

Nongenomic bronchodilating action elicited by dehydroepiandrosterone (DHEA) in a guinea pig asthma model



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ABSTRACT

Primates secrete large amounts of the precursor steroid dehydroepiandrosterone (DHEA); in humans, its levels are low during childhood and start declining after the fourth decade. It has been postulated that the progressive decline in DHEA levels may be related with the severity of asthma associated with age. To determine whether DHEA may regulate the airway smooth muscle (ASM) activity, isolated tracheal rings with and without epithelium from male guinea pigs were isometrically recorded to characterize the response of ASM to DHEA at different concentrations on KCl- and carbachol (CCh)-induced contraction as well as on ovalbumin (OVA)-induced contraction in sensitized guinea pigs. Additionally, we used barometric plethysmography in sensitized guinea pigs in order to compare changes of the lung resistance increased by the antigen challenge to OVA in the absence and presence of different doses of DHEA. DHEA concentration-dependently abolished the contraction to KCl, CCh and OVA, and no differences were found in preparations with and without epithelium. DHEA-induced relaxation was not modified by the suppression of protein synthesis or transcription, pharmacological inhibition of nitric oxide (NO) synthase, nor by antagonist of β_2 -adrenergic receptors or an inhibitor of the 3β -HSD enzyme. Likewise, Ca^{2+} -induced contraction in Ca^{2+} -free depolarized tissues was antagonized by DHEA, and the contraction to the L-type voltage-dependent calcium channel activator (Bay K 8644) was inhibited by DHEA. Furthermore, DHEA prevented OVA-induced increases in lung resistance. These results indicate that DHEA-induced relaxation in ASM is a nongenomic (membrane) action and is not produced after its bioconversion. The data suggest that DHEA-induced relaxation is an epithelium- and NO-independent mechanism that involves a blockade of voltage-dependent calcium channels and possible non-selective cation channels.

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1. Introduction

Asthma is a worldwide common chronic disease. It is well known that childhood asthma frequency before puberty is higher in boys than in girls [1–10]. Contrastingly, during adulthood, asthma prevalence augments among women when compared to men;

furthermore, this ailment exhibits a more severe form in female asthma [1,5,11–17]. Notably, asthma severity decreases among males during adolescence but prevalence increases once again in this gender during the fifth or sixth decade [10]. Therefore, it seems that decreasing serum androgen levels in men may contribute to an increase in asthma [10,18]. In general, it is accepted that female sex hormones (estrogens and progesterone) aggravate asthma and other allergic diseases, whereas male sex hormones (androgens) suppress such diseases (for review see [8]).

Even though asthma incidence is determined by a large number of variables, its relation to sex and age is, indeed, not well understood. Obviously, the endocrine status may be an important factor to take into account, due to the fact that many changes are closely related to the age-associated decline in hormones. For instance, dehydroepiandrosterone (DHEA) and its sulfated derivative (DHEA-S), the most abundantly produced adrenal steroids, particularly in primates, and all the other hormones that decline with age appear to be associated with physiological functions and overall human health. In this respect, DHEA decreases with age in both men and

Abbreviations: DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; ASM, airway smooth muscle; CCh, carbachol; KCl, potassium chloride; OVA, ovalbumin; NO, nitric oxide; KHS, Krebs–Henseleit bicarbonate solution; BK, bradykinin; E_{max} , maximal effect; IC_{50} , inhibitory concentration 50; L-NAME, N^o-nitro-L-arginine methyl ester; DMSO, dimethylsulfoxide; KCl-Ca^{2+} $\emptyset$, KCl-Ca^{2+} -free solution; R_L , lung resistance; EAR, early asthmatic response; LAR, late asthmatic response; SOCE, store-operated Ca^{2+} entry; ROCC, receptor-operated Ca^{2+} channel; VDCC, voltage-dependent Ca^{2+} channels.

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women; thus, one of the main characteristics of DHEA and DHEA-S is their age-dependent pattern of secretion. Large amounts of DHEA are produced during fetal development, but after birth, this production falls sharply and remains low for several years; then the synthesis resumes and the plasmatic levels peak during the second decade of life [19,20]; nonetheless, the DHEA levels may be lower for women when compared with men. Towards the third life decade, an age-dependent decline in DHEA's plasmatic concentrations starts and progresses with aging in both genders [21].

Regarding the relationship between DHEA and asthma, available data are scant. Preliminary reports have documented that DHEA levels in postmenopausal, asthmatic women were lower when compared with an equal number of age-matched, postmenopausal, non-asthmatic women [22]. Additionally, asthmatic patients, with severe bronchospasm, had lower DHEA and/or DHEA-S levels [23–25]. Although these findings certainly reinforce the view that the low levels of DHEA may affect the asthma symptoms, very few formal studies have evaluated its effects on the respiratory system. DHEA has been considered an important immunomodulating and anti-inflammatory hormone that reverses cytokine imbalances associated with asthma. Therefore, DHEA may prevent and attenuate allergic inflammation in the airways of different animal models of asthma [26–28]. Subsequently, it has been suggested that DHEA supplementation may show some therapeutic benefits for asthmatic patients [29,30]. Additionally, we have shown that DHEA is capable of inducing an acute nongenomic uterine and vascular relaxation in humans and animals [31–33], and we hypothesized that the airway smooth muscle (ASM) may be also a target of the nongenomic relaxing effect induced by DHEA. To test this hypothesis, we examined the potential bronchodilating effects of DHEA on ASM in order to investigate what role it might play in preventing the development of bronchospasm during an antigenic challenge. For this purpose, we analyzed the potential bronchodilating effect induced by DHEA on high potassium (KCl)-solution or carbachol (CCh)-elicited contraction in ASM preparations from non-sensitized (unchallenged control guinea-pigs) vs. ovalbumin (OVA)-sensitized guinea pigs (an experimental model of asthma). The role of airway epithelium in these DHEA-induced relaxations was also investigated. Accordingly, the response to DHEA was also evaluated on OVA-induced contraction in guinea pigs tracheas from sensitized animals. To confirm that DHEA-induced relaxation is operative *in vivo*, the steroid was tested in the lung resistance of OVA-sensitized animals. In order to shed further light on the mechanism(s) involved in DHEA-induced bronchodilation, we also explored the possible participation of: (i) the product of DHEA bioconversion in its relaxing effect; (ii) β_2 -adrenoceptors; (iii) epithelium-derived relaxing factor (nitric oxide; NO); (iv) nuclear transcription/translation mechanisms; and (v) voltage-dependent Ca^{2+} channels (VDCC).

