Estrogen effects on human airway smooth muscle involve cAMP and protein kinase A

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Townsend EA, Sathish V, Thompson MA, Pabelick CM, Prakash YS. Estrogen effects on human airway smooth muscle involve cAMP and protein kinase A. Am J Physiol Lung Cell Mol Physiol 303: L923–L928, 2012. First published September 21, 2012; doi:10.1152/ajplung.00023.2012.—Clinically observed differences in airway reactivity and asthma exacerbations in women at different life stages suggest a role for sex steroids in modulating airway function although their targets and mechanisms of action are still being explored. We have previously shown that clinically relevant concentrations of exogenous estrogen acutely decrease intracellular calcium ([Ca2+]i) in human airway smooth muscle (ASM), thereby facilitating bronchodilation. In this study, we hypothesized that estrogens modulate cyclic nucleotide regulation, resulting in decreased [Ca2+]i, in human ASM. In Fura-2-loaded human ASM cells, 1 nM 17β-estradiol (E2) potentiated the inhibitory effect of the β-adrenoceptor (β-AR) agonist isoproterenol (ISO; 100 nM) on histamine-mediated Ca2+ entry. Inhibition of protein kinase A (PKA) activity (KT5720; 100 nM) attenuated E2 effects on [Ca2+]i. Acute treatment with E2 increased cAMP levels in ASM cells comparable to that of ISO (100 pM). In acetylcholine-contracted airways from female guinea pigs or female humans, E2 potentiated ISO-induced relaxation. These novel data suggest that, in human ASM, physiologically relevant concentrations of estrogens act via estrogen receptors (ERs) and the cAMP pathway to nongenomically reduce [Ca2+]i, thus promoting bronchodilation. Activation of ERs may be a novel adjunct therapeutic avenue in reactive airway diseases in combination with established cAMP-activating therapies such as β2-agonists.

sex steroid; bronchial smooth muscle; bronchodilation; bronchoconstriction; calcium; asthma; β-adrenoceptor

Asthma, a respiratory disease characterized by airway inflammation and hyperresponsiveness, is more common in adult women compared with men (9, 24, 27, 32). Furthermore, adult women experience changes in symptom severity and the number of exacerbations with changes in hormonal status (different phases of the menstrual cycle, pregnancy, and menopause) (1, 6, 7, 16, 18). Thus clinical evidence suggests a role for sex steroids (especially female sex steroids) in modulating airway reactivity. What is less clear is whether estrogens or progesterone are detrimental or beneficial to the airway. Whereas clinical evidence would suggest the former, whole animal studies have found reduced airway hyperresponsiveness in the absence of estrogens or estrogen receptor (ER) signaling (3, 4, 10, 23). We previously demonstrated that in human airway smooth muscle (ASM) cells, clinically relevant concentrations of 17β-estradiol (E2) reduce intracellular Ca2+ concentration ([Ca2+]i) (32). Thus it is possible that estrogens promote bronchodilation by acting on ASM.

β-AR agonists are the most effective bronchodilators available to date and are a mainstay of treatment for acute asthma exacerbations (13, 22). Although β2-agonists are generally effective, tachyphylaxis with repeated use as well as reduced efficacy are well-recognized limitations. Accordingly, there is considerable interest in development of adjunct and novel approaches to enhancing bronchodilation.

Isolation of human ASM cells. Studies were conducted using ASM cells enzymatically dissociated from lower-order bronchi (3rd to 6th generation) from lung samples incidental to patient (female only) surgery at Mayo Clinic, Rochester (deidentified samples; typically from lobectomies and pneumonectomies; approved and considered exempt by Mayo Institutional Review Board). Visibly normal airway, as confirmed by a pathologist’s review, was excised and rapidly transferred to the laboratory in ice-cold Hanks Balanced Salt Solution (HBSS). ASM cells were enzymatically dissociated as previously described (32) using a papain dissociation kit (Worthington Biochemical, Lakewood, NJ). Cells were maintained at 37°C (5% CO2, 95% air) using phenol red-free DMEM/F12 medium (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum until ~80% conflu-
ent. Phenol red-free media was used to avoid ER activation. All experiments were performed in cells from passages 1–3 of subculture that had been serum starved for 48 h before experimentation. Periodic assessment of ASM phenotype was performed by verifying stable expression of smooth muscle actin and myosin, and agonist receptors, with lack of fibroblast marker expression.

