

Estrogen Reduces Carbachol-Induced Constriction of Asthmatic Airways by Stimulating Large-Conductance Voltage and Calcium-Dependent Potassium Channels

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Both the incidence and severity of asthma in women are influenced by fluctuations in estrogen (E_2) levels, raising the possibility that E_2 s may reduce the hyperresponsiveness that is characteristic of asthma. We examined the effect of E_2 and its downstream signaling pathways in isolated mouse bronchial and tracheal rings passively sensitized either with serum from patients with atopic asthma (ATR) or with serum from control subjects (CTR). ATR exhibited significantly higher sensitivity to carbachol than CTR. Pretreatment of ATR with E_2 shifted the carbachol concentration–response curve (CCRC) toward that of CTR. The E_2 effect was abolished by the nitric oxide synthase inhibitor, L-nitroarginine methyl ester, the soluble guanylyl cyclase inhibitor, quinoxalin-1, or the protein kinase G inhibitor, KT5823. Inhibition of the large-conductance, calcium-activated potassium (BK_{Ca}) channel activity with iberiotoxin also attenuated the E_2 effect on ATR. In patch-clamp studies, E_2 increased by 50-fold the BK_{Ca} channel activity in freshly isolated airway smooth muscle cells. This increase was completely blocked by KT5823. These studies suggest that, at physiologic concentrations, E_2 can prevent cholinergic-induced constriction of asthmatic tracheal rings by activating the nitric oxide–cGMP–protein kinase G pathway to increase BK_{Ca} channel activity.

Keywords: airway smooth muscle, asthma, estrogen, large conductance voltage and calcium-dependent potassium channels, nitric oxide

Asthma affects $\sim 5\%$ of the population of the United States, and the incidence of this disease continues to increase despite efforts to enhance therapeutic measures (1). Although asthma affects people of all ages, there is a clear age dependency and sexual dimorphism. Asthma is most prevalent before age 10, with male sufferers outnumbering females by 2:1; however, after puberty, more females than males are afflicted by asthma (1). These findings suggest the interesting possibility that sex hormones may influence both the incidence and severity of asthma.

There is increasing evidence that estrogen (E_2) influences airway function during a woman's reproductive years, when levels of sex hormones undergo cyclical variations (2–4). Premenstrual worsening of asthma was described more than 70 yr ago (5), with nearly half of women with asthma exhibiting increased symptoms during this "low-estrogen" (E_2 blood levels of ~ 0.01 nM) phase (6, 7). Similarly, there is an exacerbation of asthma symptoms

(8) and a decline in pulmonary function (7–9) as hormone levels decrease in the premenstrual luteal phase. Conversely, the frequency and severity of asthma lessens near ovulation when E_2 levels are highest (E_2 blood levels of ~ 1 nM) or after exogenous estradiol (8, 9) and oral contraceptives (10, 11). Furthermore, forced expiratory volume and vital capacity are higher in the early luteal phase, when E_2 and progesterone levels are high (12). Overall, women with asthma have lower estradiol levels compared to women without asthma (13), thus reinforcing the notion that a negative correlation exists between sex hormone levels and asthma symptomatology.

Although it is becoming increasingly clear that E_2 affects the incidence and severity of asthma, there is virtually no direct evidence of potential mechanisms by which E_2 might influence airway reactivity. Therefore, the objective of the present study was to investigate the cellular mechanisms by which E_2 modulates contractility of airway smooth muscle (ASM). We measured carbachol-stimulated mouse airway ring contraction *ex vivo*, and also studied the excitability of single ASM cells via the patch-clamp technique. To simulate the asthmatic state, we exposed these tissues and cells to human serum from either patients with asthma or control subjects without asthma. Our findings demonstrate that E_2 can both relieve and prevent airway hyperresponsiveness associated with asthma, and, therefore, may represent a heretofore-unexplored means of treating this increasingly common and debilitating disease.

MATERIALS AND METHODS

Animals and Procedures

Experiments were approved by the Institutional Committee for Animal Use in Research and Education and adhered to the American Physiological Society standards of humane animal experimentation. C57BL6 mice (Harlan, Indianapolis, IN; 20–30 g) were killed by cervical dislocation. The tracheas and left and right mainstem bronchi were removed, cleared from adherent connective tissue, and cut into transverse rings measuring 3 mm in length. Each ring was mounted between two stainless steel clips in a Myodata 2.02 organ bath system (DMT-USA, Atlanta, GA) filled with aerated Krebs solution (glucose 11.1 mM, KCl 4.7 mM, NaCl 118 mM, $CaCl_2$ 2.5 mM, $MgCl_2$ 0.5 mM, NaH_2PO_4 1 mM, and $NaHCO_3$ 25 mM) maintained at 37°C. The tracheal or bronchial rings were set at optimal length by equilibration against a passive preload of 0.6 g, as previously determined for these types of experiments (14). Only one agonist was tested in one ring, but different rings from the same animal were tested under different conditions.

Responses were expressed as percentage of the maximal contractile response for carbamylcholine chloride (carbachol, 10^{-4} M) in each ring. Cumulative concentration–response curves (CCRC) to carbachol were produced and expressed as the percentage of maximal contractile response to carbachol in each ring. The efficacy of carbachol was defined as the maximal force generated (F_{max}), and estimated from the final plateau level of the CCRC. Potency was characterized as the agonist (carbachol) concentration eliciting half-maximal response (EC_{50}), i.e., the concentration producing a half maximal contractile force ($F_{max}/2$), and was determined graphically. A geometrical mean EC_{50} and the

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SEM were then calculated by computer from the average CCRC of all CCRCs in each experimental group.

Sensitization of ASM Tissue

Bronchial or tracheal rings were incubated for 24 h at room temperature in either (1) human serum containing immunoglobulin (Ig) E > 1,000 U/ml obtained from allergic patients with moderate to severe asthma and 4–5/6+ radioallergosorbent test (RAST)–positive (specific IgE concentration of more than 17.5 Phadebas RAST U/ml) to *Dermaphagoides pteronyssinus*, *Dermatophagoides farinae*, and ragweed, and positive skin test to these antigens; or (2) human serum from individuals without atopy or asthma with normal serum IgE levels (< 70 U/ml) as previously described (15). High-IgE sera were pooled from 18 patients with asthma (12 men and 6 women, 2 of which were girls under 10 yr of age and none of which were pregnant). Because 14 of the 18 sera were from male or prepubertal female subjects, the amount of E₂ and progesterone concentrations in the pooled sera, although not measured, was probably insignificant for the present studies; furthermore, because all high-IgE sera were pooled, all tracheal rings incubated with serum from asthmatic patients (ATR) were exposed to sera of identical makeup. Sera were drawn from all 18 subjects during the first visit to the allergy clinic for screening tests; none of the subjects were on prescription medication before blood withdrawal.