2. Materials and methods

2.1. Animals

Adult male guinea pigs of the Hartley strain (450–600 g) were obtained from the Instituto Nacional de Enfermedades Respiratorias, Mexico City. The animals were housed in our animal facility in conventional conditions and fed with Purified Guinea Pig Diet pellets (Harlan Inc.) and water *ad libitum*. The protocols were approved by the Institutional Care and Use Committee of the Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México. The experiments were conducted in accordance with the published Guiding Principles in the Care and Use of Animals, approved by the American Physiological Society. The guinea pigs were grouped in: non-sensitized and ovalbumin (OVA)-sensitized guinea pigs.

2.1.1. OVA-sensitized guinea pigs procedure

To obtain the asthmatic model, guinea pigs were sensitized at day 1 by administration of 0.5 ml i.p. and 0.5 ml s.c. of $60 \mu\text{g ml}^{-1}$ OVA and 1 mg ml^{-1} $\text{Al}(\text{OH})_3$ (aluminum hydroxide as adjuvant) dissolved in saline (0.9% NaCl). At day 8, the animals received an antigenic challenge with 3 mg ml^{-1} OVA in saline solution nebulized during 5 min, delivered by an ultrasonic nebulizer (WH-2000; Yuehue Medical Instrument Factory Co., Ltd., China). On day 15, animals received another antigenic challenge with 1 mg ml^{-1} OVA in saline solution nebulized for 1 min. The response to the antigen was always established by deep abdominal breathing. The onset of severe dyspnea was selected as the experimental endpoint for this study. Finally, the guinea pigs were subjected to experimentation on days 21–25. Exceptional guinea-pigs with a poor response to antigen were excluded from the study.

2.2. Tracheal rings preparation and contractile force measurements *in vitro*

In order to compare the effect of androgens on non-sensitized (control group) and sensitized (asthmatic group) guinea pigs, we used the same experimental protocol for the two groups. The guinea pigs were deeply anaesthetized with sodium pentobarbital (35 mg kg^{-1} , i.p.) and then killed by exsanguination. The trachea was immediately placed in Krebs–Henseleit bicarbonate solution (KHS) with the following composition (mM): NaHCO_3 (25), NaCl (120), KCl (4.7), KH_2PO_4 (1.2), MgSO_4 (1.2), CaCl_2 (2.5) and glucose (11); this solution was aerated continuously with a gas mixture of 95% O_2 and 5% CO_2 to maintain pH 7.4 and constant temperature at 37°C . The trachea was cut in eight tracheal rings of approximately 0.3 cm of length (each one containing three cartilaginous rings).

To determine the role of the airway epithelium in the relaxing effect induced by DHEA, tracheal rings from non-sensitized animals were used in two ways: (i) preparations with epithelium: tracheas were carefully handled to avoid epithelium injuring; and (ii) preparations without epithelium: the luminal surface of tracheal rings was rubbed by using a dental drill bur with cylinder round end (K-File Colorinox 140). The presence and absence of epithelium was evaluated functionally in all experiments (as described below).

Each tracheal ring was suspended horizontally between two stainless steel wires, and was placed in a temperature-controlled (37°C) organ bath containing 10 ml KHS continuously gassed with O_2/CO_2 throughout the experiment for isometric tension recordings. One wire was attached to a fixed support at the bottom of the chamber, and the other end to an isometric force transducer (FTO3C; Grass Instrument, Quincy, MA, USA) connected to a computerized data acquisition and analysis system (MP 150; Biopac Systems, CA, USA). The resting force was adjusted to 10 mN (1 g). Under these conditions, tissues were equilibrated for 1 h before conducting the experiments. Then, the bronchocontractile responses to 60 mM KCl were induced after replacing KHS with an equimolar substitution of 60 mM KCl and 64.7 mM NaCl. KCl caused a tonic contraction which was repeated three times in order to verify the reproducibility of the response to obtain a maximum stable tonic contraction during 15 min.

The epithelial condition was pharmacologically corroborated as reported previously [34]. Bradykinin (BK) induces epithelium-dependent relaxation; on this basis, 320 nM BK was added of each preparation at basal tone. The absence of epithelium (rubbed tissues) was verified when BK induced an increase of the basal tone ($\leq 0.3 \text{ g}$); whereas the presence of epithelium was confirmed when BK elicited a diminution of the basal tone in the tracheal rings with epithelial integrity. Then, the tissues were washed with KHS.

2.2.1. DHEA response on ASM contraction

After the three KCl pre-stimuli, preparations with and without epithelium from non-sensitized and sensitize animals were contracted either with KCl (60 mM) or CCh (0.3 μ M) for 90 min (control response). Later on, tissues were washed with KHS. Likewise, different tracheal rings only from sensitized animals with epithelium were contracted with 1 μ M OVA. The tissues contracted by these different agents (KCl, CCh or OVA) induce a tonic contraction through different mechanisms; these contractions were stable with constant amplitude for a long time (recorded more than 2 h). After a stable tension was attained ($\sim$ 15 min), different concentrations (9, 20, 42, 91 and 200 μ M) of DHEA, dissolved in absolute ethanol, were added cumulatively with an interval of 15 min between each addition and the response was also evaluated on this period. Subsequently, the tissues were washed to re-equilibrate for 60 min before inducing the last contraction to KCl or CCh, recorded during 90 min (second control response to verify tissue viability); this was not the case of OVA-induced contraction which never returns to the baseline after washout. The concentration–response curves to DHEA were plotted, and the inhibitory concentration 50 (IC_{50} ; DHEA concentration to inhibit 50% of KCl-, CCh-, or OVA-induced contraction) was calculated to compare DHEA potency and the sensitivity of each contraction to DHEA-induced relaxation. Likewise, the highest concentration to induce the maximal relaxing effect (E_{max}) on KCl-, CCh- or OVA-induced contraction was obtained for DHEA to determine its efficacy. In a separate group of experiments, the effect of the vehicle of DHEA (ETOH; absolute ethanol) on the different tonic contractions was determined after exposure to ETOH; each concentration added never exceeded 0.1% (v/v). The concentration–response curves to DHEA on contractions induced by different bronchoconstrictors were determined independently in tracheal rings from different animals ($n=6$). The IC_{50} and E_{max} values in each group were compared.

