Rapid effects of estrogen on intracellular Ca\(^{2+}\) regulation in human airway smooth muscle

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Townsend EA, Thompson MA, Pabelick CM, Prakash YS. Rapid effects of estrogen on intracellular Ca\(^{2+}\) regulation in human airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 298: L521–L530, 2010. First published January 22, 2010; doi:10.1152/ajplung.00287.2009.—The severity of asthma, a disease characterized by airway hyperresponsiveness and inflammation, is enhanced in some women during the menstrual cycle and during pregnancy but relieved in others. These clinical findings suggest that sex steroids modulate airway tone. Based on well-known relaxant effects of estrogens on vascular smooth muscle, we hypothesized that estrogens relax airway smooth muscle (ASM), thus facilitating bronchodilation. In ASM tissues from female patients, Western and immunocytochemical analyses confirmed the presence of both estrogen receptor (ER) isoforms, ER\(\alpha\) and ER\(\beta\). In fura 2-loaded, dissociated ASM cells maintained in culture, acute mediated, at least in part, via decreased Ca\(^{2+}\)/H\(^{+}\)-selective agonist (R,R)-THC had a greater reducing effect on [Ca\(^{2+}\)]\(_i\) compared with the ER\(\beta\)-selective agonist DPN. The effects of E\(_2\) on [Ca\(^{2+}\)]\(_i\) were mediated, at least in part, via decreased Ca\(^{2+}\) influx through \(\alpha_1\)-type channels and store-operated Ca\(^{2+}\) entry but not via Ca\(^{2+}\)-activated K\(^+\) channels, receptor-operated entry, or sarcoplasmic reticulum uptake. Overall, these data support our hypothesis that estrogens relax ASM and suggest a potentially novel therapeutic target in airway hyperresponsiveness.

ASTHMA, A DISEASE CHARACTERIZED by airway hyperresponsiveness and inflammation, is more prevalent among adult women compared with men (11, 32, 45). Clinical data reveal an increased severity and frequency of exacerbations in women (2, 41). Cyclical variations in airway reactivity termed menstrual asthma have been estimated to affect approximately 40–50 percent of women with preexisting disease (8, 37). Although these clinical data would suggest that sex steroids such as estrogens may have a detrimental effect in asthma, closer examination reveals that asthma exacerbation is actually greater during the late luteal phase when estrogen levels are lowest (8, 16, 18). Furthermore, some postmenopausal women experience decreased airway function (3). Finally, although asthma is the most prevalent airway disease endured by pregnant women (approximately 4–8%; Refs. 29, 51) during the third trimester of pregnancy (when circulating estrogen levels are high and the gravid uterus would be expected to compress the lungs), only one-third of women with preexisting disease will experience exacerbation of symptoms, and another one-third actually experience improvement in asthma symptoms (2, 24). Taken together, these contrasting clinical data suggest that sex steroids modulate airway tone. What is not clear is which sex steroid (estrogen vs. progesterone) and whether these hormones are detrimental or beneficial.

In both normals and asthmatics, airway tone represents a balance between bronchoconstriction and bronchodilation, mediated by airway smooth muscle (ASM) contractility vs. relaxation. ASM contractility is determined by both regulation of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) and force (28, 40). Previous studies have already demonstrated that increased airway tone in diseases such as asthma involve, at least in part, increased [Ca\(^{2+}\)]\(_i\) (25, 43, 50). Therefore, it is possible that sex steroids such as estrogens and progesterone modulate airway tone by altering [Ca\(^{2+}\)]\(_i\) in ASM.

There are currently limited data on the role of sex steroids in regulation of airway tone or their effects on ASM, especially in humans. Measurements of airway resistance in mouse models of asthma have found both downregulation (5, 13, 31) as well as upregulation (6) of airway hyperresponsiveness by estrogens. Other studies in guinea pig have found that progesterone can induce ASM relaxation (44). However, in humans and mouse models, airway function is actually reduced when progesterone levels are high (20, 52). Based on the clinical data that asthma exacerbation is greater when estrogen levels are lower, we focused on estrogens and hypothesized that estrogens facilitate bronchodilation.