cAMP assay. ASM cells grown to 80% confluence were nonenzymatically harvested and centrifuged at 200 g for 2 min, washed with PBS, and recentrifuged. The cell pellet was resuspended in HBSS containing 5 mM HEPES and 0.5 mM IBMX, pH 7.4. This cell suspension was then treated according to manufacturer’s instructions (LANCE Ultra cAMP Kit, Perkin-Elmer, Beverly, MA). The cell suspension (5 μl containing ∼1,000 cells) was added to 5 μl of agonist solutions and allowed to incubate for 30 min at room temperature in an OptiPlate 384-well plate. The detection mix containing Eu-cAMP tracer and ULight-anti-cAMP was added and allowed to incubate for 1 h. The assay was read on a Molecular Devices Flexstation 3 system (LANCE settings; 340 nm Ex/665 nm Em; Sunnyvale, CA). Cyclic AMP standards included with the kit and cell suspensions stimulated with HBSS (containing IBMX) only served as standard curve and internal controls, respectively.

Western blot analysis. Standard immunoblotting techniques were used for detection of ERα (Santa Cruz Biotecology, Santa Cruz, CA), ERβ (Santa Cruz SC-53494), and β2-AR (Santa Cruz SC-9042) and detected using horseradish peroxidase-conjugated secondary antibodies in ASM cell lysates subjected to immunoprecipitation. Primary antibody (2 μg, rabbit anti-β2-AR, Santa Cruz SC-9042) was used per 200 μl of whole cell lysate and incubated overnight at 4°C with gentle rotation. Protein A agarose beads (50 μl) were added to the sample and incubated for 4 h at 4°C. Proteins were recovered as described previously (32). These samples were then processed as described for Western analysis (β2-AR; no. 2100065; Sigma-Aldrich, St. Louis, MO). Blots were imaged on a Kodak ImageStation 4000mm (Carestream Health, New Haven, CT) and quantified using densitometry.

[Ca2+]i imaging. Techniques for real-time Ca2+ imaging have been previously described (29, 32). Briefly, ASM cells were loaded with the fluorescent Ca2+ indicator Fura-2 AM (5 μM, 30 min, room temperature) and imaged using a Metaffluor-based real-time microscopy system (Nikon Instruments TE2000 inverted microscope; ×40/1.3 NA oil-immersion lens; 1 Hz; acquisition of 510 nm emissions following alternative excitation at 340 vs. 380 nm). In all experiments involving [Ca2+]i measurements, 10 μM histamine was used as an agonist to induce [Ca2+]i increases.

Force measurements. All animal experiments were approved by Columbia University’s Animal Care and Use Committee, and animal care was in accordance with the guidelines published by the American Physiological Society. Female Hartley guinea pigs (~400 g) were obtained from Charles River and were euthanized with intraperitoneal injection of pentobarbital. The trachea was quickly removed and placed on ice in Krebs-Hensleit buffer of the following composition in millimolar: 118 NaCl, 5.6 KCl, 0.5 CaCl2, 0.24 MgSO4, 1.3 NaH2PO4, 25 NaHCO3, and 5.6 glucose, pH 7.4. The epithelium was removed by gentle abrasion with cotton. Detailed methods have been previously described (14). Briefly, tracheal rings were suspended in 4-ml-water jacketed organ baths (37°C; Radnoti Glass Technology, Monrovia, CA) and connected to a Grass FT03 force transducer (Grass Telefactor, West Warwick, RI) using silk sutures and adjusted to a resting tension of 1 g and allowed to equilibrate for 1 h with buffer exchanges every 15 min. Two complete acetylcysteine (ACH) dose-response curves were constructed (100 nM-100 μM) and then thoroughly washed. Rings were then contracted with −EC50 ACH. Following stable force generation with ACH, samples were treated with vehicle or 50 nM E2 for 20 min, followed by an isoproterenol (ISO) dose response curve (100 μM-10 μM; half-log increments). Measurements are expressed as a percentage of remaining contraction of ACH-plateau force. Parallel studies were conducted in isolated, epithelium-denuded bronchial rings from female patients, obtained as described above for cells. To avoid confounding effects of variable chronic inflammation and/or cigarette smoke exposure, medical histories were reviewed, and samples were taken only from female patients without these problems.

