Pathol. Oncol. Res. (2017) 23:329–334
DOI 10.1007/s12253-016-0105-2

ORIGINAL ARTICLE

Estrogen Receptors in Regulating Cell Proliferation of Esophageal Squamous Cell Carcinoma: Involvement of Intracellular Ca^{2+} Signaling

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Received: 22 May 2016 / Accepted: 24 August 2016 / Published online: 5 September 2016

Abstract Esophageal cancer is a deadly disease in the esophagus with a poor prognosis. Over 90 % of esophageal cancer is esophageal squamous cell carcinoma (ESCC) and its pathogenic mechanisms remain unclear. Epidemiology study found a strong gender difference with a sex ratio of 8–9:1 in favor of males, but the molecular mechanisms for so striking gender difference are poorly understood so far. In the present study, we demonstrated the expression of estrogen receptors in human ESCC cells. 17β-E2 but not 17α-E2 was found to dose-dependently suppress the cell proliferation of human ESCC cells, which was attenuated by estrogen receptor antagonist ICI1 82,780. Furthermore, 17β -E2 but not 17α-E2 10 nM markedly induced both intracellular Ca^{2+} release and extracellular Ca^{2+} entry into ESCC cells, which was again attenuated by estrogen receptor antagonist ICI182,780. Taken together, our data clearly demonstrate that estrogen exerts anti-proliferative action on human ESCC cells likely through estrogen receptor-Ca^{2+} signaling pathway, which may provide a reasonable explanation on the striking male predominance of ESCC.

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Keywords Estrogen receptor · Esophageal squamous cell carcinoma · Gender difference · Cell proliferation · Ca^{2+} signaling

Introduction

Esophageal cancer is an aggressive malignant disease with a poor prognosis and 5-year survival rate about 15 %. It is the sixth leading cause of cancer-related death in the world [1]. There are two major histological types of esophageal cancer, squamous cell carcinoma and adenocarcinoma, each of which has distinct etiological and pathological characteristics. Both types of esophageal cancer remain equally virulent. Although the incidence of adenocarcinoma is continuously increasing both in the United States and in the Europe, more than 90 % of esophageal cancers worldwide are squamous cell carcinoma [2]. In China, esophageal squamous cell carcinoma (ESCC) represents over 99 % of esophageal cancer cases [3]. Unlike esophageal adenocarcinoma that is more dominant in Western countries, ESCC has been studied less and the pathogenic mechanisms remain unclear [4, 5]. It was noted clinically a strong gender difference with a sex ratio of 8–9:1 in favor of males [5, 6]. Furthermore, the female patients with ESCC have a better prognosis than males [5]. However, the molecular mechanisms for so striking gender differences of ESCC in term of incidence, mortality rate and prognosis are poorly understood so far [5, 6].

It is well known that estrogen contributes to many physiological and pathological processes in both men and women through activation of estrogen receptors (ER) [4, 7, 8]. Estrogen also plays pivotal roles in several types of human malignancies, such as breast cancer and colon cancer [5, 6, 8]. It has been demonstrated that the surgical removal of ovaries could retard the growth of breast tumors and that females are

less prone to tumors at non-conventional target organs of sex steroid hormones, e.g., gastrointestinal tract [5]. Although several reported studies suggest the possible roles of estrogens in development of ESCC [5, 6], controversy still exists regarding the biological action and clinical significance of estrogens in ESCC [6]. The role of ER in the pathogenesis of ESCC and the underlying mechanisms remain to be elucidated.

Therefore, in the present study, we attempted to reevaluate estrogen actions through ER in ESCC. We first detected the ER expression in human ESCC cells, and then evaluated their roles in the regulation of cancer cell proliferation to characterize their potential biological functions. We further determined if Ca^{2+} signaling plays a role in the biological functions of ER in ESCC.

Material and Methods

Materials

17β-estradiol (17β-E2) and 17α-estradiol (17α-E2) were purchased from Sigma Chemical Co. (St. Louis, MO). ICI 1 82,780 was from Tocris (Ellisville, MI). All other chemicals were obtained from Fisher Scientific (Santa Clara, CA). Fura-2 acetoxymethyl ester (AM) was from Molecular Probes, Inc. (Eugene, OR). Human esophageal squamous cell carcinoma cells (EC109 cells) were purchased from American Type Culture Collection.