There is already a precedent for estrogen-induced relaxation of smooth muscle in the well-recognized vasodilatory effects of estrogens (34, 36). In this regard, as with vascular smooth muscle, estrogen may act via both acute (likely nongenomic) and genomic mechanisms in modulating ASM tone. In vascular smooth muscle, both of these effects are mediated through estrogen receptors (ERs), primarily ER\(\alpha\). Previous studies have shown that the ER\(\alpha\) and ER\(\beta\) isoforms are expressed in human lung (35); however, their functional role is unclear. Although ER expression has been noted in the lung, specific ER expression in ASM or its function have not been established. In the present study, we determined the expression of ERs in normal human ASM and their role in acute (nongenomic) regulation of [Ca\(^{2+}\)]\(_i\) during agonist stimulation, thus setting the foundation for examining the role of estrogens in asthmatic ASM. In this regard, signaling pathways activated by the two ER isoforms are likely different (e.g., in other tissues, ER\(\beta\) modulates ER\(\alpha\) signaling, which, in turn, involves multiple signaling pathways such as mitogen-associated kinases and Src-phosphatidylinositol pathway (19, 30)); however, we did not specifically examine these differential signaling pathways here.
MATERIALS AND METHODS

Isolation of Human ASM Cells

Studies were conducted using ASM cells enzymatically dissociated from 3rd to 6th generation bronchi of lung samples incidental to patient surgery at Mayo Clinic, Rochester, MN (approved and considered exempt by Mayo Institutional Review Board). This study was limited to tissues obtained from adult female patients, although no attempt was made to determine whether these patients were in menopause or not. Lung samples excised for emphyema or other infectious or severe inflammatory causes were excluded. Visibly normal airway, as confirmed by pathological review, was excised and rapidly transferred to the laboratory in ice-cold HBSS. ASM cells were enzymatically dissociated as previously described by our group (46). Briefly, epithelium and connective tissues were removed by blunt dissection, and smooth muscle tissue was carefully minced followed by papain and collagenase dissociation and ovomucoid/albunin separation as per manufacturer’s instructions (Worthington Biochemical, Lakewood, NJ). Cell pellets were resuspended and seeded into 75-cm² tissue culture flasks or eight-well Lab-Tek chambers (Nalge Nunc International, Rochester, NY). Cells were maintained at 37°C (5% CO₂-95% air) using phenol red-free DMEM/F-12 medium (phenol red is known to activate ERs, albeit weakly; Invitrogen, Carlsbad, CA) supplemented with 10% FBS until ~80% confluent. Before experiments, the cells were washed in PBS, and medium was changed to phenol red-free DMEM/F-12 lacking serum for 48 h. All experiments were performed in cells from passages 1 to 3 of subculture. Periodic assessment of smooth muscle myosin heavy chain (or other contractile protein) expression, and lack of fibroblast markers, was performed to rule out dedifferentiation during the cell-processing period. ER expression in passaged cells was compared with expression in ASM tissue from which they were derived and found to be comparable.

Isolation of ASM Cell Fractions

Human ASM cells were obtained as described above and plated on 100-mm plates under the same conditions. Cells were grown to 80% confluence and harvested. These whole cell lysates were then separated into heavy (nuclear/Golgi), cytosolic, and membrane fractions using the Fraction-PREP Cell Fractionation System (BioVision, Mountain View, CA) according to the manufacturer’s protocol.

Immunoprecipitation of Proteins from Cell Fractions

The above fractions were subjected to immunoprecipitation for either ERα or ERβ. Primary antibody (2 μg; mouse anti-ERα: cat. no. 2512, Cell Signaling Technology; mouse anti-ERβ: cat. no. SC-53494, Santa Cruz Biotechnology) was used per 200 μl of sample fraction and incubated overnight at 4°C with gentle rotation. Protein G agarose beads (50 μl) were added to the sample and incubated for 4 h at 4°C. Proteins were recovered through centrifugation and eluted from the beads at 100°C for 5 min. These samples were then processed as described below for Western analysis (goat-anti-ERβ SC-6822 was used for overnight incubation for ERβ detection). Purity of cell fractions was confirmed via Western analysis of proteins exclusive to each fraction (heavy: rabbit anti-i-C-Jun SC-1694; membrane: rabbit anti-Gαi2,3 SC-262; cytosol: rabbit anti-ERK42 SC-94).

Western Blot Analysis

Proteins were separated by SDS-PAGE (10% gradient gels; Criterion Gel System; Bio-Rad, Hercules, CA) at 200 V for 1 h and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) for 60–75 min. Membranes were blocked for 1 h at room temperature with 5% milk in Tris-buffered saline (TBS) containing 0.1% Tween (TBST) and then incubated overnight at 4°C with 1 μg/ml rabbit anti-ERα (SC-542; Santa Cruz Biotechnology) or mouse anti-ERβ (SC-53494; Santa Cruz Biotechnology). GAPDH (cat. no. 2118; Cell Signaling Technology) was used for normalization. Following three washes with TBST, primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and signals developed by SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical, Rockford, IL). Blots were imaged on a Kodak Image Station 4000MM (Carestream Health, New Haven, CT) and quantified using densitometry. Human ASM samples were processed as described above. Samples of postpartum rat uterus were used as positive controls for ER expression. To determine applicability of the primary antibodies to other human tissues, human pulmonary artery samples were also analyzed.

