

Estradiol Decreases the Acetylcholine-elicited Airway Reactivity in Ovariectomized Rats through an Increase in Epithelial Acetylcholinesterase Activity

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Estrogen replacement therapy (ERT) is frequently prescribed for postmenopausal women. Epidemiological data suggest that sex hormones may play a role in the expression of asthma, but the mechanism(s) whereby this influence is mediated remain(s) unclear. To better understand the role of physiologic doses of estrogens in airway function, we tested the hypothesis that 17 β -estradiol (E_2 , 10 μ g/kg per d for 21 d) given to oophorectomized female rats modifies airway responsiveness to cholinergic agonists, compared with oophorectomized rats given placebo. *In vivo*, the concentration of inhaled acetylcholine (ACh) required to double pulmonary resistance (EC_{200RL}) in anesthetized spontaneously breathing tracheotomized rats was calculated as an index of airway responsiveness. E_2 -treated rats were less responsive to ACh than placebo-treated rats (EC_{200RL} , 9.40 ± 1.48 vs. 1.52 ± 0.85 mg \cdot ml $^{-1}$, respectively). *Ex vivo* airway responsiveness was evaluated with the cumulative concentration–response curve (CCRC) of isolated tracheal segments. Compared with placebo, E_2 treatment significantly increased the EC_{50} of ACh ($p = 0.01$) but did not alter the CCRC to carbachol. Removing the epithelium or treatment with physostigmine abolished the difference in EC_{50} of ACh between the groups. Acetylcholinesterase (AChE) activity of homogenized whole trachea was 1.4-fold greater in the E_2 -treated group compared with placebo ($p = 0.02$), whereas no difference was found in homogenized epithelium-free trachea. We conclude that E_2 treatment decreases airway responsiveness to ACh in ovariectomized rats at least in part by increasing AChE activity dependent on the presence of the epithelium.

Keywords: acetylcholinesterase; airway reactivity; estradiol; rat

Estrogen replacement therapy (ERT) is frequently prescribed for postmenopausal women in order to prevent osteoporosis and symptoms related to menopause and to reduce the risk of cardiovascular disease. These benefits are related to nonreproductive actions of estrogens on the cardiovascular and immune systems, brain, and bone. Side effects must be taken into consideration, however, when envisaging their administration to healthy women.

Epidemiological data suggest a role for estrogens in the etiology and/or evolution of asthma (1–3). With the onset of puberty, asthma incidence becomes higher in females than in males and remains higher throughout the reproductive years (4). In addition, long-term use and/or high doses of postmenopausal estrogen therapy have been reported to increase the subsequent risk of asthma (5). In contrast, others have suggested that estrogen treatment can be beneficial in asthma (6).

However, little is known about the mechanisms by which estrogens may influence airway reactivity.

To investigate the effect of physiological doses of estrogens on airway reactivity and the mechanisms involved, we gave a chronic physiological dose of 17 β -estradiol (E_2) to oophorectomized female rats to see whether it could modify the *in vivo* airway responsiveness to inhaled aerosolized acetylcholine (ACh). We then studied *ex vivo* the contractile response of isolated trachea to cholinergic agonists and to KCl. As estradiol was found to decrease airway responsiveness to ACh, the mechanism(s) responsible for this effect was (were) investigated.

METHODS

Animals and Procedure

Experiments were performed according to the recommendations of the French Accreditation of Laboratory Animal Care. Seven-week-old female Wistar rats (Janvier, France) were anesthetized intraperitoneally with a solution of xylazine (7 mg/kg) and sodium pentobarbital (30 mg/kg). A bilateral ovariectomy was performed, and a pellet of either 17 β -estradiol (0.05 mg for 21 d, that is, 10 μ g/kg per d; Innovative Research of America, Sarasota, FL) or placebo was implanted subcutaneously. An additional group was sham-operated (intact ovaries).

