = 3$), analyzed using one-way ANOVA, followed by a Bonferroni post hoc test.

Figure 5. Thapsigargin-evoked Ca^{2+} response in Lipofectamine-transfected cells, nonspecific short inhibiting RNA-transfected cells, and estrogen receptor α -knockdown cells

Representative tracings of the Ca^{2+} signal from cells in response to thapsigargin (1 μM) in Ca^{2+} -free Hank's balanced salt solution, followed by the addition of extracellular Ca^{2+} (1.2 mM). A) Inhibition of sarco(endo)plasmic reticulum Ca^{2+} ATPase with thapsigargin induced a higher Ca^{2+} release and a higher Ca^{2+} influx in 17 β -estradiol-treated Lipofectamine-transfected cells as compared with vehicle-treated control cells. B) 17 β -estradiol-treated nonspecific short inhibiting RNA-transfected cells also showed a higher thapsigargin-evoked Ca^{2+} release and influx. C) 17 β -estradiol-treated estrogen receptor α -knockdown cells had the same Ca^{2+} profile as vehicle-treated estrogen receptor α -knockdown cells. Comparisons were made using area under the curve in thapsigargin-evoked Ca^{2+} release (D) and influx (E) due to estrogen receptor α knockdown. Significant difference among groups are indicated ($\bullet$ — $\bullet$, $P < 0.05$, $n = 3$), analyzed using one-way ANOVA, followed by a Bonferroni post hoc test.

Figure 6. Thapsigargin-evoked Ca²⁺ response in 5 min 17 β-estradiol-treated cells

A) Representative Ca²⁺ traces from cells treated for 5 min with either vehicle or 17 β-estradiol in response to thapsigargin (1 μM) in Ca²⁺-free Hank's balanced salt solution. B) The mean ± standard deviation of the area under the curve from the transient thapsigargin response in Ca²⁺-free Hank's balanced salt solution and Ca²⁺ response upon the addition of extracellular Ca²⁺ (1.2 mM). There was no significant difference among individual treatment groups ($P > 0.05$, $n = 5$), analyzed using a t-test.