Cell Culture

Human esophageal squamous-cell carcinoma cells (EC109 cells) were maintained in RPMI 1640 supplemented with 10 % fetal calf serum (FCS, GIBCO-BRL) and 10 units/ml penicillin/streptomycin in an incubator at 37 °C and 5 % CO_2. These EC109 cells were grown to 70–80 % confluence in medium containing 10 % FCS.

Total RNA Extraction and Semiquantitative RT-PCR Analysis

Total RNA from EC109 cells was isolated with TRIZOL reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Total RNA samples were resuspended in water and quantified by $OD_{260/280}$. Five micrograms of total RNA were reverse transcribed into cDNA. After inactivation at 70 °C for 10 min, 1 μl of the reverse transcribed reaction mixture (20 μl) was used as the template for PCR containing 0.2 mM 2-deoxynucleotide 5′-triphosphate, 3 mM $MgCl_2$, 500 mM KCl, 20 mM Tris·HCl (pH 8.0), 0.2 μM oligonucleotide primers as shown below, and 1 U *Taq* polymerase (Invitrogen). Primers were synthesized by Integrated DNA Technologies (Coralville, IA). Specific sense and antisense primers for mouse ERα (GenBank accession no. NM007956) were 5′-AAGGGCAGTCACAATGAACC-3′ and 5′-GCCA GGTCATTCTCCACATT-3′. The predicted size of the PCR-amplified product for ERα was 155 bp. Specific sense and antisense primers for mouse ERβ (GenBank accession no. NM207707) were 5′-GAAGCTGGCTGACAAG GAAC-3′ and 5′-AACGAGGTCTGGAGCAAAGA-3′. The predicted size of the PCR-amplified product for ERβ was 187 bp. Human glyceraldehyde-3-phosphate dehydrogenase sense and antisense primers were 5′-ACCACAGTCCATGCC ATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′. The samples were amplified in an automated thermal cycler (GeneAmp 2400; Applied Biosystems, Foster City, CA). DNA amplification conditions included an initial 3-min denaturation step at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 57 °C, 40 s at 72 °C, and a final elongation step of 10 min at 72 °C. The products were electrophoresed on a 1.5 % agarose gel, stained with ethidium bromide, and then photographed under UV light. To confirm band identity, the RT-PCR products were also subjected to restriction enzyme analysis.

Western Blot Analysis

Cells were washed three times with ice-cold PBS and then lysed with total lysis buffer [150 mM NaCl, 10 mM Tris·HCl (pH 7.8), 1 mM EDTA, 0.5 % Triton X-100, and 1 mM sodium orthovanadate] containing protease inhibitors (1 μg/ml leupeptin and 100 μg/ml PMSF) and incubated at 4 °C for 30 min with constant shaking. The cells were then scraped into microcentrifuge tubes and centrifuged at 12,000 *g* for 15 min to remove insoluble material. The protein content in each sample was determined and normalized. The protein extracts were mixed with sodium dodecyl sulfate sample buffer and boiled for 5 min. The protein extracts were separated by SDS-PAGE (10 %) and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). ERα and ERβ protein expression was detected by immunoblotting with polyclonal antibodies specific against ERα or ERβ as described previously [9]. Protein extracts of uterine artery endothelial cells (UAEC) were used as positive controls.

Cell Proliferation Assay

Cells were treated for 24 and 48 h with drugs or DMSO. Cell proliferation was assessed at the corresponding time points using the colorimetric MTT-assay (Fivephoton Biochemicals) according to the manufacturer's protocol. The extinction measurements were calculated relative to the negative control at the corresponding time points. At least three independent experiments were performed.

Measurement of $[Ca^{2+}]_{cyt}$ by Digital Ca^{2+} Imaging

The cytosolic free Ca^{2+} concentrations in single human EC109 cells ($[Ca^{2+}]_{cyt}$) were measured by fura-2 fluorescence ratio digital imaging. Briefly, EC109 cells grown on coverslips were loaded with 5 μM fura-2 AM dissolved in 0.01 % Pluronic F-127 plus 0.1 % DMSO in physiological salt solution (PSS, described below) at room temperature (22 °C) for 60 min and then washed in PSS for 30 min. Thereafter, the coverslips with EC109 cells were mounted in a perfusion chamber on a Nikon microscope stage. The ratio of fura-2 fluorescence with excitation at 340 or 380 nm ($F_{340/380}$) was followed over time and captured with an intensified charge-coupled device camera (ICCD200) and the MetaFluor Imaging System (Universal Imaging, Downingtown, PA). The PSS solution used in digital Ca^{2+} measurement contained the following (in mmol/l): 140 Na^+, 5 K^+, 2 Ca^{2+}, 147 Cl^-, 10 HEPES, and 10 glucose. For the Ca^{2+}-free solution, Ca^{2+} was omitted and 0.5 mM EGTA was added to prevent possible Ca^{2+}contamination. The osmolalities for all solutions were ~284 mosmol/kgH_2O.