Immunofluorescence Microscopy

ER expression in ASM cells was determined using immunofluorescence as described for other proteins (47). ASM grown on glass slides to 50% confluence were fixed with 2% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 in 0.1 M TBS for 10 s. After washing gently with TBS, cells were blocked in 4% normal donkey serum for 1 h at room temperature and incubated overnight at 4°C with primary antibody (1 μg/ml rabbit anti-ERα SC-542, goat anti-ERβ SC-6822). After thoroughly washing with TBS, cells were incubated with appropriate Cy3 or Alexa Fluor 488 fluorescent secondary antibodies (1:200 dilution; donkey anti-rabbit or anti-goat IgG; Jackson ImmunoResearch) for 1 h at room temperature.

Labeled ASM cells were visualized using an Olympus Fluoview laser scanning confocal microscope mounted on an Olympus BX50WI equipped with Ar and Kr lasers and appropriate filters. Cells were imaged at 1,024 × 1,024 pixels and a 0.4-μm optical section thickness using a ×40 oil-immersion lens and different hardware zooms.

Real-Time [Ca²⁺], Imaging

The techniques for [Ca²⁺]ᵢ imaging of human ASM cells has been previously described (47). ASM cells were incubated in 5 μM fura-2 AM (Molecular Probes, Eugene, OR) for 45 min at room temperature and visualized using a fluorescence imaging system (MetaFluor; Universal Imaging, Downingtown, PA) on a Nikon Diaphot Inverted Microscope (Fryer Instruments, Edina, MN) equipped with a Photometric Cascade digital camera system (Roper Scientific, Tucson, AZ). [Ca²⁺]ᵢ responses of ~15 cells per visual field were obtained using individual, software-defined regions of interest (×40 oil-immersion lens, 512 × 512 pixel resolution, 0.33-Hz image acquisition of 510-nm emissions following alternative excitation at 340 vs. 380 nm). Cells were initially perfused with 2.5 mM Ca²⁺-free HBSS, and baseline fluorescence was established. Actual [Ca²⁺]ᵢ levels were estimated using previously described calibration techniques for fura 2 (39, 47).

Materials

(R,R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8- chrysenediol [iR]-THC and diaphydropontinirile (DPN) were obtained from Tocris Biosciences (Ellisville, MO). 17β-Estradiol (E₂), ICI-182,780 (ICI), and other chemicals were obtained from Sigma Chemical (St. Louis, MO) unless mentioned otherwise. Tissue culture reagents, including DMEM/F-12 and FBS, were obtained from Invitrogen.

Statistical Analysis

Twelve bronchial samples from different female patients were used to obtain ASM cells. [Ca²⁺]ᵢ experiments were performed in at least 25 cells each for 4 different bronchial (patient) samples, although not all protocols were performed in each sample obtained. In experiments where responses were compared in the presence or absence of specific drugs, paired t-test was used, whereas population studies were compared using unpaired i-test or 1-way ANOVA with repeated measures.
as appropriate. Bonferroni correction was applied for multiple comparisons. Statistical significance was established at $P < 0.05$. All values are expressed as means ± SE.

RESULTS

Expression of ERs in Human ASM

Western blot analysis of ASM (i.e., epithelium denuded, ASM isolated) from bronchial samples of female patients revealed that both full-length ER$\alpha$ and ER$\beta$ are expressed to a considerable extent, even compared with tissues known to have these isoforms such as postpartum rat uterus ($n = 6$; Fig. 1). Furthermore, human pulmonary artery also showed expression of these ER isoforms, albeit to different extents compared with ASM (Fig. 1A). Overall, in human ASM, ER$\alpha$ expression (calculated relative to GAPDH expression) was greater than that of ER$\beta$ (Fig. 1B). In other tissues, lower molecular weight ER isoforms (that lack specific domains) have been reported (15, 26). Such isoforms were also noted in human ASM (data not shown).

Immunostaining of enzymatically dissociated ASM cells demonstrated plasma membrane, cytosolic, as well as diffuse nuclear expression of ER$\alpha$. In comparison, ER$\beta$ was expressed at the plasma membrane also but to a lesser extent; however, distinct expression of this isoform was detected at the nucleus.