Materials. 2’,5’-Dideoxyadenosine was obtained from Tocris Biosciences (Ellisville, MO). E2, ISO, KTS720, forskolin, and other chemicals were obtained from Sigma Chemical unless mentioned otherwise. Tissue culture reagents, including Dulbecco’s Modified Eagle’s Medium F-12 (DMEM/F12) and FBS, were obtained from Invitrogen.

Statistical analysis. Seven bronchial samples from different female patients were used to obtain ASM cells. [Ca2+]i experiments were performed in at least 25 cells, each from four different bronchial (patient) samples although not all protocols were performed in each sample obtained. In all cases using ASM cells, n represents numbers of patients. The guinea pig trachea studies involved five animals with eight rings/animal. 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 t-test or one-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

Estrogen potentiates ISO effects on [Ca2+]i, regulation. Ca2+ imaging of Fura-2-loaded ASM cells showed that exposure to E2 (1 nM) for 15 min significantly reduced by ~40% increases in peak [Ca2+]i, induced by histamine, consistent with our previous finding (32) (Fig. 1A; P < 0.05). Exposure for 15 min to 100 nM ISO also significantly reduced [Ca2+]i responses to histamine to comparable levels (Fig. 1A; P < 0.05). The additional presence of E2 (1 nM) during ISO exposure resulted in a larger, ~65% reduction in peak [Ca2+]i, which was significantly different from either E2 or ISO alone (Fig. 1, A and C; n = 5, P < 0.05 compared with vehicle as well as to E2).

Estrogen effects on ASM involve adenyl cyclase. In a separate set of experiments, ASM cells were pretreated with the AC inhibitor, 2’,5’-dideoxyadenosine (20 μM) for 15 min before and during exposure to E2, ISO, or a combination of E2 and ISO (concentrations as above). Cells were then stimulated with 10 μM histamine. The AC inhibitor completely prevented the previously observed reduction of [Ca2+]i, responses induced by ISO (Fig. 1, B and C; n = 5; P < 0.05 for inhibitor effect). However, a residual 20–25% reduction in [Ca2+]i persisted in groups treated with E2 (Fig. 1, B and C; P < 0.05).

ER activation increases cAMP. In a first set of experiments, baseline cAMP accumulation by human ASM cells was evaluated using increasing concentrations of forskolin (1 nM to 100 μM, half-log increments) and time-resolved fluorescence resonance energy transfer (TR-FRET). Cyclic AMP standards were used to construct a TR-FRET-concentration curve and calculate the amount of cAMP (Fig. 2A). Baseline cAMP by the forskolin method showed a dynamic range of 30 nM to 10 μM (Fig. 2B). Subsequently, the effects of E2 (10 nM), ISO (100 μM), or the combination of E2 and ISO were evaluated. All three treatments showed a substantial increase in cAMP compared with vehicle (HBSS only) controls (Fig. 2C; n = 5–8, *P < 0.05). The E2 and ISO combination showed an additive effect in total cAMP produced (Fig. 2C; n = 5–8, #P < 0.05 compared with E2 or ISO alone). It should be noted
that lower concentration of ISO was used in this analysis based on pilot studies showing saturation of the cAMP assay when 100 nM concentrations were used.

Estrogen effects on ASM are PKA dependent. In Fura-2-loaded ASM cells, pretreatment with the PKA antagonist KT5720 (100 nM) for 15 min before and during E2 treatment completely prevented E2 effects on the [Ca^{2+}]_i responses to histamine. KT5720 treatment alone (i.e., no E2) did not substantially alter the [Ca^{2+}]_i response to histamine (Fig. 3: n = 4, P < 0.05).