Statistical Analysis

All data are expressed as means ± SE for a series of *n* experiments. Data were analyzed by one-way ANOVA followed by the Student-Newman-Keuls post hoc test or by Student's *t*-tests for unpaired samples with GraphPad Prism 3.0 (San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

Expression of ER in EC109 Cells

Two specific ERs, ERα and ERβ, have been identified in mammalians to date. ER were also detected in ESCC [5, 6]. We soughed to assess if ERα and ERβ were expressed in human esophageal squamous cell carcinoma cells (EC109 cells). This was done using RT-PCR analysis to determine the expression of mRNAs specific for the two subtypes of ERs in these cells. Figure 1a clearly shows that transcripts for two subtypes of ERs, ERα and ERβ, were readily detected in EC109 cells. By using Western blot analysis with antibodies, ERα protein was detected in EC109 cells (Fig. 1b). The apparent molecular mass of ERα protein migrates on a SDS-PAGE similarly to that in the sheep uterine artery endothelial cells (UAEC, as a positive control) [9] with an apparent molecular mass of approximately 67 kDa. ERβ protein was also detected in EC109 cells and migrated on SDS-PAGE similarly to that in UAEC with an apparent molecular mass of approximately 56 kDa (Fig. 1b). Therefore, both types of ERα and ERβ were expressed in human esophageal squamous cell carcinoma cells.

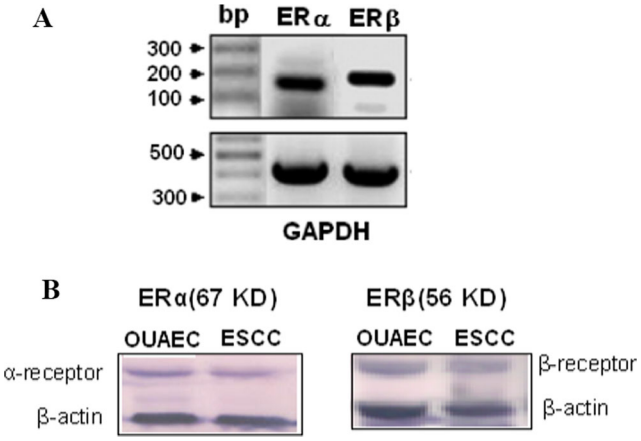

Fig. 1 Expression of ERs (ERα and ERβ) in human esophageal squamous cell carcinoma (ESCC) cells. **a** RT-PCR was performed to determine the expression of ER mRNA. Five micrograms of total RNA isolated from ESCC cells were used in each reaction. DNA ladder (bp) was indicated on the *left*. The predicated product sizes of ERα and β are 155 and 187 bp, respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. **b** Western blot was performed to determine the expression of ER proteins. Anti-ERα and anti-ERβ antibodies recognized two proteins with molecular masses of 67 and 56 kDa, respectively in ESCC cells. Sheep uterine artery endothelial cells (OUAEC) were used as positive controls and β-actin was used as a loading control. These data are representative of three experiments conducted on different times with identical results

ER-Induced Anti-Proliferative Action of EC109 Cells

17β-E2 has been found to mediate intestinal epithelial cell proliferation through specific ER [10, 11]. To test the role of ER in regulation of cell proliferation in human esophageal squamouscell carcinoma, we used the MTT assay to test the effect of 17β-E2 on proliferation of EC109 cells. They were treated with different concentrations of 17β-E2 for different time courses, and then cell proliferation was measured by MTT assay. As shown in Fig. 2a, at the concentration of 1 nM, 17β-E_2 treatment for 24 h markedly suppressed EC109 cell proliferation, which reached the maximal action at the concentration of 10 nM since increasing concentration to 50 nM did not further increase its anti-proliferative effect. 17β-E2 treatment for 48 h further markedly suppressed EC109 cell proliferation (Fig. 3a). To test if the anti-proliferative effect of 17β-E2 is specific, 17α-E2, an inactive biological isoform was used, but it had no effect on cell proliferation (Figs. 2 and 3b). To test if the anti-proliferative effect of 17β-E2 is specific through estrogen receptors, ICI 1 82,780 (1 uM) was used to block estrogen receptors. As shown in Figs. 2 and 3b, after pretreatment with ICI1 82,780, the anti-proliferative effects of 17β-E2 for 24 and 48 h were prevented.