Drug Treatments

Rings were exposed to E₂ and/or progesterone for 30 min before the start of the tension studies or before lysis for protein estimation. Receptor, enzyme, or channel inhibitors (ICI182780, L-nitroarginine methyl ester [L-NAME], quinoxalin-1, KT5823, iberiotoxin) were added to the E₂/progesterone mixture for the same amount of time.

Cell Culture

Mouse tracheal smooth muscle segments were harvested and grown in smooth muscle growth medium containing Amniomax C-100 (Gibco-BRL, Carlsbad, CA), supplemented with penicillin and streptomycin. Cultures of smooth muscle cells that formed around the tissue were trypsinized (0.05% trypsin, 0.53 mM EDTA), passaged, expanded, and genotyped to confirm their identity. Cells were stained with smooth muscle α -actin (Clone 1A4; Sigma, St. Louis, MO) and checked for classic “hill-and-valley” morphology to confirm their smooth muscle status. Cells (passages 1 and 2) were plated onto 12-mm coverslips for patch-clamp experiments. To ensure that cells retained a contractile phenotype in culture, cover slips were treated with a high-potassium depolarizing solution and cell contraction was observed under a microscope (IMT-2; Olympus Co., Tokyo, Japan). Only contractile cells were employed in patch-clamp studies.

Patch-Clamp Studies

For cell-attached patch studies, the recording chamber contained the following solution (mmol/liter): 140 KCl, 10 MgCl₂, 0.1 CaCl₂, 10 HEPES, and 30 glucose (pH 7.4; 22–25°C). Activity of single potassium channels was recorded (pCLAMP 6.0.4; Axon Instruments, Foster City, CA) in cell-attached patches by filling the patch pipette (2–5 M Ω) with Ringer solution (mmol/liter): 110 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, and 10 HEPES. Voltage across the patch was controlled by clamping the cell at 0 mV with the high-concentration extracellular potassium solution. Average channel activity (average NPo; expressed as number of channels $\times$ single-channel open probability) in patches with multiple large-conductance voltage and calcium-dependent potassium (BK_{Ca}) channels was determined as described previously (16). NPo calculations were based on 10–15 s of continuous recording during periods of stable channel activity. Single-channel data were recorded at a potential of +40 mV where BK_{Ca} channel openings are easily distinguished from other channel species to permit more accurate statistical analysis.

Western Blotting

Culture medium of confluent tracheal smooth muscle cells was replaced for 24 h with medium containing either (1) 10% human serum containing > 1,000 U/ml IgE obtained from allergic patients with moderate to severe asthma (as above), or (2) 10% human serum from individuals

without atopy or asthma (control subjects). Cells were then treated with drugs, manually dissected, and placed in ice-cold homogenization buffer containing protease inhibitors (mM): 50 Tris-HCl, pH 7.7; 0.1 EDTA; 1 EGTA; 250 sucrose; 0.1% 2-mercaptoethanol; 10% glycerol; 1 phenylmethylsulfonyl fluoride; 1 pepstatin A; 2 leupeptin; and 0.1% aprotinin. The cells were homogenized with a glass/glass homogenizer, and the homogenate was centrifuged at 1,000 $\times$ g for 10 min, followed by a second centrifugation at 14,000 $\times$ g for 15 min, at 4°C. The supernatant was used for Western blot analysis as described in detail previously (17), fractionated on 8% polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibodies against type 1 nitric oxide (NO) synthase (NOS1; 1:1,000) and phosphoNOS1 (pNOS1; 1:500). The bound antibody was detected by electrochemiluminescence (ECL; Amersham, Little Chalfont, UK).

Drugs

KT5823 was purchased from Calbiochem (San Diego, CA). ICI182780 was purchased from Tocris (Ellisville, MD). All other agents were purchased from Sigma.

Statistical Analysis

All data were expressed as mean $\pm$ SEM. Statistically significant differences between two groups were evaluated by Student's *t* test. Comparisons among multiple groups were made by the one-way analysis of variance test. A probability of less than 0.05 was considered to indicate a significant difference.

RESULTS

Effect of Carbachol on Tracheal Rings

Carbachol caused a concentration-dependent contraction of tracheal rings (Figure 1A). The carbachol response was significantly enhanced in ATR compared to rings incubated with sera from control subjects (CTR). The EC₅₀ of carbachol in the former group decreased almost 3-fold in comparison with the EC₅₀ of the CTR, reflecting a profound increase in the carbachol-induced contraction ($P < 0.001$). Similarly, carbachol induced more than a 2-fold greater Fmax in ATR (Fmax = 7.46 ± 1.52 g, $n = 18$) compared to that in CTR (Fmax = 3.44 ± 0.66 g, $n = 9$) ($P < 0.025$; see Figure 1B).

Effect of E₂ on Isometric Contraction of Tracheal Rings

A 30-min treatment with E₂ completely prevented the enhancement of carbachol-induced contractility of ATR. CCRCs to carbachol in ATR shifted to the right in the presence of 100 nM E₂ ($n = 19$, $P < 0.01$), bringing them to the levels of CCRCs from CTR (Figure 1C). The effect of E₂ was abolished in the presence of ICI182780, the nonselective E₂ receptor antagonist, which decreased the EC₅₀ 2-fold, back to the level found in the absence of E₂ ($n = 8$, $P < 0.05$; Figure 1C).

Different concentrations of E₂ (1, 10, and 100 nM) had no effect on the CCRC induced by carbachol in CTR ($P < 0.4$; Figure 2A). Similarly, the maximal contraction remained the same between control rings and rings treated with 100 nM E₂ (Fmax = 3.45 ± 0.65 g and 3.08 ± 0.73 g, respectively; Figure 2B). Conversely, different concentrations of E₂ (1 nM, $n = 4$; 10 nM, $n = 9$; and 100 nM, $n = 19$) shifted the CCRC to carbachol to the right in ATR. The Fmax was also reduced in rings that were pretreated with 100 nM E₂ (from 7.46 ± 1.51 g to 4.07 ± 0.61 g, $P < 0.05$; Figure 2D).