2.3. The potential role of NO in the bronchodilating effect of DHEA

The potential role of NO in DHEA-induced relaxation was separately explored in a set of experiments. Tracheal rings with epithelium from non-sensitized animals were incubated with the NO synthase inhibitor, N^{ω} -nitro-L-arginine methyl ester (L-NAME, 10 μ M), for 30 min on KCl-induced contraction, before eliciting the relaxation to the same cumulative concentrations of DHEA. The bronchodilating effect of DHEA was observed during 30 min. The concentration–response curve to DHEA as well as its potency and efficacy in tracheal rings without any pretreatment was compared with L-NAME pretreatment.

2.4. Mechanism of action

To investigate the mechanisms of action in the bronchodilating effect to DHEA, we analyzed the involvement of: nuclear transcription/translation mechanisms; the product of DHEA bio-transformation; β_2 -adrenoceptors; and VDCC.

2.4.1. Inhibitors of protein synthesis, transcription and 3β -HSD activity

In other experiments, the tracheal rings with epithelium were pre-incubated with the protein synthesis inhibitor cycloheximide (40 μ M) or the transcription inhibitor actinomycin D (10 μ M) for 30 min on the KCl-induced contraction. DHEA was then added at the same concentrations and its effect was compared in the presence and absence (control group) of those inhibitors. This protocol was designed to discard that DHEA might induce a genomic action. Additionally, since DHEA can be bioconverted into androgens or estrogens, the involvement of androgen or estrogen in the acute bronchodilation to DHEA was examined by the same

protocol: KCl pre-contracted tissues were pre-incubated, during 60 min, with 1 μ M Epostane (WIN 32,729); a pure inhibitor of the 3β -hydroxy steroid dehydrogenase (3β -HSD) activity which is an essential step in the biosynthesis of active estrogens and androgens. Then, DHEA, at selected concentrations, were added in a cumulative concentration curve. The comparisons were made with the concentration–response curve of DHEA without any pretreatment. In addition, the effect produced by the final volume of vehicle (0.1% ETOH; absolute ethanol) for inhibitors plus the DHEA vehicle was analyzed in independent experiments.

2.4.2. Potential interaction of DHEA with the β_2 -adrenergic receptors

To assess the possible interaction of DHEA with the β_2 -adrenergic receptors, the tracheal rings with epithelium contracted with CCh were incubated with 10 μ M propranolol (a β_2 -adrenergic receptors antagonist) for 30 min. Then, each concentration of DHEA was added and its effect (evaluated as described before) was compared in the absence of that antagonist. Previously, we corroborated that 10 μ M propranolol elicited an antagonist effect in ASM of guinea pig. For this purpose, on the steady tone of the CCh-induced contraction, salbutamol (10 μ M; β_2 -adrenergic agonist) was added, and an inhibition of the contraction tone was observed. Then, on a second CCh contraction, the rings were incubated for 30 min with 10 μ M propranolol, before adding salbutamol. Propranolol at 10 μ M abolished the salbutamol-induced relaxation (data not shown). On this basis, DHEA relaxing effect on CCh-induced contraction was evaluated in the presence of 10 μ M propranolol under the same experimental protocol.

2.4.3. Potential calcium channel antagonism

In another assay, DHEA was tested in tracheal rings depolarized with a KCl– Ca^{2+} -free solution (KCl– Ca^{2+} 0; a depolarizing 60 mM KCl solution modified by addition of 2 mM EGTA and without $CaCl_2$). A transient contraction was obtained to KCl– Ca^{2+} 0 and, once the baseline was reached, 2.5 mM $CaCl_2$ was added to evoke a reproducible tonic contraction, which was recorded for $\sim$ 30 min (control). Afterwards, the tissues were washed with KCl– Ca^{2+} 0 to re-equilibrate for 60 min were then pre-incubated with different noncumulative concentrations of DHEA (9–91 μ M) for 10 min before the addition of $CaCl_2$ at 2.5 mM. Under these conditions, the contraction induced by $CaCl_2$ was recorded for 30 min, which was compared with the corresponding control. Subsequently, the tissues were washed and a $CaCl_2$ -induced contraction was elicited again. The washout was done three times with KCl– Ca^{2+} 0 after all $CaCl_2$ contractions. Additionally, different tissues were pre-incubated with 100 μ M dihydropyridine-calcium channel activator (Bay K 8644, dissolved in 0.1% ethanol) 10 min after the contraction to KCl was induced. After 30 min, the cumulative concentration–response curve to DHEA was performed. The effect of DHEA was recorded as described before, and compared with its effect without Bay K 8644 and estimated in terms of diminution of the IC_{50} and E_{max} values of the control. Moreover, in different preparations, the concentration eliciting E_{max} of DHEA (200 μ M on KCl contraction) was added 10 min after the 100 μ M Bay K 8644 induced contraction had been elicited at the basal tone. Obviously, the response produced by the final volume of the DHEA vehicle or Bay K 8644 vehicle (0.1% absolute ethanol) was analyzed in independent experiments under the same experimental conditions.

2.5. Effect of DHEA on OVA-induced bronchospasm in vivo

Lung resistance (R_L) was measured by using the isovolumetric method in a closed-chamber plethysmograph (Buxco Electronics Inc., Wilmington, NC, USA). Sensitized guinea pigs (after 21–25