After experiments (17–19 d after pellet implantation), the uterus was removed, drained of fluid, stripped of remaining fat and mesentery, and weighed.

Drugs were obtained from Sigma (La Verpillère, France).

Measurement of Airway Responsiveness to Acetylcholine

Pulmonary mechanics were studied in anesthetized, spontaneously breathing animals. The necks were opened, and the rats were tracheotomized. A 3-cm length of polyethylene tubing connected to a Fleisch pneumotachograph (no. 00; Fleisch, Lausanne, Switzerland) was inserted in the trachea to measure air flow (\dot{V}). Tracheal pressure, esophageal pressure (measured through a catheter inserted in the esophagus), and air flow were recorded on a polygraph (Pneumomultitest; EREMS, Toulouse, France). Pulmonary resistance (RL) was determined by the method of Mead and Whittenberger (7).

Aerosols were generated for 60 s, using a Hudson nebulizer (model 1700; Hudson Oxygen Therapy, Temecula, CA) with an output of 0.18 ml/min connected to one side of a Plexiglas box.

After an initial measurement of RL , rats were administered an aerosol of saline and increasing concentrations of ACh until RL reached at least 200% of the saline control value. ACh responsiveness was expressed as the concentration of ACh required to increase RL to 200% of the value measured after saline aerosolization (EC_{200RL}).

Measurement of Tracheal Isometric Contraction

Animals were anesthetized and perfused, through a polyethylene catheter inserted into the right ventricle, with saline at 100-mm Hg pressure to remove blood from lungs. The trachea was removed and cut into transverse rings measuring 3 mm in length. Each ring was mounted between two stainless steel clips in vertical 5-ml organ baths of a computerized isolated organ bath system (IOS UF-1; Phymep, Paris, France) filled with modified Krebs solution as described else-

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where (8). The tracheal rings were set at optimal length by equilibration against a passive preload of 2 g, as previously determined for these types of experiments (9). As a general rule, only one agonist was tested in one ring, but different rings from the same animal could be tested under different conditions. Duplicate airway rings from the same animal were studied under each experimental condition, and a mean cumulative concentration–response curve (CCRC), representative of each individual, was obtained.

CCRCs were made by using the spasmogen KCl (3×10^{-3} to 10^{-1} M), substituted for NaCl in an equimolar amount in the presence of atropine (10^{-6} M). The response was expressed as a percentage of the maximal contractile response observed with ACh (5×10^{-2} M) in the ring under consideration.

CCRCs were produced to the cholinergic agonists ACh and carbamylcholine chloride (carbachol, CCh). CCRCs to ACh were determined in the presence of physostigmine at a concentration of 5×10^{-8} M for 5 min, which did not alter the resting tension of the tracheal rings (results not shown). CCRCs to ACh were also produced after removing epithelium by gently rubbing the luminal surface with wet gauze.

The mechanical response to cholinergic agonist was expressed as a percentage of the maximal contractile response observed in the considered ring with the considered agonist. The efficacy of an agonist was defined as Fmax, that is, the plateau (maximum) level of the contractile force on the CCRC. The potency was characterized as the EC₅₀, the concentration producing a contractile force of Fmax/2, as graphically determined. A geometric mean EC₅₀ and the SEM were then calculated for each mean curve.

Each tracheal ring was fixed in formalin for histological examination.