Effect of E2 on $[Ca^{2+}]_i$ Response to Agonist

In the first set of studies, fura 2-loaded human ASM cells were exposed to 1 $\mu$M histamine, resulting in the typical transient $[Ca^{2+}]_i$ elevation to a peak level followed by a lower plateau that was still greater than baseline $Ca^{2+}$ (Fig. 3A). Baseline $[Ca^{2+}]_i$ levels ranged from 100 to 150 nM. With exposure to histamine, the amplitude of the $[Ca^{2+}]_i$ response was ~750 nM. Following a thorough washout of histamine with 2.5 mM $Ca^{2+}$ HBSS for at least 15 min, cells were exposed to 1 nM E2 for 15 min. This resulted in a small but insignificant rise in $[Ca^{2+}]_i$ basal levels. Subsequent exposure

Fig. 1. Expression of estrogen receptors (ERs) in human (h) airway smooth muscle (ASM). A: full-length ER isoforms -$\alpha$ (ER$\alpha$-66) and -$\beta$ (ER$\beta$-56) are expressed in both ASM and pulmonary artery (PA) from lungs of female patients. Rat uterus (Ut.) was used as a positive control in both cases. B: densitometric analysis of relative ER expression normalized to GAPDH levels shows ER$\alpha$ is more prominent than ER$\beta$ in ASM. Values are means ± SE. *Significant difference between ER$\alpha$ and ER$\beta$ ($P < 0.05$). AU, arbitrary units.

Fig. 2. Localization of ERs in ASM cells. A: immunocytochemical staining of enzymatically dissociated ASM cells from female patients followed by confocal imaging demonstrated expression of both ER$\alpha$ (Cy3-conjugated secondary antibody; red) and ER$\beta$ (Alexa Fluor 488-conjugated secondary antibody; green). Top shows secondary staining controls, whereas middle and bottom shows ER$\alpha$ and ER$\beta$ expression (left, middle) as well as colocalization (merged images with yellow) at 2 different magnifications. ER$\alpha$ and ER$\beta$ are present at the plasma membrane as well as in the cytosol and nucleus, albeit to different extents. B: ER expression was determined in whole ASM cell lysates separated into heavy (nuclear/Golgi), membrane, and cytosolic fractions. Complementary to immunocytochemical staining, ER$\alpha$ was expressed by all 3 fractions, whereas ER$\beta$ expression was limited to the heavy fraction. Purity of the cell fractions was tested by blotting for proteins exclusive to each fraction. B represents original scan contrasts and brightness, which differed between blots.

Overall, ER$\alpha$ expression was more ubiquitous than ER$\beta$ with ER$\beta$ preferentially localizing to the nucleus (Fig. 2A). These results were confirmed by Western analysis of cell fractions illustrating ER$\alpha$ expression in heavy (nuclear/Golgi), cytosolic, and membrane fractions, whereas ER$\beta$ expression was expressed largely in the heavy fraction (Fig. 2B).

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to histamine resulted in a significantly smaller $[\text{Ca}^{2+}]$, response compared with vehicle controls ($n = 4$; $P < 0.05$; Fig. 3, A and B). To determine whether these results were specific to histamine, the above experiment was repeated in separate sets of cells using ACh (1 $\mu$M) as an alternative agonist and KCl (110 mM) as a nonreceptor agonist that changes membrane potential. In general, the inhibitory effects of E2 on $[\text{Ca}^{2+}]$ responses to ACh persisted ($n = 4$; $P < 0.05$; Fig. 6A); however, there was no difference in the peak $[\text{Ca}^{2+}]$, response between E2-treated cells and vehicle controls with KCl depolarization (data not shown). Further analysis of $[\text{Ca}^{2+}]$, response to KCl showed that E2 extended the time required to reach peak $[\text{Ca}^{2+}]$, levels ($n = 4$; $P < 0.05$; Fig. 6B).

In a subsequent set of studies, seven concentrations of E2 that span the physiological range were used to establish a dose-response relationship for effects on $[\text{Ca}^{2+}]$, response to histamine. Because of known tachyphylaxis with histamine, as well as the number of interventions, these studies had to be performed as comparisons of cell populations. ASM cells were exposed to 0 (vehicle control), 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, and 1 $\mu$M E2 for 15 min and then stimulated with 1 $\mu$M histamine. Compared with vehicle controls, there was considerable decrease in amplitude of $[\text{Ca}^{2+}]$, responses for all E2 concentrations, forming a biphasic dose-response curve (Fig. 4). All E2 concentrations in the physiological range (100 pM to 10 nM) significantly decreased the $[\text{Ca}^{2+}]$, amplitude, with the maximum reduction in $[\text{Ca}^{2+}]$, occurring at 1 nM E2 ($n = 4$; $P < 0.05$). Accordingly, for subsequent studies, we used 1 nM E2 to further explore estrogen signaling.