Effect of E₂ on Isometric Contraction of Bronchial Rings

Carbachol induced contraction of first order bronchial rings ($n = 5$). The carbachol effect was intensified 10-fold when the rings were incubated overnight with medium containing sera from patients with asthma ($n = 10$), shifting the curve to the left ($P < 0.001$). A 30-min treatment of the bronchial rings with 100 nM E₂ shifted the average CCRC back toward the right ($n = 9$,

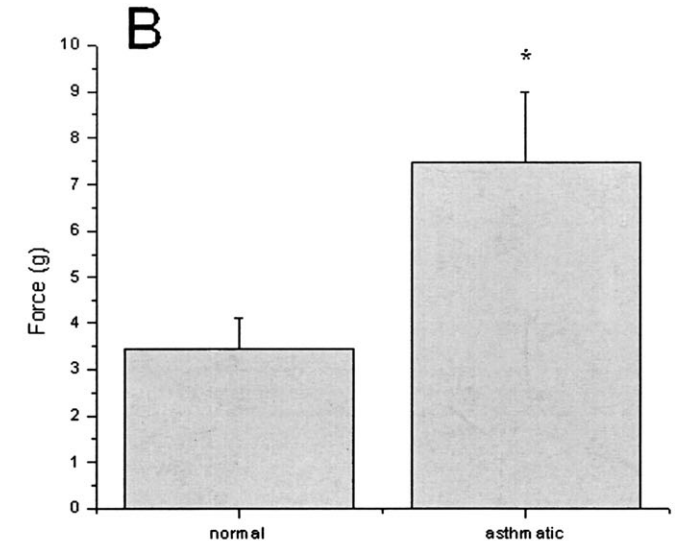
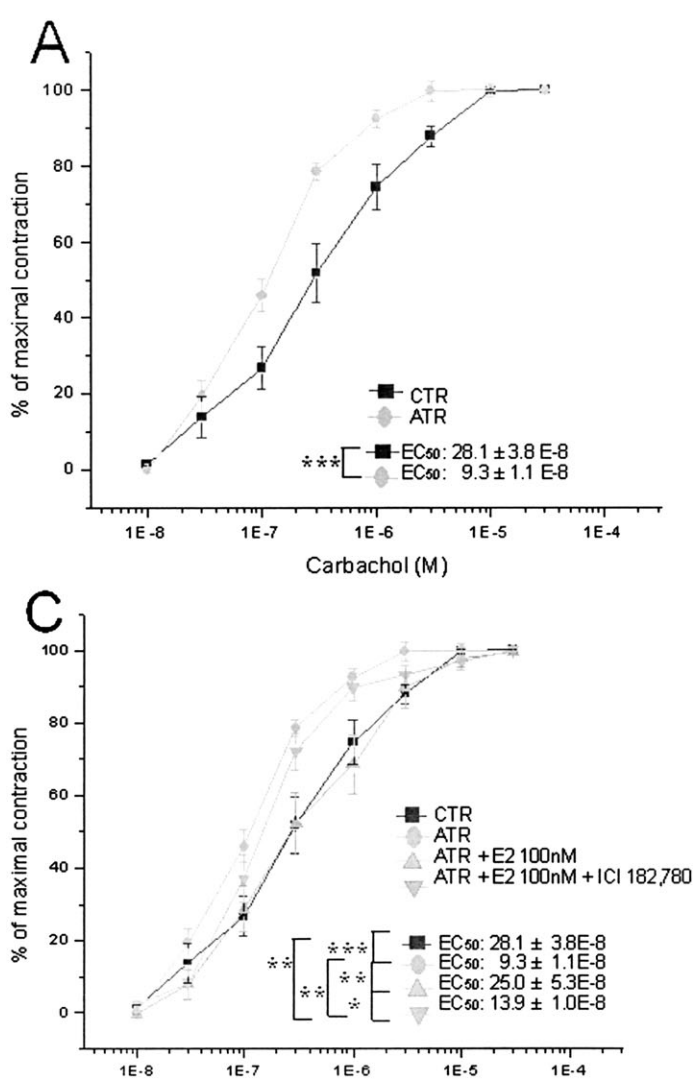


Figure 1. Effects of carbachol on active tension in isolated mouse trachea rings incubated overnight with serum from normal subjects (CTR) or from patients with asthma (ATR). (A) Data expressed as percent of maximal contraction of each ring. Results are expressed as means $\pm$ SEM. *n* (number of tracheal rings in each group): CTR (closed squares, *n* = 9) and ATR treated with vehicle (closed circles, *n* = 18). (B) Maximal force (F_{max}) of contraction to carbachol is much higher in ATR than in CTR. (C) Effects of E₂ on carbachol-induced contraction of tracheal rings. CTR (closed squares, *n* = 9), ATR treated with vehicle (closed circles, *n* = 18), ATR treated with 100 nM E₂ (closed triangles, *n* = 19), or ATR treated with 100 nM E₂ and 10 μ M of the E₂ receptor antagonist ICI 182780 (closed inverted triangles, *n* = 8).

$P < 0.01$ compared with the CCRC to carbachol of ATR without E₂; Figure 3).

Effect of E₂ and Progesterone on Isometric Contraction of Tracheal Rings

In early pregnancy, the circulating blood concentrations of E₂ and progesterone are ~ 10 nM and 140 pM, respectively (18). Treatment of ATR with this combination of hormones increased the EC₅₀ to carbachol 3-fold and shifted the CCRC to carbachol to the right ($P < 0.02$, *n* = 10; Figure 4A). The CCRC to carbachol of CTR was not altered by either E₂ or progesterone alone or by the E₂/progesterone combination ($P > 0.05$; data not shown). In late pregnancy, the circulating blood concentrations of E₂ and progesterone can reach ~ 100 nM and 260 pM, respectively (18). A 30-min treatment of ATR with this combination of hormones induced a 14-fold shift to the right of the CCRC to carbachol as compared with the CCRC of ATR treated with vehicle (*n* = 18, $P < 0.001$; Figure 4B). The CCRC to carbachol of rings treated with the combination of these hormones increased 3-fold and shifted to the right even when compared with CCRC to carbachol in CTR ($P < 0.01$; Figure 4B).

Downstream Mediators of the Effects of E₂

L-NAME, the nonspecific inhibitor of NOS, completely inhibited the effect of E₂ on carbachol-induced constriction of ATR ($P < 0.05$, *n* = 3; Figure 5A). L-NAME decreased the EC₅₀ to

carbachol by 50% and shifted the CCRC to the left of the CCRC of CTR ($P < 0.005$). Western blot analysis revealed equal expression of type I or neuronal NOS (NOS1) in lysates of tracheal smooth muscle cells treated with medium containing sera from subjects either with or without asthma (Figure 5B). However, there was a significant decrease in NOS1 Ser-1416 phosphorylation (pNOS1) of cells incubated with sera from subjects with asthma, which was prevented by treatment with E₂ (Figure 5B).