Measurement of Cholinesterase Activity

After exsanguination, the trachea was excised and cut longitudinally into two equal parts, one of which was rubbed to remove the epithelium. Each part was then homogenized separately, using a glass-to-glass homogenizer, in high-salt extraction buffer containing detergent (10 mM Tris-HCl [pH 7.4], 1% Triton X-100, 1 M NaCl). The homogenates were centrifuged at $10,000 \times g$ for 15 min, and the supernatant was removed to measure AChE activity by the method of Ellman and coworkers (10). Briefly, the reaction was performed at 20° C in a 1-ml total volume (0.1 ml of supernatant, 0.9 ml of a reaction mixture containing 25 mM phosphate buffer [pH 7.0], 0.3 mM dithionitrobenzoate, and 1 mM acetylthiocholine as a substrate). The production of 5-thio-2-nitrobenzoate was measured at 412 nm with continuous recording of optic density (DO). Acetylcholinesterase (AChE) activity was measured in the presence of the specific inhibitor of butyrylcholinesterase activity, tetraisopropyl pyrophosphoramidate (iso-OMPA, 10^{-4} M, preincubated for 15 min). It was verified that the nonspecific cholinesterase inhibitor physostigmine (5×10^{-8} M, preincubated for 5 min) abolished cholinesterase activity. Acetylcholinesterase activity was normalized, using the total protein concentration determined by the method of Lowry and coworkers (11).

Analysis of Data

Data are expressed as means \pm SEM. Comparisons between two groups were made by an unpaired *t* test. Values of $p < 0.05$ were considered to be significant.

RESULTS

Effect of 17 β -Estradiol on Animal Characteristics

Animal body weight gain was lower in the rats given E₂ at 10 μ g/kg per d compared with controls (15 vs. 53%, respectively). Uterus weight was 4.5-fold greater in the E₂ treatment group than in the control group (604 ± 39 vs. 132 ± 4 mg, respectively), indicating an effect of E₂ on this target sexual organ (Table 1).

Effect of E₂ Treatment on Airway Responsiveness to ACh *in vivo*

As summarized in Table 2, both minute ventilation (expressed per kilogram body weight) and RL before aerosol challenge (basal values) were similar in placebo and E₂-treated groups.

TABLE 1. EFFECT OF 17 β -ESTRADIOL ON CHARACTERISTICS OF OOPHORECTOMIZED RATS

	Placebo (n = 20)	E ₂ , 10 μ g/kg per d (n = 20)	<i>t</i> Test (<i>p</i> value)
Animal weight at castration, g	203 \pm 5.8	216 \pm 7.9	NS (0.41)
Increase in weight by Day 17–19, %*	52.8 \pm 3	15.4 \pm 1.3	< 0.0001
Uterus weight, mg	132 \pm 4	604 \pm 39	< 0.0001

* Expressed as a percentage of the weight at castration.

The mean concentration–response curves for the placebo and E₂-treated groups are shown in Figure 1. The responsiveness of the E₂-treated group to ACh was significantly lower than that of the placebo group (EC_{200RL}, 9.40 ± 1.48 vs. 1.52 ± 0.85 mg \cdot ml⁻¹, respectively).

Effect of E₂ Treatment on Isometric Contraction

In isolated intact rings, the CCRC induced by the depolarizing agent KCl did not differ between E₂-treated and control groups for either the EC₅₀ or the Fmax (Table 3 and Figure 2). ACh and CCh induced similar maximal contraction in both groups, indicating that E₂ treatment had no effect on efficacy of the agonists, whereas the EC₅₀ in response to ACh were about 40-fold greater than those in response to CCh (Figure 3). The response to CCh was similar in rings from E₂-treated rats and those from control rats. In contrast, the CCRC to ACh was shifted rightward (about 2-fold) by E₂ treatment (Figure 3).

CCRCs induced by ACh did not differ between sham-operated and E₂-treated groups for either the EC₅₀ (182.0 ± 51.2 vs. 191.6 ± 37.2 , respectively, n = 7, NS) or the Fmax (2.20 ± 0.51 vs. 2.16 ± 0.48 , respectively, n = 7, NS). In contrast, placebo-treated ovariectomized rats elicited EC₅₀ values in response to ACh that were lower than those of sham-operated rats (88.0 ± 13.8 vs. 182.0 ± 51.2 , respectively, n = 7, $p = 0.04$).

Physostigmine (5×10^{-8} M, preincubated for 5 min) shifted the CCRC to ACh to lower concentrations by approximately 1.5 log units in both groups ($p < 0.0001$) but did not modify Fmax (Figure 3). Interestingly, physostigmine abolished the difference between E₂-treated and placebo-treated groups in response to ACh.