**Effect of ER Inhibition on E2 Effects**

To ascertain whether the blunting of $[\text{Ca}^{2+}]$, responses by E2 was mediated by ERs, in an additional set of experiments, 1 $\mu$M isoform-nonselective ER antagonist ICI (Faslodex) was administered 15 min before 1 nM E2 exposure and was continued during the subsequent 15 min in combination with E2 ($n = 4$). In the presence of ICI, the previously observed blunting of $[\text{Ca}^{2+}]$, response by E2 was abolished, and $[\text{Ca}^{2+}]$, response to histamine approximated that of vehicle controls ($n = 4$; Fig. 3).

**Effect of ER Isoform-Selective Agonists**

E2 is a nonspecific ER agonist and does not differentiate between ER$\alpha$ and ER$\beta$ activation. To determine which receptor subtype is involved in modulation of $[\text{Ca}^{2+}]$, ER$\alpha$- and ER$\beta$-selective agonists were used. (R,R)-THC is a nonsteroidal, ER$\alpha$-selective agonist (EC$_{50}$ = 10 nM; Ref. 53) but also an ER$\beta$-selective agonist. DPN is an ER$\beta$-selective agonist (EC$_{50}$ = 0.85 nM; Ref. 33). The protocol described above to test E2 effects was modified by replacing E2 with either (R,R)-THC or DPN.

Acute exposure of ASM cells to 10 nM (R,R)-THC decreased $[\text{Ca}^{2+}]$, response to ~50% of vehicle control ($n = 4$; $P < 0.05$; Fig. 5). The reduction of $[\text{Ca}^{2+}]$, by (R,R)-THC approximated the observed effects of E2. In contrast, 10 nM DPN decreased the $[\text{Ca}^{2+}]$, response to a much lesser extent than (R,R)-THC and was statistically comparable with vehicle control (Fig. 5; $n = 4$). The combination of (R,R)-THC and DPN did not appear to have additive effects on $[\text{Ca}^{2+}]$, responses.
These experiments were repeated using ACh and KCl as described previously. In the case of ACh, the results were qualitatively similar to those with histamine. [Ca$^{2+}$]$_i$ was decreased in both cases by (R,R)-THC to 50% of control, whereas DPN did not decrease [Ca$^{2+}$]$_i$ significantly (n = 4; P < 0.05; Fig. 5A). Addition of iberiotoxin resulted in a ~50% increase in plateau Ca$^{2+}$ levels compared with vehicle controls (likely representing Ca$^{2+}$ influx in response to membrane depolarization; Fig. 7B). E2 in the presence of iberiotoxin reduced these elevated plateau levels by ~50% compared with iberiotoxin alone (P < 0.05). The extent of E2-induced decrease in Ca$^{2+}$ was comparable with E2 effects on plateau Ca$^{2+}$ in cells not exposed to iberiotoxin.

In parallel studies, we examined E2 effects on store-operated Ca$^{2+}$ entry (SOCE). We (1, 39) have previously established the

Mechanisms of E2 Effects on [Ca$^{2+}$]$_i$ Regulation

Ca$^{2+}$ influx. Previous studies (including our own) in vascular smooth muscle have demonstrated that estrogens decrease Ca$^{2+}$ influx (27, 48). Accordingly, we tested the role of influx mechanisms in estrogen effects in human ASM. The protocol described above for ICI was modified by replacing this compound with the t-type Ca$^{2+}$ channel inhibitor nifedipine (1 µM). In the presence of nifedipine, we found that 1 nM E2 effects on [Ca$^{2+}$]$_i$ plateau levels to 1 µM histamine were considerably smaller, compared with effects with E2 alone (P < 0.05; n = 4; Fig. 7A). However, when similar studies were conducted in the presence of 100 nM iberiotoxin (to inhibit BK$_{Ca}$ channels), E2 effects were largely unaffected (n = 4; P < 0.05; Fig. 7B). Addition of iberiotoxin resulted in a ~50% increase in plateau Ca$^{2+}$ levels compared with vehicle controls (likely representing Ca$^{2+}$ influx in response to membrane depolarization; Fig. 7B). E2 in the presence of iberiotoxin reduced these elevated plateau levels by ~50% compared with iberiotoxin alone (P < 0.05). The extent of E2-induced decrease in Ca$^{2+}$ was comparable with E2 effects on plateau Ca$^{2+}$ in cells not exposed to iberiotoxin.