days of treatment) were anesthetized with sodium pentobarbital (35 mg kg^{-1} , i.p.), and the depth of anesthesia was maintained with hourly administration of additional doses of pentobarbital as needed. Each animal received pancuronium bromide (0.06 mg kg^{-1} , i.v.) to avoid spontaneous respiratory movements. After cannulating the trachea, each animal was mechanically ventilated through a rodent ventilator (7025; Ugo Basile Biological Research Apparatus, Varese, Italy) with a tidal volume of 10 ml kg^{-1} and 48 breaths min^{-1} . Right jugular vein and left carotid artery were cannulated for drug administration (DHEA or OVA) and for arterial pressure recording through a dynograph (BPA 400A; Digi-Med Blood Pressure Analyzer, Louisville, KY, USA), respectively. A water-filled cannula was positioned into the middle third of the esophagus in order to measure intraesophageal pressure as a surrogate of intrapleural pressure. Pressures obtained from the esophageal and tracheal cannulas were recorded through a differential pressure transducer (TRD4510; Buxco Biosystem). Pressure inside the plethysmograph chamber was also recorded through another differential pressure transducer. This last signal was converted to a pseudo-flow signal through software (XA v2.1; Buxco Biosystem) which calculated the relationship between signals from both transducers to obtain R_L through the formula: $R_L = \Delta P / \Delta V$, where ΔP is the pressure change and ΔV is the flow change. The R_L of the animals was recorded for 3 min (basal R_L value). After 15 min, the guinea pigs received $75 \mu\text{g kg}^{-1} \text{ min}^{-1}$ OVA i.v., administrated in $40 \mu\text{l}$ saline solution, and the R_L was recorded for 30 min. To determine if the bronchospasm produced by OVA could be prevented by the DHEA, sensitized guinea pigs were pretreated, 15 min before the antigenic challenge, with non-cumulative bolus injection of DHEA at different i.v., independent doses (35 , 110 , or $350 \mu\text{mol kg}^{-1} \text{ min}^{-1}$; each independent dose was administrated in $40 \mu\text{l}$ dimethylsulfoxide (DMSO); $11 \mu\text{mol kg}^{-1} \text{ min}^{-1}$ equivalent to 4 IU min^{-1}). R_L of sensitized guinea pigs was compared in the absence (control response) and presences of DHEA administration or in the pretreatment with the i.v. bolus injection to the same dose-volume of the DHEA vehicle, DMSO, which did not change the R_L value after the antigen challenge.

2.6. Data presentation and statistical analysis

All data in the text and figures are expressed as mean $\pm$ SDM. Differences with $P < 0.05$ were considered to be significant. All experiments were repeated at least six times. In the tension studies, n represents one guinea pig. The relaxing effect of DHEA on CCh-, KCl- or OVA-induced contraction, and the preventive effect of Ca^{2+} -induced contraction are referred as percentage of inhibition of each contraction. The concentration for each compound is expressed as the final concentration in the organ bath. The efficacy of the DHEA was expressed as E_{max} (maximal effect induced by DHEA at the highest concentration tested). The potency of DHEA was calculated from the cumulative concentration–response curve by straight-line regression and obtained through the IC_{50} values (IC_{50} ; DHEA concentration required to induce 50% relaxation or prevention of the contraction induced in each protocol) on: (i) KCl-, CCh- or OVA-induced contraction in isolated tracheal rings, and the prevention of Ca^{2+} -induced contraction by DHEA in depolarized isolated tracheal rings; and (ii) prevention of R_L in sensitized guinea pigs *in vivo*. Bronchospasm was expressed as the changes in R_L (% increase). Nonpaired Student *t*-test was used to compare the responses between two groups. To compare IC_{50} between groups, we used a one-way ANOVA followed by Tukey multiple comparisons test. The comparison between treatments vs. control we used one-way ANOVA followed by Dunnett's multiple comparison test.

2.7. Drugs and chemicals

With the exception of Epostane (WIN 32,729; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Bay K 8644 (INC Pharmaceuticals, Costa Mesa, CA, USA) and sodium pentobarbital (Pfizer, Mexico), the remaining compounds were purchased from Sigma (St. Louis, MO, USA) and included: dehydroepiandrosterone (DHEA; 3β -hydroxy-5-androsten-17-one), carbachol chloride (CCh), bradykinin (BK), ovalbumin (OVA), pancuronium bromide, aluminum hydroxide ($\text{Al}(\text{OH})_3$), dimethylsulfoxide (DMSO), N^{ω} -nitro-L-arginine methyl ester (L-NAME), propranolol ((RS)-1-(isopropilamino)-3-(naftalen-1-iloxy)propan-2-ol), salbutamol, the protein synthesis inhibitor (cycloheximide), the transcription inhibitor (actinomycin D). DHEA, Epostane, Bay K 8644, protein synthesis, and transcription inhibitors were dissolved in absolute ethanol to the concentration needed for each experiment; $10 \mu\text{l}$ aliquots were added to the 10 ml bath chambers, which never exceeded 0.5% (v/v) of vehicle, and the remaining drugs were dissolved in distilled water. Actinomycin D, salbutamol and BK were kept in the dark until use in order to avoid light-induced degradation.

3. Results

3.1. Effects of DHEA on isolated tracheal rings

DHEA in the concentration range of 9 – $200 \mu\text{M}$ fast and strongly inhibited the tone of the contraction induced by KCl and CCh as well as the tone of OVA-induced contraction in sensitized guinea pigs in a concentration-dependent manner, an effect observed within 2 min after each concentration was added (Fig. 1, top). After washout, the amplitude and tone of the next contraction induced by the respective agent, was completely recovered (data not shown), and thus, the DHEA action disappeared. These concentration–response curves did not significantly differ from those obtained in tracheal ring without epithelium (Fig. 1B and D), and from non-sensitized and sensitized animals (Fig. 1A, C and E). Regarding the sensitivity of each contraction to DHEA-induced relaxation, the IC_{50} values indicated that: OVA-induced contraction in sensitized animals was more sensitive than CCh-induced contraction in non-sensitized and sensitized animals, and these two last contractions turned out to be significantly more sensitive than KCl-induced contraction in non-sensitized and sensitized animals (Fig. 1F). However, the E_{max} values show that DHEA is more efficient to relax CCh-induced contraction in sensitized animals than KCl-induced contraction in non-sensitized animals and OVA-induced contraction in sensitized animals (Fig. 1F). The inhibition on the contraction tone induced by different agents reveals the ability of DHEA to induce relaxation in ASM. The vehicle used with the DHEA (0.1% ETOH at each concentration) did not significantly affect ($< 5.7\%$ of relaxation, $n \geq 6$) the tone of KCl, CCh or OVA contraction, but the effect induced by DHEA was significantly different from the vehicle control ($P < 0.01$).