Removal of the epithelium shifted the CCRC to ACh leftward to lower concentrations by approximately 0.5 log unit ($p < 0.001$). Removing the epithelium abolished the difference in EC₅₀ between the E₂-treatment group and the placebo group, but did not modify Fmax (Figure 3).

Effect of E₂ on Acetylcholinesterase Activity

A significant increase in AChE activity was observed in the E₂-treated group compared with the placebo group when the whole trachea was studied ($20.7 [\pm 1.3] \times 10^{-3}$ vs. $14.9 [\pm 1.6] \times 10^{-3}$ DO/min per mg of protein, respectively, n = 7, $p = 0.02$). However, after removing the epithelium, no difference

TABLE 2. EFFECT OF 17 β -ESTRADIOL ON AIRWAY RESPONSIVENESS*

	Placebo (n = 5)	E ₂ , 10 μ g/kg per d (n = 5)	<i>t</i> Test (<i>p</i> value)
\dot{V}_E baseline, ml/min per kg	340.5 \pm 18.8	325.9 \pm 24.9	NS (0.56)
RL baseline, cm H ₂ O \cdot s \cdot ml ⁻¹	0.73 \pm 0.22	0.78 \pm 0.15	NS (0.63)
EC _{200RL} , mg \cdot ml ⁻¹	1.52 \pm 0.85	9.40 \pm 1.48	0.003

* Expiratory volume per minute (\dot{V}_E) and pulmonary resistance (RL) were measured at baseline in anesthetized animals, that is, before any aerosol challenge. EC_{200RL} values are concentrations of ACh required to increase RL to 200% of baseline.

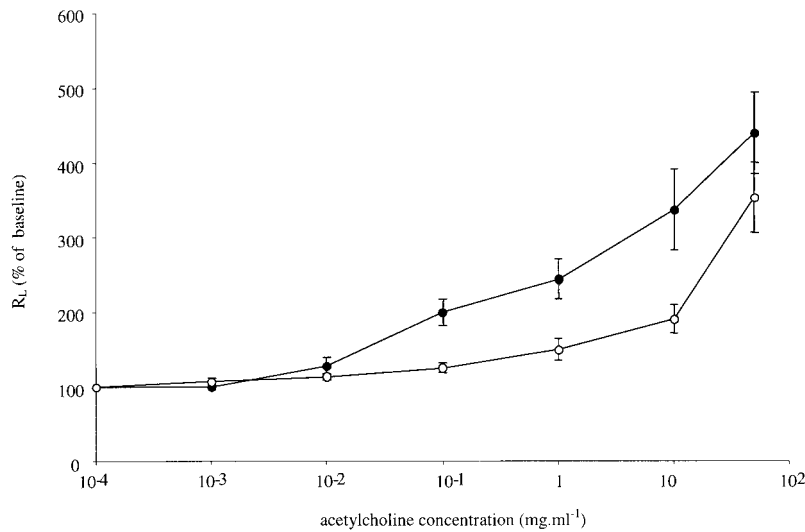


Figure 1. Mean dose–response curves for placebo-treated (solid circles, n = 5) and E₂ (10 µg/kg per d)-treated (open circles, n = 5) oophorectomized rats. Pulmonary resistance (R_L), expressed as a percentage of the preinhalation value, is displayed on the ordinate; acetylcholine concentration is displayed on the abscissa.

in AChE activity was observed between the E₂-treated and placebo groups ($14.0 \pm 1.3 \times 10^{-3}$ vs. $14.1 \pm 1.6 \times 10^{-3}$ DO/min per mg of protein, respectively, n = 7, NS).