E2 -Mediated Ca^{2+} Signaling in EC109 Cells

ER activation stimulates several intracellular signaling pathways, such as Ca^{2+}, cAMP, PKC and gene regulation [12, 13].

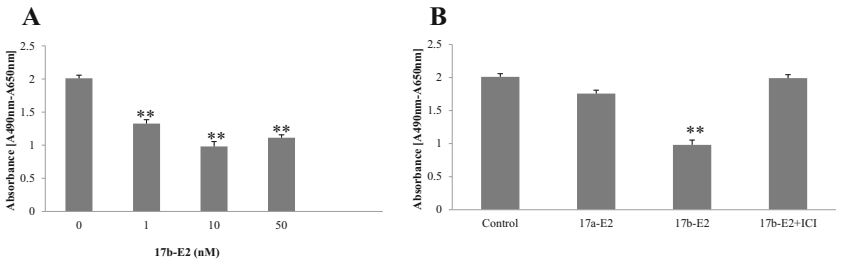

Fig. 2 Cell proliferation after treatment with E2 agonist and antagonist for 24 h. Treatment of EC109 cells with different concentrations of 17b-E2 (1, 10 and 50 nM) for 24 h reduced cell proliferation (**a**). Treatment of EC109 cells with 17a-E2 (10 nM) did not affect cell proliferation, and ICI (1 uM) prevented the anti-proliferative action of 17b-E2 (**b**). The results are the means of 6 experiments ± SEM. *$P < 0.05$ and **$P < 0.01$ vs. control

It is well known that Ca^{2+} signaling plays an important role in gene regulation and cell proliferation [14, 15]. To test if Ca^{2+} signaling plays a role in the anti-cell proliferation of E2, we first used digital Ca^{2+} imaging to assess the ability of E2 to mobilize Ca^{2+} in EC109 cells. As illustrated in Fig. 4, 17β-E2, but not 17α-E2, at 10 nM significantly increased $[Ca^{2+}]_{cyt}$, suggesting a specific action. 17β-E2 induced a transient increase in $[Ca^{2+}]_{cyt}$ in Ca^{2+}-free solutions (Fig. 4a), but a sustained increase in $[Ca^{2+}]_{cyt}$ in Ca^{2+}-containing solutions (Fig. 4b). These data suggest that 17β-E2 induces both Ca^{2+} release from the intracellular Ca^{2+} store and extracellular Ca^{2+} influx in EC109 cells. To further investigate if Ca^{2+} signaling is involved in ER-mediated activation, ICI 1 82,780 (1 uM) was used (Fig. 5). After ICI 1 82,780 pretreatment, 17β-E2-induced Ca^{2+} signaling in EC109 cells was significantly attenuated (compare Fig. 5a vs. b), suggesting an ER-specific action.

Discussion

The incidence of human esophageal squamous cell carcinoma (ESCC) has a striking male predominance; however, the cellular and molecular mechanisms underlying these gender differences are poorly understood. It was reported that menopausal hormone therapy is significantly associated with lower risk of ESCC and that the women who took both estrogen and progestin had a lower risk of developing ESCC than those who took a placebo [16] [17]. Recent studies suggest that ER may be involved in the development and progress of ESCC [18]. However, the clinical and biological significance of ER in ESCC has not been clarified so far.

In the present study, we first examined the expression of ER subtypes in ESCC. We demonstrated for the first time the expression of both ERα and ERβ subtypes in ESCC not only at the levels of mRNA but also proteins. Our data are consistent with the previous reports that both subtypes of ER are expressed in the biopsy tissues from ESCC patients [18], which is closely associated with the prognosis, possibly through altering cell proliferation of carcinoma cells. Our study has provided further support for an important role of ER in the development of ESCC.