3.2. Effect of selected inhibitors on DHEA response

The importance of NO or β_2 -adrenoceptors in mediating DHEA-induced ASM relaxation was investigated by obtaining a series of complete concentration–response relationships on KCl- or CCh-induced contraction in tracheal rings with epithelium. In these experiments, pretreatment with the NO synthase inhibitor (L-NAME) or a β_2 adrenoceptor antagonist (propranolol) resulted in no significant differences when compared to the DHEA response curves ($P > 0.05$). In addition, the inhibitors of protein synthesis (cycloheximide) and transcription (actinomycin D) had no effect

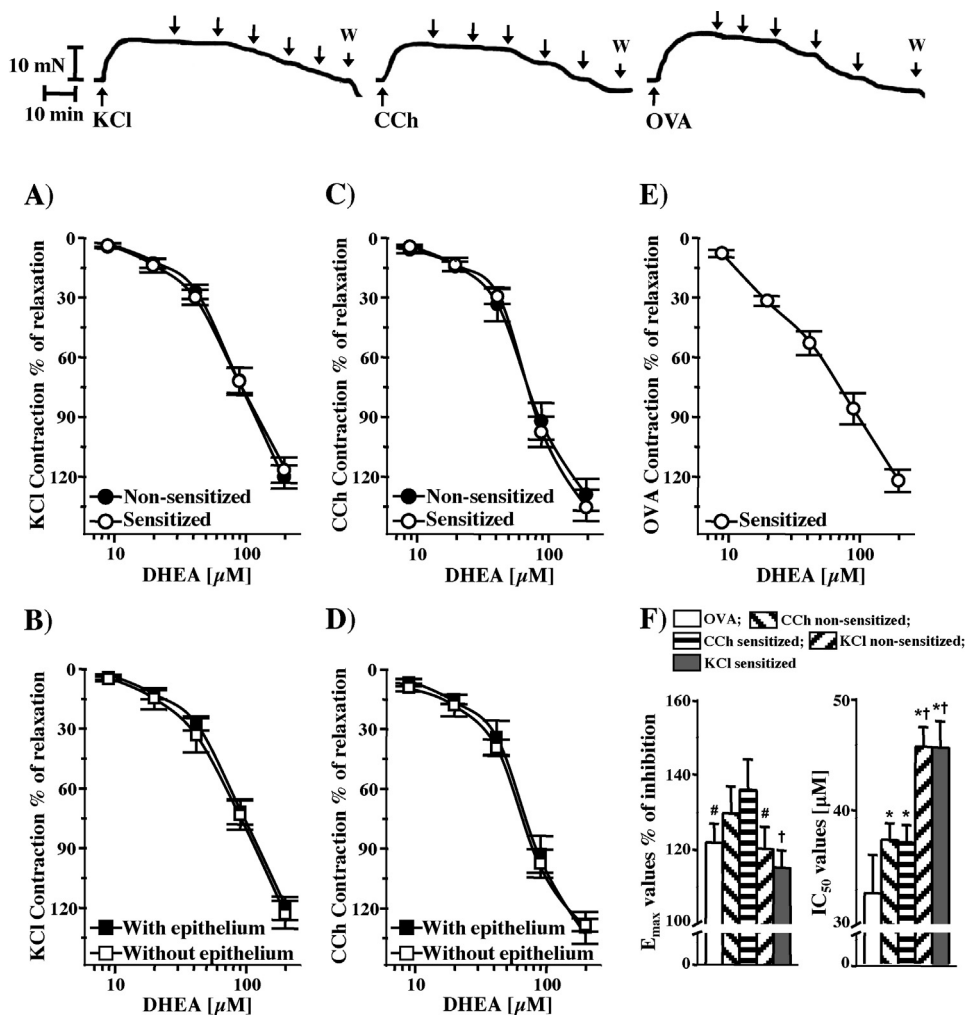


Fig. 1. Relaxing effect elicited by DHEA on different contractions in guinea pig tracheal smooth muscle. Upper panel, representative recordings of DHEA-induced relaxation in tracheal rings on the tonic contraction elicited by: high potassium solution (KCl 60 mM); carbachol (CCh 0.3 μM) and; ovalbumin (OVA 1 μM) in sensitized guinea pigs. Arrows indicate the DHEA addition at increasing concentrations (9, 20, 42, 91 and 200 μM); W represents the time of washout with Krebs–Henseleit solution. Comparison of the concentration–response curves to DHEA on: (A) KCl-induced contraction in non-sensitized vs. sensitized guinea pigs; (B) trachea with vs. without epithelium; (C) CCh-induced contraction in non-sensitized vs. sensitized guinea pigs; (D) trachea with vs. without epithelium; and (E) OVA-induced contraction in sensitized guinea pigs. (F) Maximal effect (E_{max}) and inhibitory concentration 50 (IC_{50}) values obtained from the concentration–response curves (A)–(E). Statistical significances: * $P < 0.05$ as compared with OVA; † $P < 0.05$ as compared with CCh in sensitized and non-sensitized animals; # $P < 0.05$ as compared with CCh in sensitized animals. Each symbol or bar represents the mean $\pm$ SDM ($n = 6$).

on the concentration–response curve to DHEA ($P > 0.05$). Similarly, pretreatment with the inhibitor of 3 β -HSD enzyme did not block the DHEA relaxing response ($P > 0.05$). Thus, with the exception of Bay K 8644 treatment ($P < 0.001$), identical concentration–response curves to DHEA were obtained with and without the remaining treatments ($P > 0.05$). Obviously, the IC_{50} and E_{max} values did not significantly differ from that obtained after each pretreatment (Table 1).

3.3. Effect of DHEA on influx Ca^{2+} through voltage-dependent Ca^{2+} channels

After tracheal rings depolarization with KCl– Ca^{2+} solution, a tonic contraction was induced rapidly by $CaCl_2$, which was: (i) antagonized when tissues were pre-incubated with DHEA; and (ii) reversible after washout (Fig. 2A). Fig. 2B shows that DHEA induced a concentration-dependent blockade on the $CaCl_2$ contractions in both non-sensitized and sensitized guinea pigs which were not significantly different ($P > 0.05$). DHEA at the highest concentration tested (91 μM) induced a nearly complete prevention of $CaCl_2$ contractions (>98% of prevention in both non-sensitized

and sensitized, $n = 6$). For the sake of comparison, the prevention of $CaCl_2$ -induced contraction to DHEA turned out to be significantly higher than its inhibitory effect on KCl–CCh and OVA-induced contraction ($P < 0.05$). This finding reveals that DHEA is a powerful

Table 1
Maximal effect and IC_{50} values in tracheal rings with epithelium from non-sensitized animals in response to DHEA.