Physostigmine (5×10^{-8} M, preincubated for 5 min) abolished most of the cholinesterase activity in intact trachea (with epithelium) of both the E₂-treated and placebo groups ($0.9 \pm 0.5 \times 10^{-3}$ vs. $1.1 \pm 0.8 \times 10^{-3}$ DO/min per mg of protein, respectively, n = 7, NS).

In the placebo-treated group, AChE activity related to the epithelium was about 5% of the total AChE activity. In contrast, the epithelial AChE activity in the trachea from E₂-treated rats was about 30% of the total AChE activity (Figure 4). Interestingly, calculation revealed that changes induced by E₂ corresponded to a 6-fold increase in epithelial AChE activity.

DISCUSSION

The aim of the present study was to investigate the effect of 17β-estradiol (E₂) on the airway responsiveness to cholinergic agonists in ovariectomized female rats. Compared with ovariectomized placebo-treated rats, we found that E₂ induced (1) a decrease in airway responsiveness to ACh *in vivo*, and (2) a decrease in the sensitivity to ACh of the tracheal muscle, due at least in part to (3) an enhancement of acetylcholinesterase activity dependent on the presence of the epithelium *ex vivo*.

ERT is currently prescribed for postmenopausal women after loss of their endogenous estrogen and progesterone secretion. Epidemiological studies suggest that this treatment influences either the airway reactivity or the course of asthma (5, 6). The aim of our study was to investigate the effect of physiological doses of estrogens on airway reactivity.

Our results regarding the decrease in airway reactivity *in vivo* after E₂ treatment are in agreement with the study by Lieberman and coworkers (1), in which the effect of ERT on airway reactivity to histamine was studied in 36 postmenopausal women who did not suffer from respiratory disease. The authors found a smaller decrease in the forced expiratory volume in 1 s (FEV₁) induced by histamine after 4–6 weeks of ERT compared with the histamine challenge performed before ERT.

Our control group consisted of placebo-treated castrated female rats in order to avoid any effect of endogenous progesterone and estrogens. The E₂ treatment dose of 10 µg/kg per d was chosen as it is a physiological dose (12). Both uterus weight and the body weight gain were in agreement with those previously reported (13–15). Treatment of ovariectomized rats with estradiol decreases food intake, increases energy expenditure, and consequently reduces body fat content and body weight gain (15). All tracheal rings of the *ex vivo* experi-

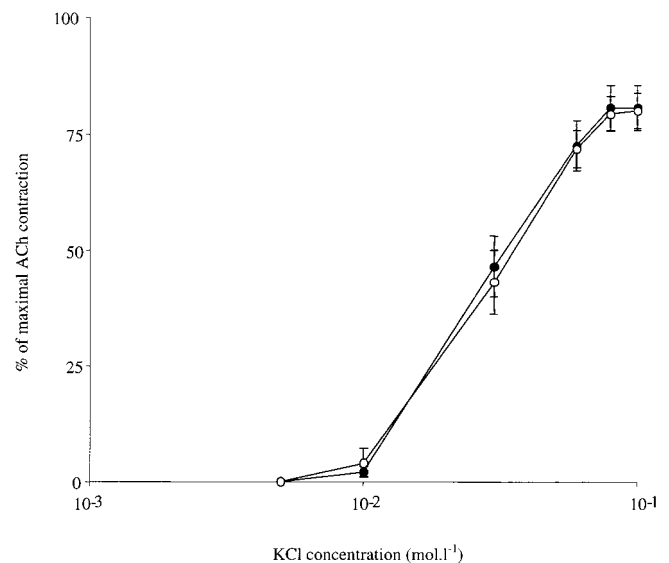


Figure 2. Mean dose–response curves representing the effect of KCl on isolated tracheal rings of placebo-treated (solid circles, n = 5) and E₂ (10 µg/kg per d)-treated (open circles, n = 5) oophorectomized rats. Data are expressed as percentages of the maximum contraction measured with ACh (5×10^{-2} M) for the considered ring.