Although the expression of ER has been demonstrated in the esophageal tissues obtained from both normal human subjects and ESCC patients, their function in normal esophagus and their biological significance in ESCC have been poorly studied. We therefore assessed the biological significance of ER in ESCC. We found that 17β-E2, an active biological isoform, but not 17α-E2, an inactive biological isoform, has marked anti-proliferative effect in human esophageal squamous carcinoma cells. 17β-E2 produced this obvious anti-proliferative action at its physiological concentrations of 1–10 nM. The anti-proliferative action of 17β-E2 is through specific ER since ER antagonist, ICI 1 82,780 was able to block this action n. Therefore, our present study has

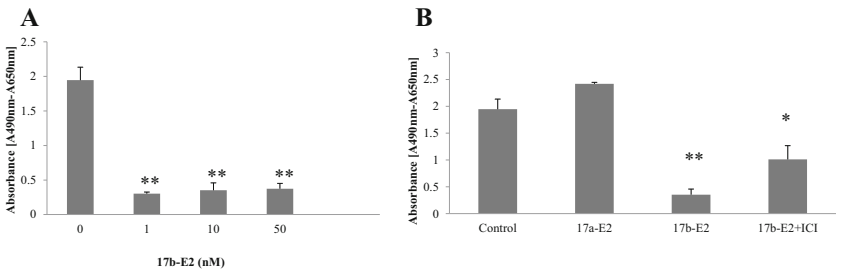

Fig. 3 Cell proliferation after treatment with E2 agonist and antagonist for 48 h. Treatment of EC109 cells with different concentrations of 17b-E2 (1, 10 and 50 nM) for 48 h reduced cell proliferation (**a**). Treatment of EC109 cells with 17a-E2 (10 nM) did not affect cell proliferation, and ICI (1 uM) prevented the anti-proliferative action of 17b-E2 (**b**). The results are the means of 6 experiments ± SEM. *P < 0.05 and **P < 0.01 vs. control

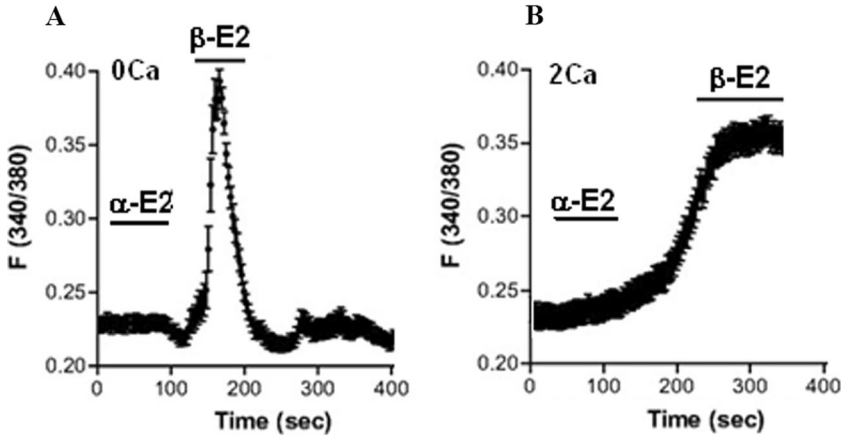

Fig. 4 Effects of E2 on $[Ca^{2+}]_{cyt}$ in human esophageal squamous cell carcinoma (ESCC) cells. After loaded with 5 μM Fura 2-AM, coverslips with cells were mounted on a Nikon microscope stage. $[Ca^{2+}]_{cyt}$ was measured using cell digital Ca^{2+} imaging during the perfusion of solutions containing E2 (10 nM). 17b-E2 induced transient $[Ca^{2+}]_{cyt}$ signaling in Ca^{2+}-free solutions (**a**), but increased sustained $[Ca^{2+}]_{cyt}$ signaling in Ca^{2+}-containing solutions (**b**). However, 17a-E2 did not significantly affect $[Ca^{2+}]_{cyt}$ signaling in Ca^{2+}-free solutions or in Ca^{2+}-containing solutions (A&B). Values are expressed as means ± SEM for 40–50 cells in each condition

demonstrated a biological significance of ER in the potential prevention/therapy of ESCC, which may also provide a reasonable explanation on the striking male predominance of ESCC [5, 6].