Contraction	Treatment	E_{max} (%)	IC_{50} (μM)
CCh	Control	129.53 $\pm$ 8.06	37.52 $\pm$ 1.49
CCh	Propranolol	130.71 $\pm$ 7.39	37.25 $\pm$ 0.95
KCl	Control	120.04 $\pm$ 5.86	45.84 $\pm$ 1.77
KCl	L-NAME	121.66 $\pm$ 8.07	45.59 $\pm$ 0.86
KCl	Epostane	121.31 $\pm$ 4.97	48.99 $\pm$ 7.56
KCl	Cycloheximide	122.62 $\pm$ 7.45	46.19 $\pm$ 2.55
KCl	Actinomycin	122.18 $\pm$ 3.37	43.41 $\pm$ 3.10
KCl	Bay K 8644	98.99 $\pm$ 7.56*	60.43 $\pm$ 5.03*

Values are mean $\pm$ SDM; n = number of animals. Half inhibitory concentration (IC_{50}) and maximal effect (E_{max}) were obtained from the concentration–response curve to DHEA on CCh- or KCl-induced contraction with and without (control) treatments.

* Value significantly different ($P < 0.001$) as compared with its control.

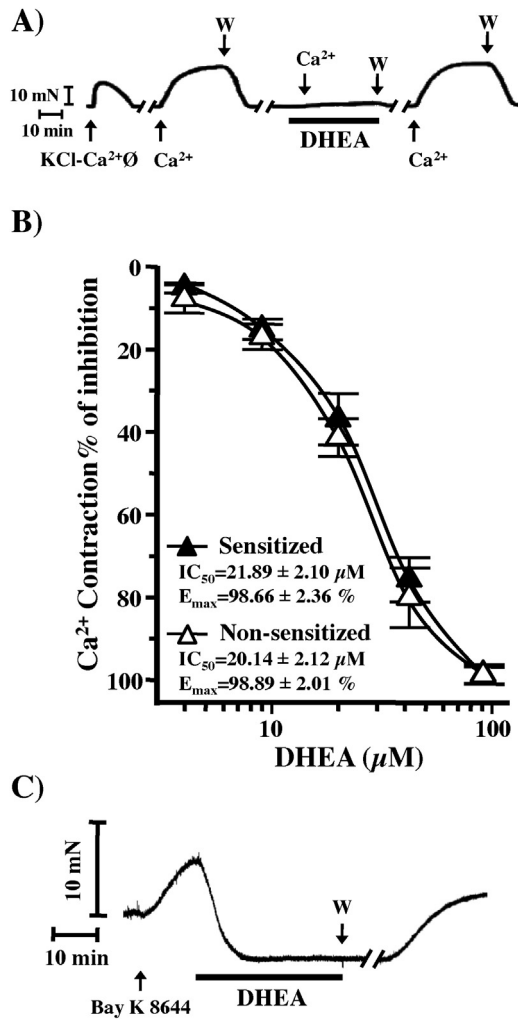


Fig. 2. Prevention of extracellular Ca^{2+} influx by DHEA. (A) DHEA antagonizes the contraction induced by 2.5 mM CaCl_2 (Ca^{2+}) in tracheal rings previously depolarized by KCl-Ca^{2+} free solution (KCl-Ca^{2+}). Ca^{2+} -induced contraction was completely prevented by DHEA at 91 μM , the solid black line indicates the incubation time with DHEA; note the total contraction recovery after washout (W) with KCl-Ca^{2+} free solution showing that the Ca^{2+} antagonistic effect of DHEA was reversible. (B) Concentration-response curves of the Ca^{2+} antagonistic effect of DHEA in non-sensitized and sensitized guinea pigs. Each point represents the mean $\pm$ SDM ($n=6$). Inhibitory concentration 50 (IC_{50}) and maximal effect (E_{max}) were expressed as the mean $\pm$ SDM ($n=6$). (C) Typical recording of the contraction induced by 100 μM Bay K 8644 (L-type VDCC channel activator) and the immediate relaxing effect to DHEA at 200 μM . W indicates washout with normal Krebs-Henseleit solution.

Ca^{2+} antagonist. The final volume of DHEA vehicle (0.1% absolute ethanol) did not significantly prevent CaCl_2 -induced contraction ($3.16 \pm 1.41\%$ of prevention, $n=6$; $P>0.05$). Furthermore, tracheal preparations pre-incubated with Bay K 8644 shifted to the left the concentration-response curve to DHEA on KCl -induced contraction (data not shown); thus, the E_{max} and IC_{50} values were significantly different ($P<0.05$) when compared with that produced by DHEA alone (Table 1). Notably, DHEA at the concentration eliciting E_{max} induced an immediate relaxant effect on the Bay K 8644-induced contraction ($>100\%$ of relaxation, $n=6$), with a loss at the basal tone; subsequently, the tissues were washed out and the basal tone was recovered (Fig. 2C).

3.4. Lung resistance measurement in response to DHEA

Fig. 3A shows the time-course of change in R_L after the challenge with OVA in the sensitized guinea pigs. After 4 min of