TABLE 3. ISOLATED INTACT TRACHEAL RING SENSITIVITY TO POTASSIUM CHLORIDE*

	Placebo (n = 5)	E ₂ , 10 µg/kg per d (n = 5)	t Test (p value)
EC ₅₀ KCl, M ($\times 10^{-2}$)	2.3 ± 0.3	2.7 ± 0.5	NS (0.27)
Fmax KCl, mg	1.81 ± 0.29	1.75 ± 0.25	NS (0.51)
Fmax ACh, mg	2.24 ± 0.31	2.13 ± 0.38	NS (0.43)

Definition of abbreviations: EC₅₀ = agonist concentration needed to obtain 50% of maximal response; Fmax = value of maximal contraction minus basal contraction and is expressed in grams; n = number of tracheal specimens for each experiment.

* For each KCl challenge, the ring was first tested with ACh to determine the maximal contractile response.

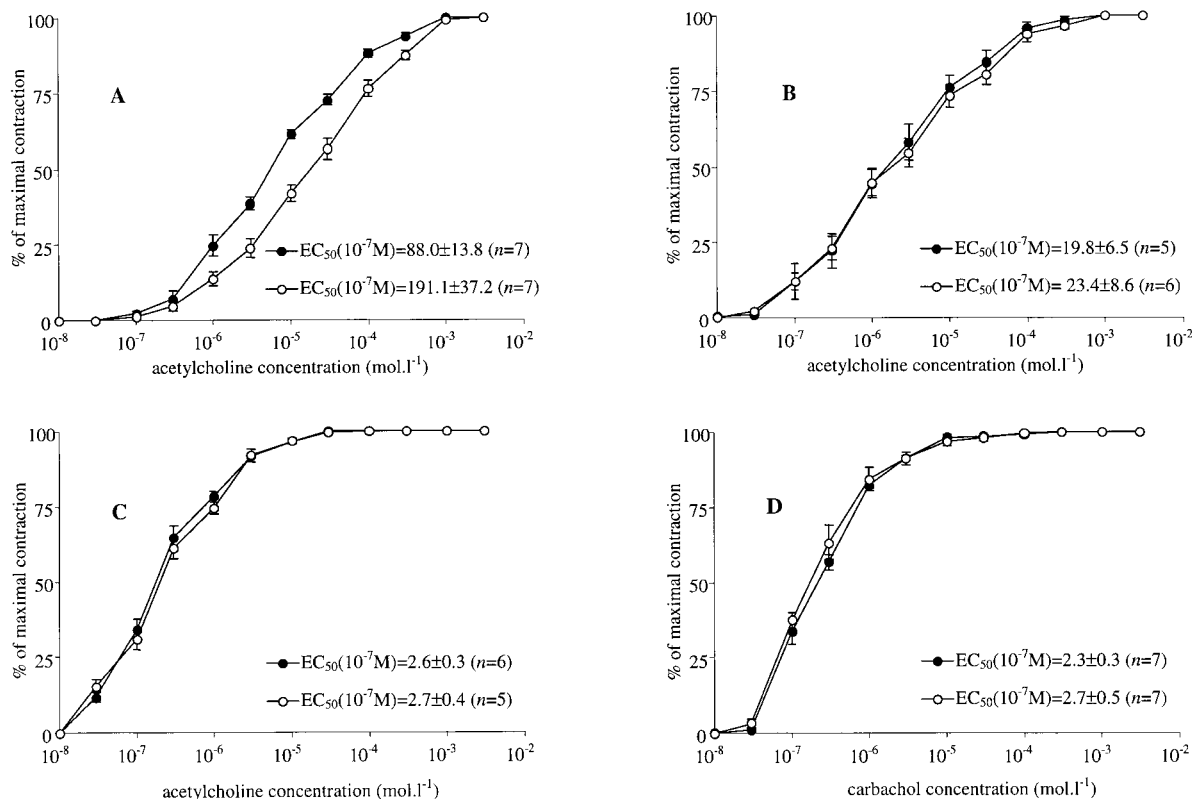


Figure 3. Effects of acetylcholine (ACh) (A–C) and carbachol (D) on active tension in isolated rings of rat trachea (solid circles, placebo treated; open circles, E₂ treated). Data are expressed as percentages of the maximum contraction measured for the considered ring. Results are expressed as means \pm SEM. EC₅₀ is the agonist concentration needed to obtain 50% of maximal response and characterizes the potency of an agonist. F_{max}, which represents the efficacy of the agonist, was about 2 g for all experiments and did not differ between groups. n = number of tracheal specimens for each experiment. E₂ treatment elicited a rightward shift of the CCRC of intact rings to ACh (A). Epithelium removal (B) abolished the differences in CCRC to ACh observed for tracheal rings with epithelium and shifted the EC₅₀ to lower doses. The CCRC constructed with intact rings preincubated with physostigmine, a nonspecific cholinesterase inhibitor (5×10^{-8} M preincubated for 5 min), elicited a leftward shift of both curves (C). Finally, the CCRC of intact rings to carbachol, a cholinergic agonist that is resistant to cholinesterase, showed a leftward shift of both curves (D).

ments were kept for microscopic examination and quantification of both epithelium and smooth muscle. The metabolic effect of estradiol does not affect either epithelium or smooth muscle cross-sectional areas (data not shown).