Estrogen potentiates relaxation effects of isoproterenol on contracted airways. In epithelium-denuded tracheal rings from female guinea pigs contracted with 1 μM ACh (~EC_{50} based on a control dose-response curve), the presence of 50 nM E2 increased the extent of relaxation induced by ISO (100 pM-100
nM, half-log increments) compared with vehicle-treated tissues (Fig. 4A; P < 0.05 for E2 effect). Indeed, in the presence of E2, relaxation to and below baseline was achieved at lower ISO concentrations (Fig. 4A). This was also reflected by greater maximum relaxation of 133.0 ± 9.2% in the presence of E2 vs. 116.7 ± 10.8% with vehicle, when additional ISO concentrations were added (Fig. 4A, n = 40 rings from 5 animals, *P < 0.05 significant compared with vehicle, #P < 0.05 significant KT5720 compared with E2 alone (n = 4).

Fig. 3. A and B: E2 effects on ASM involve PKA activation. Acute exposure to E2 decreases [Ca^{2+}]_{i} response to 10 μM histamine by ~40%. This response is abrogated by pretreatment with the PKA antagonist KT5720 (100 nM, 15 min alone, 15 min + E2). KT5720 alone had no effect on [Ca^{2+}]_{i} responses to histamine compared with vehicle. *P < 0.05 significant compared with vehicle.

We compared the efficacy of E2 to enhance ISO-induced relaxation in epithelium-denuded human bronchial rings. Complete relaxation was observed at ~10 nM in the absence of E2, which was comparable to that observed in the guinea pig (not shown). Furthermore, in the presence of E2, the extent of relaxation and sensitivity to ISO were increased, such that, at 300 pM ISO, the human bronchial rings showed greater relaxation in the presence of E2 (Fig. 4B; P < 0.05).

**ERs colocalize with β2-AR in human ASM.** Immunoprecipitation (IP) of the β2-AR with subsequent Western analysis for ER-α or ER-β revealed that both ER isoforms colocalize with β2-AR in separate IPs (Fig. 5). A strong band at 66 kDa corresponding to the full-length ER-α was visible, whereas a band of slightly lower molecular weight (56 kDa) corresponding to ER-β was also expressed. Band intensity varied between patients and was proportional to protein concentration (0.91 ± 0.26, ER-α; 0.68 ± 0.35, ER-β; Fig. 5).

**DISCUSSION**

We have previously demonstrated that clinically relevant concentrations of estrogens reduce [Ca^{2+}]_{i} in human ASM (32). The present study now demonstrates the role of cAMP and...
PKA in mediating these effects. The role of the cAMP/PKA pathway in β2-AR agonist action in bronchodilation is well recognized, as are tolerance and β2-AR desensitization with chronic use (2). Importantly, our results show that estrogens can potentiate the effects of isoproterenol in reducing [Ca²⁺], in human ASM via activation of cAMP and PKA. This potentiating effect appears to translate to enhanced bronchodilation, evidenced by increased ISO-induced relaxation of bronchial rings from both guinea pigs and humans when E₂ is present. Here, the colocalization of β2-AR and ER may play a role in the interaction between these receptors in potentiating relaxation. Our data hint at a potentially novel avenue for enhancing β₂-AR-induced bronchodilation by modulation of estrogen signaling. Given the common, cAMP-dependent mechanism for both receptors, the relevance of such an avenue lies in the possibility of reducing β₂-AR utilization and thus desensitization.