Quinoxalin-1 (ODQ), a soluble guanylate cyclase inhibitor, also inhibited by 75% the effect of E₂ ($P < 0.005$, *n* = 8; Figure 5C), and shifted the CCRC to the left, away from the CTR ($P < 0.001$) and toward the CCRC of carbachol in ATR incubated without E₂ ($P < 0.1$).

KT5823, the protein kinase G (PKG) inhibitor, also inhibited the E₂ effect on the carbachol CCRC in ATR ($P < 0.05$, *n* = 7; Figure 5D). KT5823 shifted the CCRC to carbachol by 50% toward the untreated ATR ($P < 0.01$) and away from the CTR ($P = 0.005$).

Effect of E₂ on BK_{Ca} Channels of ASM

In ATR, iberiotoxin, a BK_{Ca} channel inhibitor, attenuated the effect of E₂ ($P < 0.05$, *n* = 9; Figure 6A) and shifted the CCRC to carbachol by 50% toward the vehicle-treated, and away from the CCRC of E₂-treated rings or of CTR ($P < 0.005$).

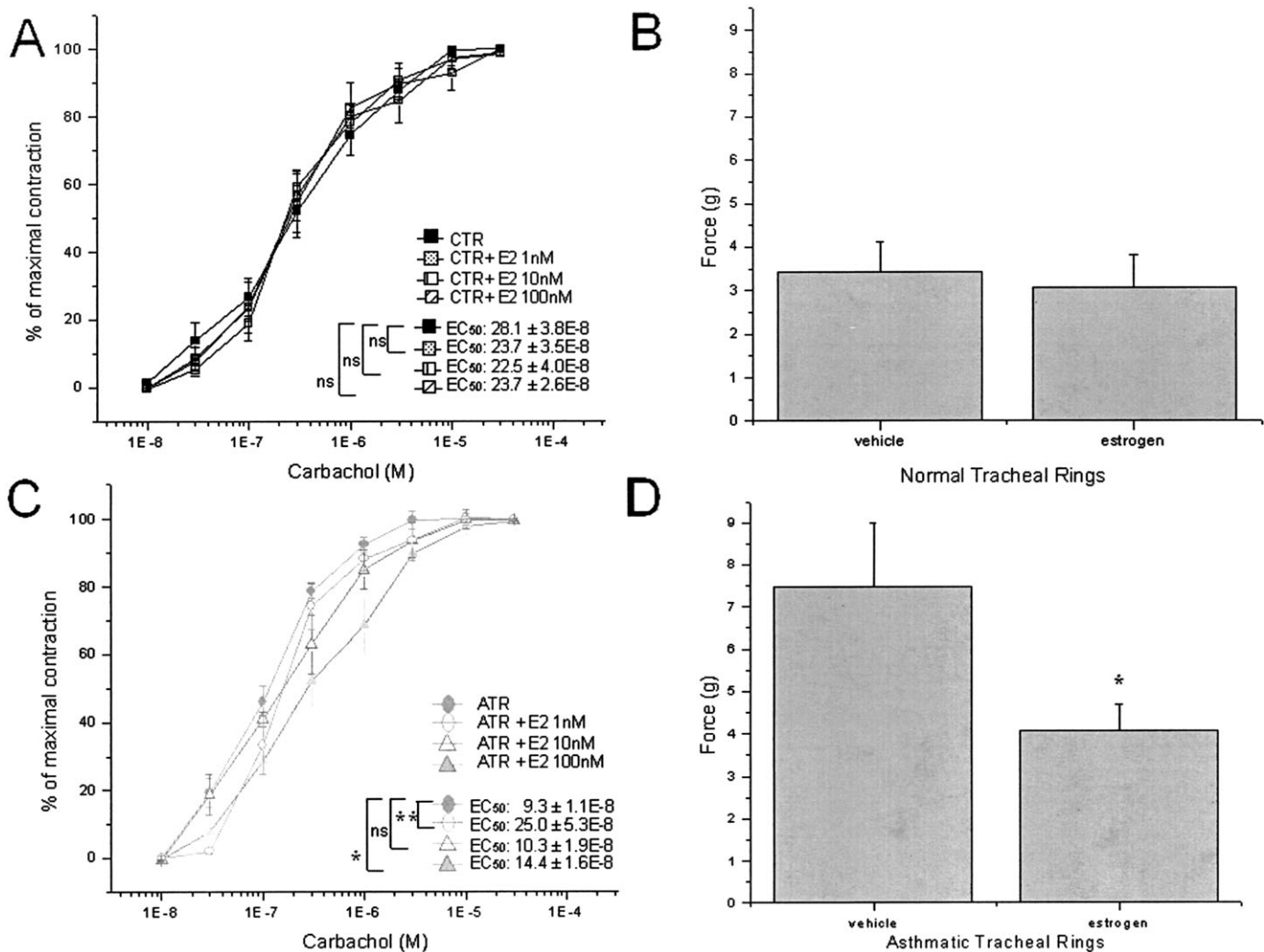


Figure 2. Effects of carbachol on active tension in isolated mouse trachea rings treated with E₂. (A) Mean dose–response curves of carbachol in CTR and then treated with vehicle (closed squares, $n = 9$), 1 nM E₂ (gray squares, $n = 7$), 10 nM E₂ (open squares, $n = 5$), or 100 nM E₂ (hatched squares, $n = 9$). (B) Effects of 30-min treatment with vehicle or 100 nM E₂ on carbachol-generated F_{max} of contraction in CTR. (C) Mean dose–response curves of the effect of carbachol in ATR and then treated with vehicle (closed circles, $n = 18$), 1 nM E₂ (open circles, $n = 19$), 10 nM E₂ (open triangles, $n = 9$), or 100 nM E₂ (closed triangles, $n = 4$). * $P < 0.05$; ** $P < 0.01$. (D) Effects of 30-min treatment with vehicle or 100 nM E₂ on carbachol-generated F_{max} of contraction in ATR ($P < 0.001$ from vehicle).