In the *in vivo* experiments, basal RL values were similar in both groups. We used spontaneously breathing anesthetized rats as preliminary experiments had demonstrated that rats became apneic and died at high ACh concentrations. In accordance with other studies, we did not determine the maximal RL, and the EC₂₀₀RL was used to investigate airway responsiveness to different agonists (16). The observed differences in EC₂₀₀RL between the placebo-treated and E₂-treated groups reflect differences in the potency of the agonist ACh.

The depolarizing agent KCl, which ultimately activates voltage-dependent calcium channels, thus inducing contraction (17), leads to an electromechanical coupling of tracheal smooth muscle. E₂ treatment did not alter the contractile response of isolated tracheal rings to KCl, suggesting that E₂ had no effect on such coupling.

The potency of ACh in rat isolated trachea is strain related (16) and age related (18). In our experiments, CCRCs to ACh of tracheal rings from sham-operated animals elicited an EC₅₀ value close to the value elicited in the E₂-treated group and within the range of reported values for age-matched animals (18).

ACh is sensitive to AChE, whereas CCh is totally resistant to hydrolysis by either AChE or nonspecific cholinesterase (19). This at least partly accounts for the leftward shift of the

CCRC in both groups (1) in response to CCh compared with ACh, and (2) in response to ACh in rings preincubated with physostigmine compared with ACh. Physostigmine has been reported to inhibit cholinesterase with IC₅₀ values of approximately 10^{-8} M (20). As the CCRCs to ACh in the presence of physostigmine were similar to the CCRC to CCh, the 5×10^{-8} M concentration of physostigmine, preincubated for 5 min and used in the present study, inhibited most of the cholinesterase activity. This was confirmed by the measurement of cholinesterase activity in homogenized intact trachea (with epithelium) preincubated for 5 min with 5×10^{-8} M physostigmine. Moreover, we found that E₂ treatment had no effect on the CCRC to CCh compared with controls but resulted in a rightward shift of the CCRC to ACh. Taken together, these observations suggest that the decreased potency of ACh after E₂ treatment may be related to increased cholinesterase activity.