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presence of this Ca\(^{2+}\) influx mechanism in human ASM cells. As previously described (1, 39), extracellular Ca\(^{2+}\) was first removed by perfusion with zero Ca\(^{2+}\) HBSS. Calcium influx through voltage-gated channels was then blocked by addition of 1 \(\mu\)M nifedipine, and the membrane was partially depolarized using 10 mM KCl. Sarcoplasmic reticulum (SR) Ca\(^{2+}\) stores were then depleted by exposure to 10 \(\mu\)M cyclopiazonic acid (CPA; inhibitor of SR Ca\(^{2+}\) reuptake). Following stabilization of [Ca\(^{2+}\)], levels, 1 nM E\(_2\) (or vehicle) was introduced. In the continued presence of nifedipine, KCl, CPA, and E\(_2\) (or vehicle), extracellular Ca\(^{2+}\) was rapidly reintroduced, triggering SOCE. The presence of E\(_2\) resulted in significantly smaller SOCE-mediated Ca\(^{2+}\) influx compared with vehicle controls (\(n = 4\); \(P < 0.05\); Fig. 7C).

We (55) have previously demonstrated that SOCE in ASM involves transient receptor potential (TRP) channels. However, Ca\(^{2+}\) influx on reintroduction of extracellular Ca\(^{2+}\) in the protocol used may also be due to other influx mechanisms that are not TRP-dependent, particularly receptor-operated calcium entry (ROC) or influx mode sodium-calcium exchange (NCX).

To determine the role of these other mechanisms in E\(_2\) effects, the above protocol was modified by introducing either 10 \(\mu\)M SKF-96365 (a potent inhibitor of ROC) or 5 \(\mu\)M KB-R7943 (NCX inhibitor) for 15 min following CPA exposure but before reintroduction of extracellular calcium. E\(_2\) (1 nM) was added thereafter. ROC inhibition decreased the observed influx following introduction of extracellular Ca\(^{2+}\); however, even in the presence of SKF-96365, 1 nM E\(_2\) inhibited the influx to the same extent as it did without SKF-96365 being present (Fig. 6C; \(n = 4\); \(P < 0.05\)). NCX inhibition slightly decreased the extent of influx, and E\(_2\) continued to inhibit the influx to the same extent as that without KB-R7943 being present (\(n = 4\); \(P < 0.05\); Fig. 7C).

SR Ca\(^{2+}\) reuptake. The initial protocol for E\(_2\)-treated cells (e.g., Fig. 3) was repeated, and the decay time constant of the [Ca\(^{2+}\)] responses with or without 1 nM E\(_2\) was evaluated by single exponential decay fit (calculated from peak [Ca\(^{2+}\)] until return to baseline; \(n = 3\); Fig. 8). Although the amplitude of [Ca\(^{2+}\)] response was decreased as reported above, the rate of fall was not significantly affected by E\(_2\) (Fig. 8).

To verify lack of E\(_2\) effect on SR Ca\(^{2+}\) reuptake, additional experiments were performed in a separate set of cells in the absence of extracellular Ca\(^{2+}\). Following initial perfusion in HBSS, extracellular Ca\(^{2+}\) was removed by perfusion with zero Ca\(^{2+}\) HBSS, and Ca\(^{2+}\) influx was nonspecifically blocked with 1 mM LaCl\(_3\). This resulted in a typical, small decrease in [Ca\(^{2+}\)\(_i\)] levels. Cells were then exposed to 1 \(\mu\)M histamine in the presence or absence of 1 nM E\(_2\). Under these conditions, the [Ca\(^{2+}\)\(_i\)] responses were comparable (vehicle 363 \pm 40 nM; E\(_2\) 352 \pm 17 nM; \(n = 3\); Fig. 8). In parallel experiments, E\(_2\) effects on sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) reuptake were evaluated. In identical extracellular conditions described above, preexposure to 1 nM...
E2 did not alter the rate of decline of [Ca$^{2+}$]$_i$, following histamine exposure as calculated from [Ca$^{2+}$]$_i$ peak to baseline value as described above ($n=3$; Fig. 8).