Whole-cell currents recorded with the perforated-patch configuration before and 10 min after the addition of 1 mM tetraethylammonium (TEA), which blocks BK_{Ca} channels selectively at this concentration, revealed an inhibitory effect of TEA that was reversible upon washout (Figures 6B and 6C). The inhibition of BK_{Ca} channel activity with 1 mM TEA suppressed steady-state outward currents by 86% (+50 mV). These findings strongly suggest that BK_{Ca} channels play a dominant role in regulating ASM excitability. Patch-clamp studies on isolated tracheal smooth muscle cells revealed that 100 nM E₂ stimulated the activity of a prominent, large-conductance potassium channel within 15–20 min (Figure 6D). This channel exhibited a microscopic conductance of nearly 200 pS in symmetrical potassium gradients (0 mV), and was stimulated by intracellular calcium (data not shown). E₂-stimulated BK_{Ca} channel activity was attenuated by 300 nM KT5823, an inhibitor of PKG activity, indicating that PKG mediates this response to E₂. On average, 100 nM E₂ stimulated NPo by more than an order of magnitude (NPo, 0.003 ± 0.002 and 0.140 ± 0.004 for control and E₂, respectively; $n = 3$,

$P < 0.001$), whereas KT5823 completely reversed the effect of E₂ (0.004 ± 0.001 , $P < 0.001$).

DISCUSSION

Asthma is characterized by a chronic inflammatory state of the airways and hyperresponsiveness to bronchoconstrictors. We have sensitized ASM with serum from patients with asthma and studied the resulting hyperresponsiveness *in vitro*. The results of this study, consistent with previous clinical observations (19), suggest that sex hormones could modulate the severity of asthma, and have led to the formulation of a novel molecular model (Figure 7) that can explain, at least in part, how E₂ could inhibit bronchoconstriction and thereby reverse and/or prevent one of the most serious complications of asthma.

Despite certain differences between murine and human models of allergic airway disease, mice are used extensively to investigate airway hyperresponsiveness (20). They are small, easily bred animals that offer the availability of transgenic and gene-targeted

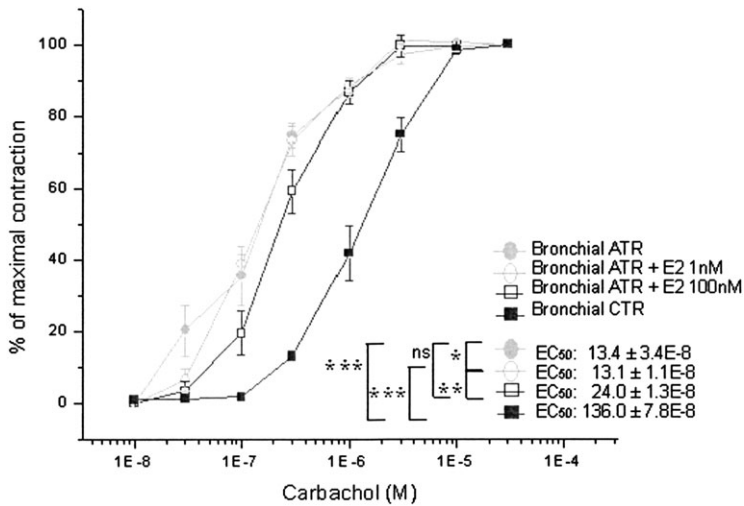


Figure 3. Effects of carbachol on active tension in isolated mouse bronchial rings incubated overnight with serum from normal subjects (closed squares, $n = 5$) or from patients with asthma and treated for 30 min with vehicle (closed circles, $n = 10$), 10 nM E₂ (open circles, $n = 7$), or with 100 nM E₂ (open squares, $n = 9$). Data are expressed as percent of the maximal contraction in each ring. Results are expressed as means $\pm$ SEM. n = number of rings in each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

phenotypes and a variety of specific reagents for phenotypic and functional analysis of the cellular and mediator responses (21). Like humans, these animals exhibit both an early- and a late-phase response to airway allergen challenge, and have the ability to develop sub-basement membrane fibrosis, smooth muscle hyperplasia, and allergen-specific responses (22). All these characteristics make mice a reasonable model of human airway disease.

We observed that ATR were hyperresponsive to carbachol in comparison to CTR. Such treatment has been shown to induce changes in the tissue's agonist-mediated constrictor and relaxant responsiveness that phenotypically resemble the proasthmatic state (15), thus rendering tissues hyperresponsive to contractile agents (23). Furthermore, exposing "asthmatic" rings to physiologic concentration of E₂ prevented the development of hyper-

responsiveness to carbachol. These findings indicate a novel protective effect of E₂ in preventing airway hyperresponsiveness in asthma.

Pregnancy is associated with a 2-fold improvement in airway asthma responsiveness and severity, with the greatest improvement observed in women who initially are the most hyperresponsive (11). In the present study, we investigated the effect of physiologic levels of sex hormones on airway reactivity in tracheal rings stimulated with either nonasthmatic or asthmatic sera by simulating the circulating concentrations of E₂ and/or progesterone observed during early and late pregnancy. Our results indicate that exposing tissues to elevated concentrations of the two hormones (10 nM and 100 nM E₂, 140 pM and 260 pM progesterone) reversed the hyperresponsiveness that occurs dur-