We found that compared with the CCRC to ACh of intact tracheal rings, epithelium removal had two effects. First, we observed a leftward shift of the CCRC in both groups, indicating that the airway epithelium modulates the contractile response to ACh. This observation is in agreement with other studies of concentration–response curves to several contractile agents in isolated tracheas of a number of animal species and bronchi from humans (21). Second, we did not observe any further difference in response to ACh due to E₂ treatment after epithelium removal. We therefore postulated that the presence of the epithelium is indispensable to the increased

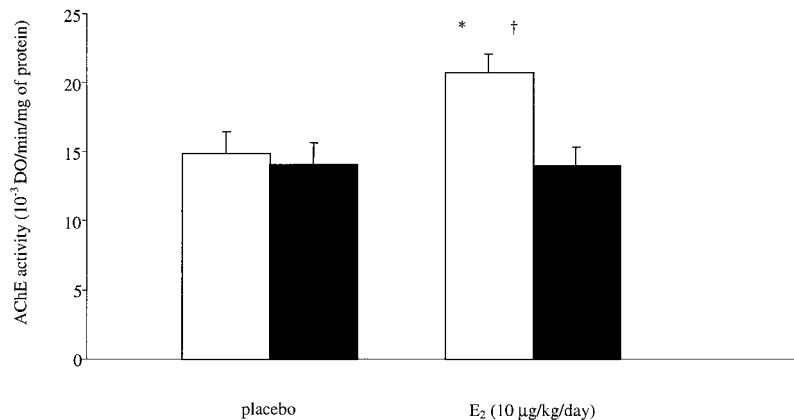


Figure 4. Effect of E₂ treatment on acetylcholinesterase (AChE) activity (expressed as 10⁻³ DO/min per mg of protein) from homogenates of rat trachea with (open columns) or without (solid columns) epithelium. Results are reported as means ± SEM for seven rats. *p = 0.01, significant difference from the same treatment group without epithelium; †p = 0.02, significant difference from the placebo group.

cholinesterase activity elicited by E₂ treatment. This hypothesis was confirmed by the enzymatic determination of acetylcholinesterase activity in the trachea.

AChE activity in the trachea exists within the epithelium, smooth muscle layer, adventitia, and in the autonomic nerve endings (22). In the guinea pig, the principal mechanism by which the epithelium inhibits tracheal contraction to ACh is via epithelium-derived AChE activity (23). However, epithelial AChE activity was not marked as compared with nonepithelial AChE activity in rat trachea when determined by histochemical staining (22). Our data are consistent with these observations, as the AChE activity related to the epithelium in the control group was only 5% of the AChE activity of the whole tracheal homogenates. In the E₂ treatment group, the epithelial AChE activity represented up to 30% of AChE activity of the whole trachea, whereas AChE activity in trachea without epithelium was similar in both groups. In other words, E₂ treatment induced a 6-fold increase in epithelial AChE activity.

The effect of E₂ on AChE activity in the respiratory tract has already been investigated. Abdul-Karim and coworkers showed that E₂ given to oophorectomized rabbits brought about a 4-fold increase in lung cholinesterase activity (24). However, these authors studied AChE activity of the whole homogenized lung, without distinguishing between trachea, bronchi, and lung parenchyma, and did not assess the functional consequences.

The effect of E₂ on AChE activity was studied more in detail in the brain, as postmenopausal women receiving estrogen replacement therapy showed a reduced risk of developing Alzheimer disease, while studies with brains displaying Alzheimer disease lesions have shown that changes occur in the expression and distribution of AChE (25). Free radicals are shown to induce an inhibition of brain AChE, which can be reversed by antioxidant compounds (26). It has been shown elsewhere that oxidative stress transforms *in vitro* purified active acetylcholinesterase to an inactivated form (27). In another *in vitro* model, E₂ was shown to decrease the interaction of AChE with amyloid β-peptide, probably as a result of its antioxidant properties (28).

In conclusion, we demonstrated that chronic treatment with a physiological dose of 17β-estradiol decreases airway reactivity in ovariectomized female rats, apparently due at least in part to an increase in AChE activity in the epithelium. To the best of our knowledge, this is the first demonstration in an experimental model of a beneficial effect of 17β-estradiol on airway responsiveness to acetylcholine and of the mechanism involved. The link between our experimental exploration of airway reactivity and the pathophysiology of asthma remains

to be established. 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 estrogens or ERT after menopause.

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