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The orl rat is more responsive to methacholine challenge than wild type

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Abstract

Background—This study presents an animal model of native airway hyperresponsiveness (AHR). AHR is a fundamental aspect of asthma and reflects an abnormal response characterized by airway narrowing following exposure to a wide variety of non-immunological stimuli. Undescended testis (UDT) is one of the most common male congenital anomalies. The orl rat is a Long Evans substrain with inherited UDT. Since boys born with congenital UDT are more likely to manifest asthma symptoms, the main aim in of this study was to investigate the alternative hypothesis that orl rats have greater AHR to a methacholine aerosol challenge than wild type rats.

Methods—Long Evans wild type (n = 9) and orl (n = 13) rats were anesthetized, tracheostomized, and mechanically ventilated at 4 weeks of age. Escalating concentrations of inhaled methacholine were delivered. The methacholine potency and efficacy in the strains were

measured. Respiratory resistance was the primary endpoint. After the final methacholine aerosol challenge, the short-acting β_2 -adrenoceptor agonist albuterol was administered as an aerosol and lung/diaphragm tissues were assayed for interleukin (IL)-4, IL-6, and tumor necrosis factor (TNF)- α . Histological and histomorphometrical analyses were performed.

Results—The methacholine concentration-response curve in the orl group indicated increased sensitivity, hyperreactivity, and exaggerated maximal response in comparison with the wild type group, indicating that orl rats had abnormally greater AHR responses to methacholine.

Histological findings in orl rats showed the presence of eosinophils, unlike wild type rats. β_2 -Adrenoceptor agonist intervention resulted in up-regulation of IL-4 diaphragmatic levels and down-regulation of IL-4 and IL-6 in the lungs of orl rats.

Conclusion—orl rats had greater AHR than wild type rats during methacholine challenge, with higher IL-4 levels in diaphragmatic tissue homogenates. Positive immunostaining for IL-4 was detected in lung and diaphragmatic tissue in both strains. This model offers advantages over other pre-clinical murine models for studying potential mechanistic links between cryptorchidism and asthma. This animal model may be useful for further testing of compounds/therapeutics options for treating AHR.

Keywords

Airway hyperresponsiveness; Hyperreactivity; Airway pharmacology; Lung inflammation; Asthma; Bronchoconstriction

1. Introduction

The orl rat is an inbred Long Evans substrain with inherited cryptorchidism or undescended testis (UDT) [1,2]. This is a distinctive model of UDT since the phenotype is similar to that seen most commonly in humans. UDT is one of the most common male congenital anomalies in humans; between 2 and 5% of full-term and 30% of premature male infants are born with UDT [3]. In orl rats, UDT prevalence is approximately 60% [4]. It is likely that clinical UDT is the result of gene-hormone-environment interactions [2,5,6]. A case-control study demonstrated that boys born with UDT were more likely to present with asthma [7]. This study interviewed mothers of affected (83 UDT cases) and unaffected boys (129 controls), and reported a 9.6% proportion of at least one episode of asthma in cases vs. 1.6% in controls. In addition, we have observed respiratory distress, a wheezing-like pattern, and a higher mortality in the orl rat, than wild type rats. A respiratory phenotype in orl rats strain has not been described. Because with an asthma-like condition, the diaphragm may be spasmodically in contraction to force air in and out the lungs, we were interested also in the evaluation of the diaphragmatic tissue.

Asthma is currently defined by the Global Initiative for Asthma as a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, and the chronic inflammation is associated with airway hyperresponsiveness (AHR) that leads to recurrent episodes of wheezing [8]. AHR is a fundamental aspect of asthma, but also may be present with other respiratory disorders. AHR reflects an abnormal response characterized by airway narrowing following exposure to a wide variety of nonsensitizing stimuli of a

chemical or physical origin. AHR reveal the ease with which airway narrowing is achieved (the sensitivity), and the extent of that response, which is represented by the hyperreactivity and maximal response [9] in the concentration-response curve. AHR may be due to the presence of airway inflammation, abnormal airway mechanics, or a combination of both; however additional mechanisms which lead to an augmentation of the stimuli (pre-junctional mechanisms) and mechanisms which lead to an increased response (post-junctional mechanisms) are complex possible mechanisms [10].

With regard to the rationale of the inflammatory biomarkers selected in this study, the overproduction of interleukin (IL)-4 has been associated with atopic and non-atopic asthma [11]. Tumor necrosis factor (TNF)- α is a pro-inflammatory cytokine that is also produced in increased amounts in asthmatic airways and may be an amplifying mediator in AHR/asthma [12,13]. IL-6, an interleukin that acts as the pro-inflammatory and anti-inflammatory cytokine, is a muscle-derived cytokine found in high levels in the tissues rats exposed to respiratory muscular work [14].