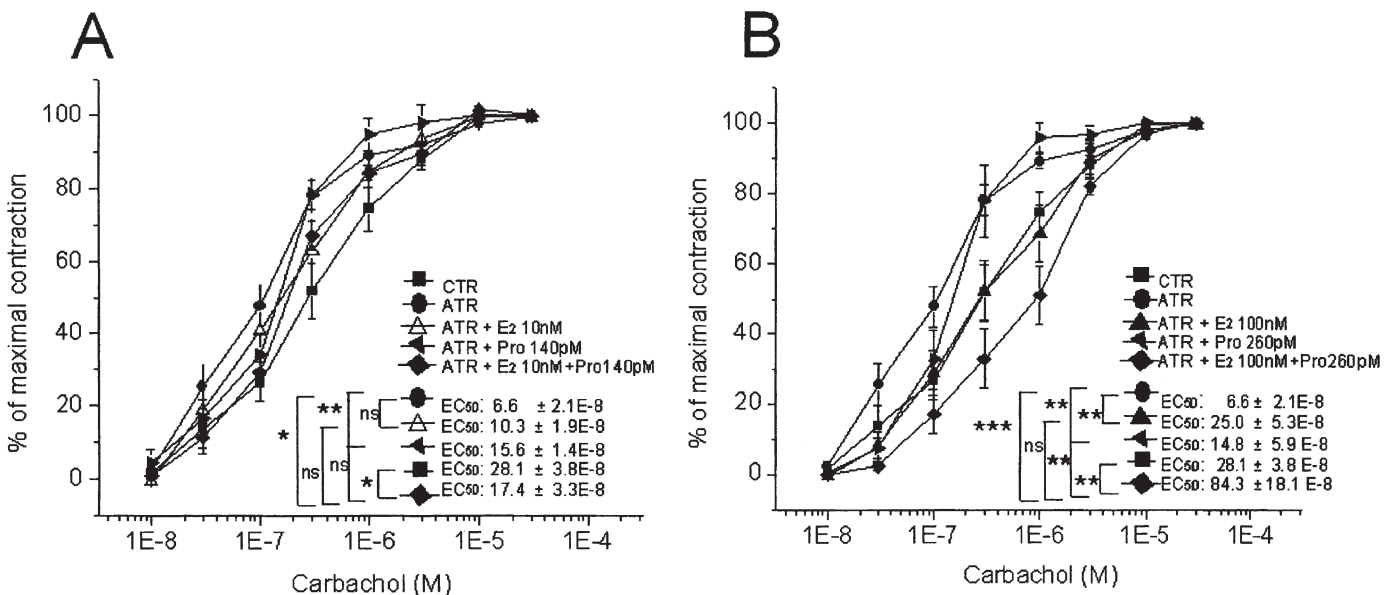


Figure 4. Effects of carbachol on active tension in isolated mouse CTR or ATR and treated for 30 min with E₂ and progesterone to simulate early and late pregnancy hormone levels. (A) Early pregnancy simulation: mean dose-response curves of the effect of carbachol on CTR (closed squares, $n = 9$), and ATR treated with vehicle (closed circles, $n = 6$), 140 pM progesterone (left-pointing closed triangles, $n = 6$), 10nM E₂ (open triangles, $n = 9$), or a combination of 140 pM progesterone and 10 nM E₂ (closed diamonds, $n = 10$). (B) Late pregnancy simulation: mean dose-response curves of the effect of carbachol on CTR (closed squares, $n = 9$) and ATR treated with vehicle (closed circles, $n = 6$), 260 pM progesterone (left-pointing closed triangles, $n = 4$), 100 nM E₂ (closed triangles, $n = 17$), or a combination of 260 pM progesterone and 100 nM E₂ (closed diamonds, $n = 18$). * $P < 0.05$; ** $P < 0.01$.

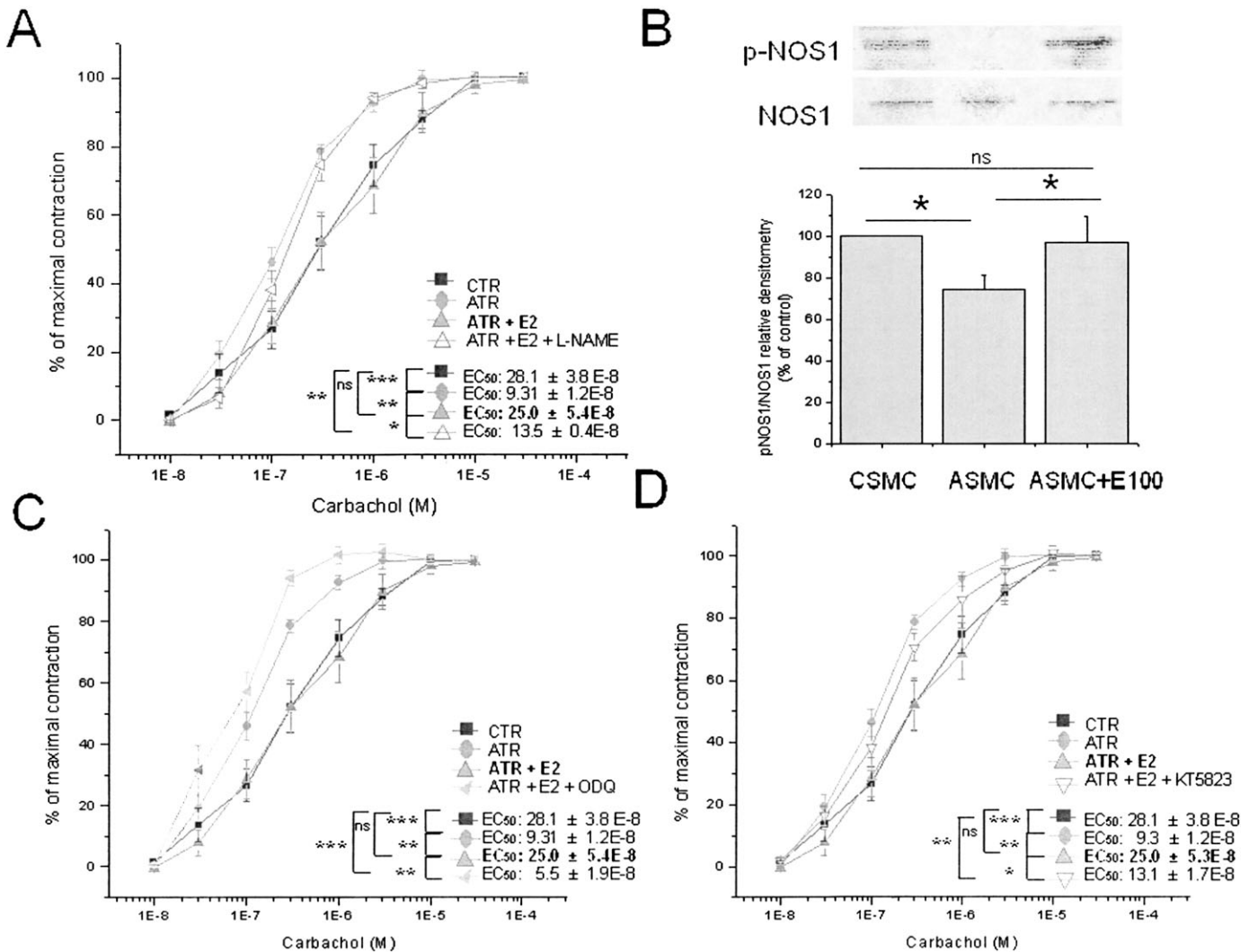


Figure 5. Effects of carbachol on active tension in CTR or ATR. (A) Mean dose-response curves of carbachol on CTR (closed squares, $n = 9$), and ATR treated with vehicle (closed circles, $n = 18$), 100 nM E₂ (closed triangles, $n = 19$), or 100 nM E₂ and 100 μ M of the nitric oxide synthase (NOS) inhibitor L-nitroarginine methyl ester (L-NAME; open triangles, $n = 3$). (B) Estrogen (E₂; 100 nM) stimulated phosphorylation of NOS1 in airway smooth muscle (ASM) cells exposed to serum from patients with asthma for 24h (ASMC) or to serum from control subjects (CSMC). Representative Western blot of phospho-NOS1 (upper blot) and of total NOS1 levels (lower blot). Graph shows the average percent of control change in relative density of pNOS1/NOS1 in four blots. (C) Mean dose-response curves of carbachol on CTR (closed squares, $n = 9$) and ATR treated with vehicle (closed circles, $n = 18$), 100 nM E₂ (closed triangles, $n = 19$), or 100 nM E₂ and 10 μ M of the soluble guanylate cyclase inhibitor quinoxalin-1 (ODQ; left-pointing closed triangles, $n = 8$). (D) Mean dose-response curves of carbachol on CTR (closed squares, $n = 9$) and ATR treated with vehicle (closed circles, $n = 18$), 100 nM E₂ (closed triangles, $n = 19$), or 100 nM E₂ and 300 nM of the protein kinase G (PKG) inhibitor KT5823 (open inverted triangles, $n = 7$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