**DISCUSSION**

ASM is the main regulator of airway hyperreactivity and inflammation and has become an important target with the recent increases in asthma diagnoses. Sex disparities in clinical findings as well as changes in airway function with the menstrual cycle, pregnancy, and menopause have raised the question of hormonal influences on ASM. The few studies that have looked at the effects of sex steroids on airway function are focused on the full-length ERs. AF-1 is not clear (19). Accordingly, in the present study, we maintained full-length ER expression, consistent with the findings of these different groups. Growing ASM cells for 48–72 h also maintained full-length ER expression, consistent with the findings of Catley et al. (7).

Recent studies, including our own, have demonstrated controlled Ca$^{2+}$ influx in response to SR Ca$^{2+}$ depletion (SOCE; Refs. 1, 42). SOCE involves members of the TRP channel including TRP canonical (TRPC) 1, 3, 4, 5, and 6 (10, 55). In addition, members of the TRP vanilloid (TRPV) family (ROC) can also contribute to Ca$^{2+}$ influx in ASM [e.g., with changing osmolarity (12, 23)]. Furthermore, some studies suggest a role for NCX in ASM (21). Accordingly, multiple [Ca$^{2+}$]$_i$ regulatory targets exist in human ASM for estrogens. The results of the present study indicate that E2 indeed inhibits several mechanisms but almost exclusively at the plasma membrane.

**Estrogen Signaling**

In humans, E2 is the most potent ER agonist. Two full-length ERs of similar estrogen binding affinities are of significance: ER$\alpha$ and ER$\beta$ both belonging to the nuclear receptor family of transcription factors (34). The role of “truncated” or shorter ER$\alpha$ isoforms (ER$\alpha$-46 and ER$\alpha$-36) that lack activation factor AF-1 is not clear (19). Accordingly, in the present study, we focused on the full-length ERs.

ERs are expressed in a wide variety of tissues such as vascular endothelium and smooth muscle. However, few studies have examined ER expression within the airway, especially in humans. ER$\alpha$ and ER$\beta$ are expressed in human bronchial epithelial cells (22), however, expression in ASM or innervation in vivo is not known. Again, based on perimenstrual fluctuations in asthma symptoms, ER expression is likely. In a recent study using gene and protein profiling techniques, Catley et al. (7) detected ER$\beta$ in human ASM cells in vitro exposed to the cytokine IL-1$\beta$. The present study is the first to directly demonstrate ER expression within ASM of bronchi of female patients. Although we did not limit selection of patients by age, hormone status, or airway disease, our findings of significant expression of both ER$\alpha$ and ER$\beta$ along with truncated isoforms suggest that ERs are likely expressed across these different groups. Growing ASM cells for 48–72 h also maintained full-length ER expression, consistent with the findings of Catley et al. (7).

The well-known transcriptional (genomic) response to estrogens is complex and cell-specific, depending (at least) on estrogen concentration and duration of exposure, coregulatory proteins, promoters in estrogen-responsive genes, active genes, and ER$\beta$-ER$\alpha$ interactions (19). Given these complexities and the relative lack of knowledge on estrogen signaling in the...
airway, we did not address the issue of prolonged estrogen exposure in ASM exposure or genomic signaling.

In other tissues, rapid effects of E2 (seconds to minutes) have been demonstrated (30, 36). It is not entirely clear whether nongenomic ER signaling involves the same intracellular/nuclear receptors as in genomic effects, but the observed effects are dependent on E2 concentration. Accordingly, in the present study, we initially selected a range of E2 concentrations that spans the physiological range (1–10 nM during menses).

**Estrogen, ERs, and effects on smooth muscle.** In the vasculature, acute estrogen exposure leads to blunting of agonist-induced [Ca\(^{2+}\)]\(_i\) responses (34). In comparison, relatively little is known about estrogen effects on [Ca\(^{2+}\)]\(_i\), in the airway. ER\(\alpha\)-deficient mice exhibit increased airway hyperresponsiveness (6), and hormone replacement in ovariectomized animals attenuates allergen-induced asthma (13), suggesting that estrogens contribute to bronchodilation (and/or decreased inflammation), consistent with our hypothesis. However, the role of estrogen signaling may be species- and model-specific, and the underlying mechanisms need further investigation. A single study in mouse tracheal and bronchial rings sensitized by serum from asthmatic humans demonstrated reductions in carbocyl-induced contractions by 30-min exposure to 100 nM E2 (14). Our study demonstrates that even 1 nM E2 can acutely and substantially reduce [Ca\(^{2+}\)]\(_i\), responses of human ASM cells to agonists such as histamine. These novel data strongly support the idea of acute, nongenomic bronchodilatory effects of estrogens in the human airway.