Both genomic and non-genomic pathways have been ascribed for mediating estrogen action [19, 20]. Genomic action starts with the binding of estrogen to its intracellular receptors and then initiating transcription. In contrast, non-genomic action is by interacting with plasma membrane receptors to activate various intracellular second messengers, including Ca^{2+} signaling [20]. Ca^{2+} signaling is a pivotal messenger, regulating many physiological functions including cell cycling, proliferation and apoptosis [14, 15]. An increase in $[Ca^{2+}]_{cyt}$ is derived from Ca^{2+} release from the intracellular stores such as the endoplasmic reticulum and Ca^{2+} entry from the extracellular space through Ca^{2+} channels and exchangers [21], leading to cell proliferation, differentiation, migration and apoptosis [22]. [23] [24] The dual roles of Ca^{2+} signaling on cell proliferation or apoptosis depend on the cell types, cell status (normal or abnormal cells), particular Ca^{2+} signature within the cells (the transit oscillation or sustained Ca^{2+} increase) etc. The apoptotic Ca^{2+} signal has been reported in many cancer cells, such as in breast cancer cells and colon cancer cells [25, 26]. An increase in concentration of $[Ca^{2+}]_{cyt}$ reaching elevated, but not cytotoxic levels is associated with activation of Ca^{2+}-dependent μ-calpain and Ca^{2+}/calpain-dependent caspase-12[27]. Activation of these proteases appears to be sufficient for the execution of apoptosis in cancer cells [14, 25, 27]. We have demonstrated that 17β-E2 rapidly increased $[Ca^{2+}]_{cyt}$ in human esophageal squamous cell carcinoma cells likely by evoking both Ca^{2+} release from the intracellular Ca^{2+} store and extracellular Ca^{2+} entry. Our data obtained from ESCC cells are in agreement with a previous report showing that 17β-E2 can rapidly stimulate Ca^{2+} entry into the other types of cells [28–30]. These effects are 17β-E2 specific because they are attenuated by ER antagonist, ICI1 82,780. All

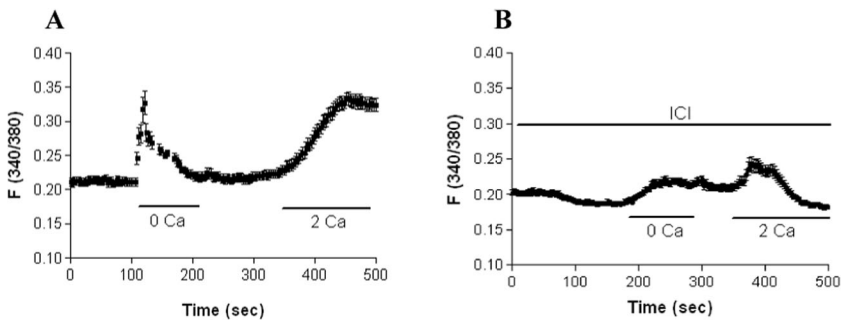

Fig. 5 Effects of E2 agonist and antagonist on $[Ca^{2+}]_{cyt}$ in human esophageal squamous cell carcinoma (ESCC) cells. After loaded with 5 μM Fura 2-AM, coverslips with cells were mounted on a Nikon microscope stage. $[Ca^{2+}]_{cyt}$ was measured using cell digital Ca^{2+} imaging during the perfusion of solutions containing E2. 17b-E2 (10 nM) induced transient $[Ca^{2+}]_{cyt}$ signaling in Ca^{2+}-free solutions and but increased sustained $[Ca^{2+}]_{cyt}$ signaling in Ca^{2+}-containing solutions (**a**). Pretreatment with ICI (1 uM) significantly prevented 17b-E2 (10 nM)-induced $[Ca^{2+}]_{cyt}$ signaling in Ca^{2+}-free solutions and Ca^{2+}-containing solutions (**b**). Values are expressed as means ± SE for for 40–50 cells in each condition

these findings, including our current data, suggest that $[Ca^{2+}]_{cyt}$ plays a role in mediating membrane ER signaling in these cells [30]. Elevated $[Ca^{2+}]_{cyt}$ mediates both acute and chronic cell activities, including gene expression, cell proliferation and apoptosis [14, 15, 25, 26]. We speculate that both genomic and non-genomic mechanisms are involved in the anti-proliferation of ER and potential esophageal protection [19, 20]. However, an in-depth understanding of the molecular mechanisms underlying the anti-proliferation of estrogen and other functions in the esophagus awaits further investigation.

Acknowledgments This work was supported by National Natural Science Foundation of China (No. 81260592). The funding institutes had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare no competing financial interest.

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