ing asthma. In the early pregnancy simulation experiments, progesterone conferred partial protection from hyperresponsiveness of ATR to carbachol; however, no further protection was observed in the progesterone and E₂ combination. In the late pregnancy simulation experiments, pretreatment of ATR with either progesterone or E₂ alone partly prevented the hyperresponsiveness to carbachol. However, the combination of E₂ and progesterone conferred a significantly higher protection. Thus, the effect of progesterone appears to be the same in either early or late pregnancy simulation, regardless of the concentration used. Conversely, the effect of increased E₂ concentration from early to late pregnancy simulation (10 versus 100 nM) is significantly greater (more protection), both when occurring alone and when in combination with progesterone. Our *in vitro* findings are consistent with the clinical observation that more than 70% of women

with asthma have fewer symptoms in the last month of pregnancy, during which hormone levels are highest (24).

There are two intracellular E₂ receptors (ER α and ER β) that mediate a variety of estrogenic actions. Classically, these receptors function as ligand-activated transcription factors to regulate the expression of E₂-responsive genes (25). In contrast, we and others have shown that E₂ may also exert rapid, nongenomic responses on endothelial and smooth muscle cells (26). At present, the nature of the E₂ effect on ASM and the ER subtype(s) involved remains unclear; however, treating tracheal rings with ICI182780, a nonselective ER antagonist (27), inhibited the effect of E₂ on ATR, thus indicating that E₂ prevents hyperresponsiveness to carbachol via activation of E₂ receptors. ER activation is known to stimulate a diverse array of intracellular signaling mechanisms, such as elevation of intracellular Ca²⁺ concentration,

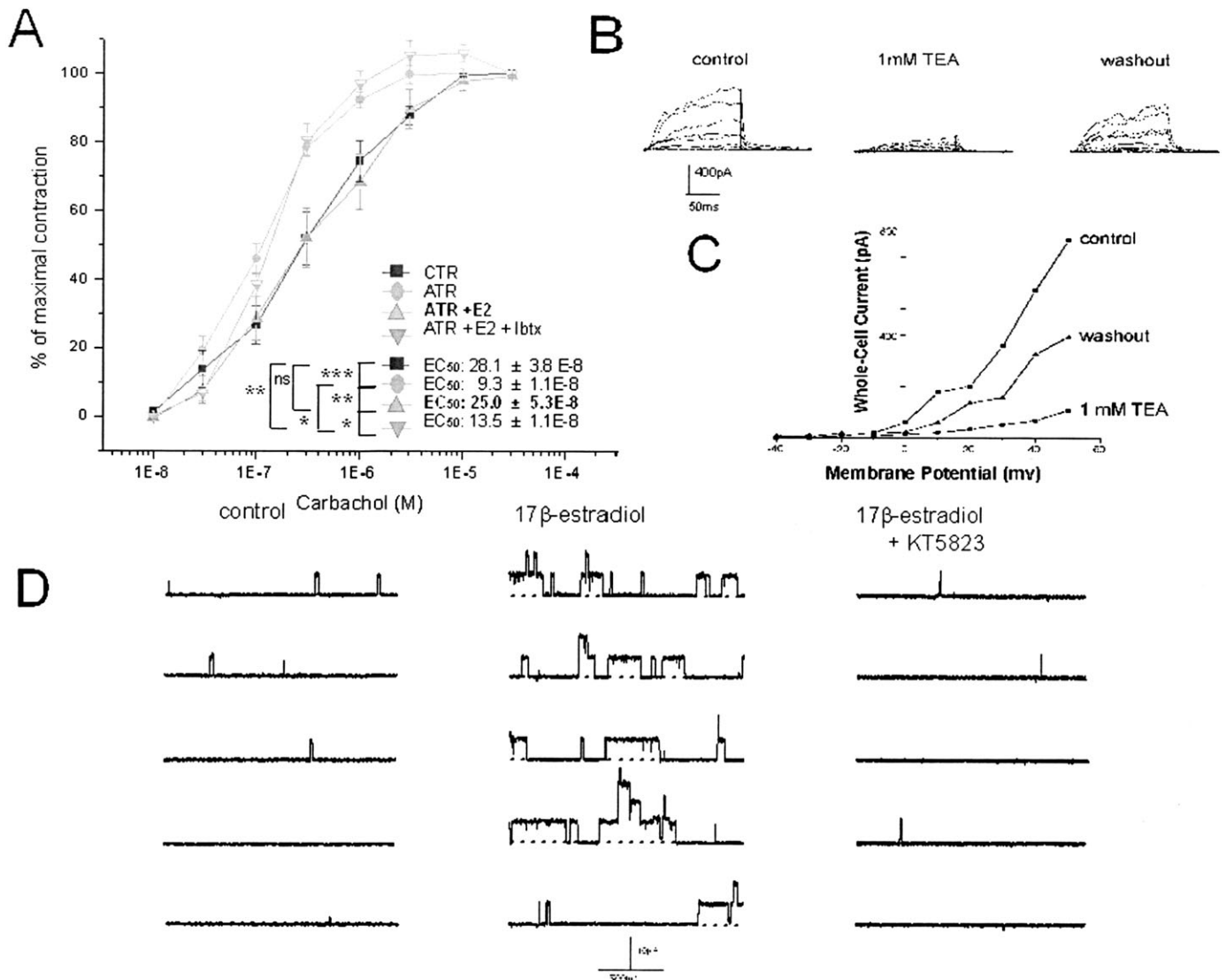


Figure 6. (A) Mean cumulative concentration–response curves to carbachol in CTR (closed squares, $n = 9$) and ATR treated with vehicle (closed circles, $n = 18$), 100 nM E₂ (closed triangles, $n = 19$), or 100 nM E₂ and 100 nM of the large-conductance, calcium-activated potassium (BK_{Ca}) channel inhibitor iberiotoxin (closed inverted triangles, $n = 9$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (B) Whole-cell currents recorded with the perforated-patch configuration ($V_{\text{hold}} = -60$ mV) before and 10 minutes after addition of 1 mM tetraethylammonium (TEA), which blocks BK_{Ca} channels selectively at this concentration. The inhibitory effect of TEA was reversible upon washout. (C) Complete current–voltage relationship for whole-cell currents before (squares) and after (circles) TEA. 1 mM TEA suppressed steady-state outward currents by 86% (+50 mV). (D) Recordings from the same cell-attached patch (+40 mV) before and 25 minutes after treatment with 100 nM E₂, and then 20 min after further addition of 300 nM KT5823, a selective inhibitor of PKG activity. Channel openings are upward deflections from baseline (closed state dashed line).