**Estrogen signaling in the airway.** Sex differences in airway function of women at different life stages have raised the question of hormonal influences, especially on ASM, the major regulator of airway tone. In this regard, the limited data on sex steroid effects on airway function are conflicting. Clinical data would suggest a worsening of airway function with increased estrogen levels. However, from an in vivo perspective, ER-α-deficient mice exhibit increased airway hyperresponsiveness (4), an effect reversed by hormone replacement in ovariectomized animals (10), consistent with a bronchodilatory role for estrogens. In vitro, we previously showed that both full-length ER-α and ER-β (32) are present in ASM of female patients and that clinically relevant concentrations of estrogens can activate these receptors to reduce agonist-induced elevations in [Ca²⁺], via inhibition of L-type Ca²⁺ channels and store-operated Ca²⁺ entry (32). The in vitro cellular data suggesting a bronchodilatory effect of estrogens are generally consistent with data in mouse tracheal and bronchial rings sensitized by serum from asthmatic humans, where carbachol-induced contractions are reduced by 100 nM E₂ (11). These effects are also similar to those observed in the vasculature (26) and occur on a rapid time scale (as also demonstrated in the present study), distinct from the well-known transcriptional (genomic) response to estrogens (17). The reasons underlying the discrepancies between the clinical and preclinical data in estrogen effects are not clear but may involve the balance between genomic vs. nongenomic effects as well as estrogen effects on the airway vs. the immune system (30). Regardless, what is important to note is that, as with the vasculature, estrogens do have nongenomic effects on time scales similar to the activation patterns for the cyclic nucleotide cascades, which lend credence to the idea that estrogen effects on [Ca²⁺], (and thus tone) may be mediated via cAMP.

The present study demonstrates that estrogen activates the cAMP/PKA pathway with downstream effects on [Ca²⁺], and relaxation. Estrogen effects on cAMP and PKA signaling in other tissues are controversial. Studies in porcine coronary arteries show that E₂ either increases (19, 31) or has no effect on cAMP levels (8). In rat heart, Wong and colleagues (33) found that estrogen inhibits L-type Ca²⁺ channel influx, blocks isoprenaline-induced increase in cAMP, and suppresses PKA activity. However, in that study, these effects appear to involve estrogen-induced downregulation of β₁AR. In the context of these previous studies in nonairway tissues, the results of the present study are novel in highlighting an estrogen-mediated elevation in cAMP and activation of PKA in human ASM. Under physiological conditions, it is likely that E₂ activates ER-α and ER-β, both of which were shown to independently colocalize with β₂-ARs in human ASM.

The downstream effects of estrogen-induced activation of the cAMP/PKA pathway is reduced [Ca²⁺], in ASM cells and potentiation of ISO-induced relaxation of precontracted ASM tissue. The cAMP-PKA pathway can certainly modulate a number of Ca²⁺ regulatory mechanisms. In neurons (25, 34), nongenomic, membrane actions of estrogen involve voltage-gated calcium channels, which are also a target in ASM (32) and thus a potential mechanism for cAMP-induced reduction in [Ca²⁺]. In a previous study also in human ASM, we have shown that store-operated Ca²⁺ entry, a mechanism inhibited by estrogens (32), is also inhibited by cAMP (28). PKA is also known to phosphorylate the Ca²⁺-sensitive large conductance potassium (BKCa) channel (21), and previous studies in murine models of asthma have shown beneficial effects of estrogen being mediated in part via BKCa channels (11); as such, it is likely that estrogen-induced increases in cAMP/PKA activity affect plasma membrane Ca²⁺ regulatory mechanisms in ASM.

**Estrogen and β₂-AR effects in ASM.** The role of the cAMP/PKA pathway in β₂-AR action in ASM is well known (13, 15). It is also recognized that frequent or sustained β₂-AR activation results in receptor internalization and desensitization (2), raising the need for alternative or adjunct avenues to sustain bronchodilatory activity. The results of the present study show that clinically relevant concentrations of estrogen can potentiate β₂-AR induced reduction in [Ca²⁺], of ASM, as well as relaxation of ASM. From a clinical perspective, there are limited data supporting a beneficial role for estrogens. For example, Gibbs and colleagues (12) showed that, in the few days before the onset of menses, women asthmatics had significantly decreased peak expiratory flow rates and increased dependence on albuterol (12), whereas exogenous estrogen has been reported to improve asthma symptoms in women (6). Importantly, our data show that acute estrogen administration potentiates the effects of isoproterenol, resulting in greater relaxation at submaximal isoproterenol concentrations as well as greater maximal relaxation compared with isoproterenol-treated tissues alone. Furthermore, we show that both estrogens and β₂-AR activate a common mechanism, cAMP/PKA, thus raising the possibility of using estrogens to enhance β₂-AR agonist effects to induce bronchodilation. Whether the combination of estrogens and β₂-AR agonists is of benefit clinically remains to be shown.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.
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