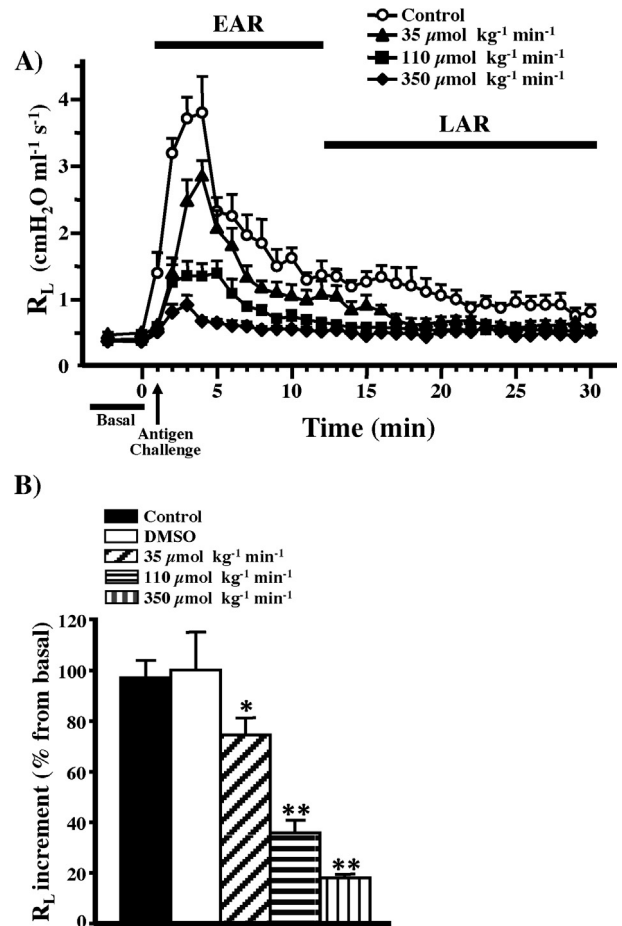


Fig. 3. Time- and dose-dependent effects of DHEA in lung resistance to antigen challenge induced by OVA in sensitized guinea pigs. (A) The different doses of DHEA were administered 15 min before the antigen challenge and evaluated during 30 min, each dose was significantly different ($P<0.05$) as compared with OVA-induced bronchospasm in the absence of DHEA (control); R_L , lung resistance; EAR, early asthmatic response; LAR, late asthmatic response. (B) Percent of diminution in R_L after 4 min of OVA-induced bronchospasm (EAR, acute phase of bronchospasm); * $P<0.05$ and ** $P<0.01$ compared with the control and vehicle group. Each value represents the mean $\pm$ SDM for 6 animals.

the challenge, an elevation of basal R_L value to $3.80 \pm 0.54 \text{ cm H}_2\text{O ml}^{-1} \text{ s}^{-1}$; $n=6$ was observed as the maximal peak of bronchospasm (early asthmatic response; EAR) but 12 min later the R_L was decreased to $1.37 \pm 0.21 \text{ cm H}_2\text{O ml}^{-1} \text{ s}^{-1}$ ($n=6$) and the basal R_L value was not recovered for 28 min (late asthmatic response; LAR); i.e., during this period the bronchospasm never returned to the basal R_L values. Pretreatment with DHEA was delayed (~ 2 min) and suppressed (25–80%) in a dose dependent manner the EAR (Fig. 3B). Furthermore, DHEA diminished ($>25\%$) the LAR, which was completely abolished at the highest doses tested. It is important to note that bronchospasm prevention was not accompanied by significant changes in blood pressure and heart rate in any case (not shown for the sake of clarity).

4. Discussion

4.1. General

In previous studies, we have shown that DHEA is capable of inducing an acute uterine and vascular relaxation in animals and humans [31–33]. In the present study, we demonstrated that DHEA may also induce ASM relaxation, a new aspect of DHEA function. The data showed that in tracheal rings from non-sensitized

and sensitized animals, DHEA elicited a concentration-dependent ASM relaxing effect on contraction induced by KCl, CCh or OVA, as well as a marked preventive effect on the Ca^{2+} -induced contraction in KCl-depolarized tissues without Ca^{2+} . In addition, we also found that sensitized guinea pigs treatment with DHEA suppressed the bronchospasm by OVA in a dose–response manner. Taken together, these results indicated that DHEA is a “bronchoactive” steroid that induces an acute ASM relaxation. The present data permit the first direct analysis of the correlation between DHEA and ASM. The relaxation induced by DHEA was immediate in onset, occurring within 2 min, and reversible after washouts, suggesting a nongenomic (membrane) action rather than the classical steroid modulation of nuclear transcription. This proposal is strengthened by our findings that the relaxation response to DHEA was not modified by inhibitors of protein synthesis and transcription. With this background, the present study reports a nongenomic relaxing action of DHEA on ASM. In line with this view, several responses induced by DHEA have been characterized as nongenomically mediated, since a no specific receptor protein had been identified or characterized for DHEA. Admittedly, it has been recently reported a membrane-bound receptor protein that interacts specifically with $[^3\text{H}]\text{DHEA}$ in endothelium, heart, liver, and kidney [35,36]. On the other hand, it has been suggested that the action of DHEA is thought to act after its biotransformation into active androgens or estrogens. However, in this study the relaxing effect of DHEA was unaffected in the presence of an inhibitor of the $3\beta\text{-HSD}$, an essential enzyme in the biosynthesis of active estrogens and androgens. Therefore, this result pointed out that the intracrine transformation of DHEA is not needed to elicit bronchorelaxation. Consequently, DHEA is not just a metabolic intermediate in the pathway for the synthesis of androgens and estrogens. These data do not contradict the fact that the ASM relaxing effect is also elicited by androgens and estrogens [37–39] or by progestins [40].

Another key finding of the current study is that the bronchodilating effect of DHEA is operative *in vivo* since this steroid may prevent and retard the bronchospasm elicited by OVA in sensitized guinea pigs. This result is consistent with a previous report where the combination of low doses of bacille Calmette–Guérin and DHEA had a synergistic effect in suppressing the development of airway hyperresponsiveness to methacholine in female mice [41]. Moreover, a clinical study has also shown that an inhaled suspension of DHEA-S delivered *via* nebulizer improved asthma control scores in subjects with poorly controlled moderate-to-severe asthma [42]. These lines of evidence and the present data suggest that DHEA is a bronchodilator that acts rapidly to alleviate bronchospasm, which could be therapeutically relevant in the treatment of asthma. Remarkably, we have now demonstrated that the prevention of bronchospasm by DHEA administered to OVA-sensitized guinea pigs about $35\text{--}350\text{ }\mu\text{mol kg}^{-1}$; equivalent to $0.01\text{--}0.1\text{ mg kg}^{-1}$, are low compared to the $0.25\text{--}0.5\text{ mg kg}^{-1}$ of DHEA and DHEA-S secreted daily in humans [43].