stimulation of adenylate cyclase activity, and enhanced NO production via NOS activity (28). Several studies have indicated that the vascular effects of E₂ involve NO production and ion channel activity (26, 29–31), and evidence from the present study has identified some important downstream elements of the E₂ signaling cascade in ASM.

Previous studies have implicated NOS1 and NOS3 deficiency after repeated allergen challenge in asthma (32). In the airway, NOS1 deficiency could induce hyperresponsiveness by promoting airway inflammation. On the other hand, NO derived from NOS1 can suppress the activation of nuclear factor κ B, thereby inhibiting expression of NOS2 as well as the production of inflammatory cytokines (33). Our data now suggest that the protective role of E₂ may be mediated by increasing NOS1 phosphorylation in asthmatic trachea. Such a protective effect of NOS activity in

the airway is supported by our previous studies demonstrating that the E₂ effects on human and pig coronary artery smooth muscle cells are also mediated by E₂-stimulated NO production (16); however, the specific isoform of NOS stimulated by E₂ in vascular smooth muscle has yet to be determined. It is not yet known how asthma reduces NOS1 phosphorylation or how this may be prevented by E₂. ¹¹⁷⁹Ser NOS phosphorylation requires Akt activation and NOS association with hsp90 (34). Asthma may affect either or both of these proteins. Interestingly, E₂ has been shown to stimulate Akt activation and NOS–hsp90 complexes in vascular smooth muscle cells (White and coworkers, personal communication).

An important effect of NO in smooth muscle is stimulation of cGMP-dependent phosphorylation. Activation of the cGMP/PKG signaling cascade stimulates BK_{Ca} channel activity in porcine

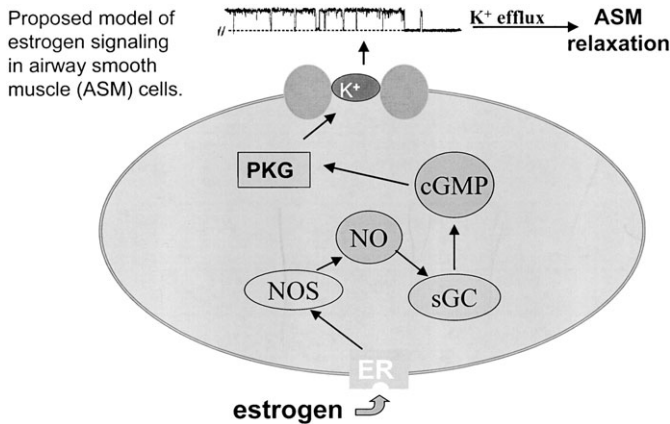


Figure 7. Proposed model of E₂ signaling in ASM cells.

(35) and human (16) arterial smooth muscle. In the present study, patch-clamp experiments of BK_{Ca} channels demonstrated that KT5823, which, at the concentration employed, inhibits PKG selectively (36), attenuates the stimulatory effect of E₂ on BK_{Ca} channel activity in ASM, implying a role for PKG in mediating this response. These results are consistent with previous studies indicating that the stimulatory effects of E₂ on potassium channels in porcine coronary arteries (37, 38) or pancreatic β -cells (39) are mediated via PKG-dependent phosphorylation. The present study provides the first direct evidence that cGMP-stimulated phosphorylation opens BK_{Ca} channels in mouse tracheal smooth muscle cells. The findings from both tension studies and single-channel recordings demonstrate that cGMP-stimulated phosphorylation opens BK_{Ca} channels, and that the BK_{Ca} channels are important targets of E₂ action in mouse tracheal smooth muscle cells. Moreover, the E₂ effect on tracheal rings was attenuated by iberiotoxin, a highly selective blocker of BK_{Ca} channels. Although E₂ was able to depress carbachol-induced constriction of tracheal rings, pretreatment of the rings with iberiotoxin abolished the E₂ effect. Furthermore, single-channel patch studies clearly identified BK_{Ca} channels in these tracheal smooth muscle cells, and demonstrated that E₂ increases the opening probability of these channels dramatically. These studies are consistent with previous reports indicating that BK_{Ca} channel is the predominant potassium channel in many smooth muscle cells types (40). Because of its large conductance and high density of expression, this channel plays an important role in setting and maintaining resting membrane potential in a variety of smooth muscle cells (41–43). Therefore, it is clear that BK_{Ca} channels expressed in smooth muscle cells are important targets of E₂ action in humans and other species.

Because this effect of E₂ occurs within minutes and not hours, it is most likely a nongenomic effect, consistent with previous clinical studies demonstrating acute effects of E₂ on coronary blood flow and/or relief of myocardial ischemia in patients of both sexes (44). Involvement of the cGMP/PKG signaling cascade in the response to 17 β -estradiol is well supported by both biochemical and functional (patch-clamp) studies that E₂ relaxes tracheal smooth muscle cells by opening BK_{Ca} channels. Pharmacologic studies also suggest a potential role for NO in the response of mouse tracheal smooth muscle to E₂; however, the overall importance of NO in mediating the direct effects of E₂ on tracheal smooth muscle remains to be clarified.

In summary, the present findings provide evidence from tissue, cellular, and molecular studies that E₂ relaxes tracheal smooth muscle cells by opening BK_{Ca} channels. These findings

constitute a cellular mechanism that can help explain why E₂ can often relieve airway hyperresponsiveness in bronchoconstrictive states, such as asthma. Further studies are needed to determine whether such an effect occurs in humans and to what extent this mechanism could represent an additional beneficial effect of endogenous E₂ or of E₂ replacement therapy, and to help explain the observed variation in asthmatic symptoms as the levels of a woman's sex hormones vary in both the nonpregnant and pregnant states.

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