Varying concentrations of E2 produced a nonclassic dose-response curve in human ASM for [Ca\(^{2+}\)]\(_i\); effects. Although the underlying mechanisms are not clear, other studies have noted the antagonizing effects of ER\(\beta\) activation on estrogen effects, mostly in the context of breast cancer, ER\(\alpha\)-dependent transcription, and ER\(\alpha\) degradation (19, 56). Given the likely complex role of ER\(\alpha\) vs. ER\(\beta\) signaling, specific signaling mechanisms were not examined. Regardless, what is important to note is that significant reductions in [Ca\(^{2+}\)]\(_i\), were observed in the physiological range of E2 concentrations.

Previous work by Montgomery et al. (36) and Levin (30) in vascular smooth muscle have tested the efficacy of selective ER agonists on relaxation compared with the nonspecific E2. In their work, the ER\(\alpha\) agonist 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT) was used. (R,R)-THC was selected as the ER\(\alpha\)-specific agonist in our study because it also acts as an ER\(\beta\) antagonist and has similar binding affinity for ER\(\alpha\) as PPT. We found that even low concentrations of ER\(\alpha\)-specific agonist produced substantial reduction in [Ca\(^{2+}\)]\(_i\), mimicking effects of the nonspecific E2, whereas an ER\(\alpha\) agonist was less effective. These data also complement the findings of Montgomery et al. (36) that PPT was more effective than E2 and DPN at relaxing mesenteric arteries, whereas DPN was less effective than both PPT and E2. Therefore, it is possible that although both isofoms are present in human ASM, nongenomic effects may be mediated via only ER\(\alpha\).

There are very little data on estrogen effects on Ca\(^{2+}\) influx in the airway. The present study found that E2 effects on [Ca\(^{2+}\)]\(_i\) were blunted by inhibiting \(\alpha\)-type channels (nifedipine). In isolated mouse tracheal or bronchial rings, Dimitropoulou et al. (14) found that estrogen effects are partly inhibited by blocking BKCa channels (similar to vasculature; Ref. 48). However, in human ASM, we were unable to detect any effect of iberio- toxin on estrogen-induced reduction of [Ca\(^{2+}\)]\(_i\). There are no data on estrogen modulation of SOCE in the airway. During these studies, we noted that on introduction of E2 when [Ca\(^{2+}\)]\(_i\) levels were elevated by the SERCA inhibitor CPA, there was an abrupt reduction in [Ca\(^{2+}\)]\(_i\), raising the possibility of accelerated NCX-mediated efflux. This effect was abolished in KB-R7943-treated cells, however, KB-R7943 had little effect on E2 modulation of SOCE-mediated influx per se, suggesting that even if NCX was present in human ASM, estrogens may not specifically inhibit this mechanism.

As mentioned above, SOCE-mediated Ca\(^{2+}\) influx occurs in ASM and involves TRPC channels. Although estrogens do not appear to modulate TRP channel expression (17), there is no information on interactions between ER activation and TRP (specifically TRPC) channels in terms of [Ca\(^{2+}\)]\(_i\), regulation. In the present study, pretreatment with SKF-96365, a potent inhibitor of receptor-operated channels, did not affect the observed E2 effect on SOCE-mediated Ca\(^{2+}\) influx, indicating that the effect of estrogens is specifically on SOCE rather than other influx mechanisms not involving TRPC.

Studies in vascular smooth muscle (27) have found no evidence for estrogen effects on SR Ca\(^{2+}\) reuptake, a potential mechanism for reducing [Ca\(^{2+}\)]\(_i\). Ovariectomy or prolonged changes in estrogen levels do alter SERCA expression but not activity (4). In the present study, we also did not find any observable impact of acute E2 exposure on SR Ca\(^{2+}\) reuptake.

**Physiological and Clinical Relevance**

The present study demonstrates that in human ASM, functional ERs exist and that estrogens acutely (likely in a nongenomic fashion) reduce agonist-induced [Ca\(^{2+}\)]\(_i\), levels, akin to the well-known effects in the vasculature. Such estrogen effects appear to involve inhibition of Ca\(^{2+}\) influx via different mechanisms. The relevance of these findings lie in the potential modulation of airway tone by ER signaling, or its dysregulation in airway disease in women. In this regard, further study is required to understand how estrogens inhibit Ca\(^{2+}\) influx. In other tissues, estrogens can increase cAMP (54), which produces bronchodilation by targeting plasma membrane mechanisms including influx channels. Whether estrogens modulate cAMP or other pathways involved in bronchodilation is a potentially clinically relevant question that will be examined in future studies.

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**DISCLOSURES**

No conflicts of interest are declared by the author(s).

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