4.2. Mechanism of bronchodilating action of DHEA

In ASM cells, Ca^{2+} influx is mediated by different pathways including store-operated Ca^{2+} entry (SOCE), receptor-operated Ca^{2+} channel (ROCC) and VDCC [44]. In this context, we observed that KCl-induced contraction was completely inhibited by DHEA, implying a reduction of extracellular Ca^{2+} influx and also suggesting an antagonism on the VDCC. In line with this proposal, we found that DHEA prevented Ca^{2+} -induced contractions in tracheal rings depolarized by high KCl– Ca^{2+} -free solution. In agreement with these data, we observed that DHEA elicited a minor relaxation in the presence of Bay K 8644 which promotes calcium influx with receptors

on the VDCCs; likewise, DHEA strongly abolished the contraction elicited by Bay K 8644. On this basis, one would expect that the relaxation elicited by DHEA is a consequence of calcium entry blockade *via* VDCCs, probably by binding to dihydropyridine site. These results indicated that DHEA inhibited contractile responses of ASM to Ca^{2+} by blocking, at least in part, the VDCC. However, the fact that DHEA may also induce an acute relaxation on CCh- or OVA-induced contraction deserves further consideration. CCh induces cytosolic Ca^{2+} increase, which consists of an initial transient due to Ca^{2+} release from sarcoplasmic reticulum Ca^{2+} stores and a subsequent sustained plateau due to extracellular Ca^{2+} influx. Therefore, DHEA may also block the SOCE and ROCC. On the other hand, the highlighted ability of DHEA to relax a bronchoconstriction immunologically induced (OVA-induced contraction in sensitized animals) pointed out that DHEA plays an important role in the functional regulation of ASM. The mechanism of action to relax OVA-induced contraction by DHEA is difficult to explain due to the fact that, upon OVA challenge, the sensitized guinea pig trachea releases histamine, peptidoleukotrienes, thromboxane A_2 , tumoral necrosis factor ($\text{TNF-}\alpha$), serotonin, and prostaglandins PGD_2 and $\text{PGF}_{2\alpha}$ among the most relevant. Interestingly, we observed that nifedipine at $1\text{ }\mu\text{M}$ (a concentration enough to block the VDCC) induced less than 10% of relaxation on OVA-induced contraction whereas DHEA induced a complete inhibition of this contraction. In this respect, we cannot rule out that this relaxing effect may employ additional mechanisms such as a blockade of SOCE and ROCC, which play an important role in the functional regulation of ASM and contribute to agonist-induced intracellular Ca^{2+} increment and tracheal contraction, as reported to be the case for acetylcholine-induced contraction [45]. On these bases, further studies are needed to define the Ca^{2+} -dependent regulation of DHEA to induce bronchodilation on ASM.

On the other hand, no difference was found in sensitivity to DHEA between non-sensitized and sensitized guinea pigs, suggesting that the pathological status might not be an important factor for the relaxing effect induced by DHEA. Thus, both non-sensitized (non-asthmatic) and sensitized (asthmatic) ASM are targets of DHEA's relaxing action. We also observe that the tracheal rings remained unaltered after epithelium removal; this finding strongly suggested an epithelium independent action. Likewise, as expected, L-NAME did not block DHEA-induced relaxation in ASM. Therefore, DHEA inhibits ASM tone *via* epithelium- and NO-independent mechanism. These findings agree with the epithelium- and NO-independent relaxing response which are elicited by the product of DHEA metabolism, testosterone and dihydrotestosterone, in isolated the guinea pig and the bovine trachea [37]. Nevertheless, these results are not in line with a previous study in the isolated rabbit trachea [38], in which testosterone elicits relaxation *via* epithelium- and NO-mediated way. The reason for this discrepancy may be related to differences between the steroid studied and perhaps species. Our results are consistent with the response of DHEA in vascular smooth muscle by producing an endothelium- and NO-independent vasorelaxation in human umbilical artery [32]. We also exclude a possible interaction of DHEA with β_2 adrenoceptors because the β_2 antagonist, propranolol, did not block the relaxing effect of DHEA.

Admittedly, μM concentrations of DHEA were tested in the *in vitro* experiments of the present study but by its nature, DHEA and other steroids are high hydrophobic substances, and are applied at rather high concentrations in isolated tissues. Consequently, μM concentrations are accepted in this type of experiments, which is consistent with previous findings indicating that in isolated guinea pig trachea, physiological levels of cortisol (around $150\text{ }\mu\text{M}$) induces bronchodilation by synergizing with the relaxing action produced by a β_2 -agonist through the nongenomic pathways of glucocorticoids [46].

4.3. Possible physiological and clinical implications

Indeed, it is important to consider that there is a correlation between the onset time of nonallergic asthma and the DHEA levels. DHEA levels are very high in the umbilical cord and in first day of life but these levels decrease progressively over the next 6 months and remain very low for the first 6 years of life; thus, asthma symptoms may be partly attributed to the lowest DHEA levels during the first 6 years of life, particularly in boys. Afterward, plasmatic DHEA levels begin an abrupt rise and continue to increase until peaking at 20–30 years [47,48], when, in men, severity of asthma is relatively stable from puberty until reaching the age of 30. Remarkably, it has been observed that nonallergic asthma usually occurs in the second half of life; initially around the age of 40 years [49], the time when circulating DHEA starts to decline. Therefore, a fall in serum DHEA may be considered at least as partly accountable for the asthma symptoms. This suggestion correlates well with the observation that the levels of DHEA and DHEA-S are reduced in patients with asthma [22–24]; hence, in asthma, it may be desirable to at least restore this hormone level, which may be considered as DHEA replacement therapy after middle age to prevent or attenuate asthma symptoms.

The age-related decline in DHEA has been implicated in the high incidence of cardiovascular and neurodegenerative diseases, cancer, diabetes, obesity, stress, viral and bacterial infections, and in overall mortality [50,51]. Notably, DHEA and DHEA-S have important immunomodulating effects on animal and humans [52,53], thus, DHEA ameliorates inflammatory responses in a number of experimental systems (reviewed by [21]). In addition, a recent study has reported that DHEA-S inhibited chemotaxis of both human neutrophils and ASM cells, suggesting that DHEA-S may attenuate airway inflammation and cell migration [54]. Taken together, the immunomodulating property of DHEA plus the acute bronchorelaxing effect that we here reported, this study provides some basic fundaments for our understanding of asthma physiopathology and the role that DHEA plays in this process.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgments

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