Native or intrinsic AHR (not antigen-induced) may be determined genetically. Most studies of inbred mice strains and back-crosses have suggested strong genetic control of airway narrowing [15–17]. It seems likely that animal models of native AHR may hold considerable potential for understanding the genetics of asthma and associated diseases. A detailed understanding of the concentration-response curves and their determinants – altered airway sensitivity, airway reactivity and maximal degree of airway narrowing – is fundamental for the translation of functional and drug response findings to the bed-side.

The aims of this pre-clinical translational study were to compare respiratory mechanics (under resting conditions, during methacholine), and following β_2 -adrenoceptor agonist treatment, bio-markers of lung inflammation, and tissue structural differences between orl rats and wild type rats. We hypothesized that orl rats would have greater AHR than wild type rats, and this outcome would be associated with biomarkers of tissue inflammation/injury and structural alterations in the lung and diaphragmatic tissues.

2. Material and methods

2.1. Animal preparation

This animal model of inhaled methacholine (methylcholine chloride; Methapharm Inc., Ontario, CA) challenge-induced constriction was approved by the Institutional Animal Care and Use Committees. Two groups, Long Evans wild type rats ($n = 9$) five females and four males, and orl rats ($n = 13$) five females and four males, and orl rats ($n = 13$) five females and eight males, were studied at 4 weeks of age (equivalent to early puberty/late childhood in humans). A control group of animals [sham-ork rats ($n = 6$)] was used. The sham-ork rats underwent the same procedures, but there were not challenged with inhaled methacholine, neither received β_2 -adrenoceptor agonist treatment. Animals were anesthetized with intraperitoneal injections of a ketamine-xylazine (1.5:1 ratio) combination (0.05 mL/10 g), without the need of a paralytic agent. Their trachea was opened and a 19-gauge commercial cannula (Buxco Research Systems, Wilmington, NC, USA) was inserted (quarter of its length) and secured with suture to avoid leak and disconnections. Oxygen saturation and

heart rate were monitored using a pulse oximeter (MouseSTAT, Smiths Medical, Waukesha, WI). Body temperature was monitored using a laser thermometer (ThermoWorks, Alpine, UT) and maintained using a far infrared warming pad (Kent Scientific Corporation, Torrington, CT).

2.2. Baseline respiratory mechanics

Animals were immediately connected to the pneumotacho-graph of the plethysmography/ventilator chamber FinePointe RC (Buxco Research Systems, Wilmington, NC, USA) and mechanically ventilated. The volume-targeted ventilator is built-in the system. Respiratory function was measured using this commercial plethysmography system, which collects invasive respiratory resistance and compliance data in anesthetized animals that are tracheostomized. Assessment herein involved measurements of flow and pressure at the tracheal opening. ECG leads were placed to monitor the heart rate. Body temperature was maintained using the heated table of the chamber. Following instrumentation, a 10-min acclimation period was performed to obtain a stable period inside the plethysmography chamber through 3 LPM of O₂, tidal volumes = 0.05 mL/10 g, respiratory rate of 90 breaths/min, and 2 cm H₂O of positive end-expiratory pressure. A baseline recording without nebulization (resting mechanics) was done at the end of the acclimation period, followed by a control recording using normal saline.

2.3. Methacholine challenge and short-acting β_2 -adrenoceptor agonist administration

Once a stable control-saline measurement was recorded, an aerosol methacholine challenge was started with 0.3, 0.7, 1.5, 3.1, 6.2 and 12.5 mg/mL of methacholine during 5 min, at a volume of 0.05 mL using a standard commercial clear Plexiglass in-line aerosol block (Buxco Research Systems, Wilmington, NC, USA) and nebulizer head (Aerogen, Inc., Galvan, Ireland). Respiratory resistance, dynamic compliance (C_{dyn}), respiratory rate and tidal volume were recorded every 5 min during the methacholine administration. At the end of the methacholine challenge, animals were treated with a short-acting β_2 -adrenoceptor agonist, albuterol sulfate inhalation solution, 0.5% (Bausch & Lomb Incorporated, Tampa, FL) at a concentration of 0.238 mg/mL using the same delivery system to evaluate for dilation response, to minimize tissue distortion and facilitate the histological evaluation. All drug delivery times were set at 5 min; response time and recovery time was set at 3 min and 1 min respectively. The methacholine challenge lasted overall 42 ± 10 min. Data derived from these measurements were stored automatically by the FinePointe RC software version 1.0 (Buxco Research Systems, Wilmington, NC, USA) which interfaces with the plethysmography chamber. Resistance was the primary endpoint and data presented herein correspond to the mean of the first 3 min recorded after each drug exposure. Data are expressed as percent of bronchoconstriction from baseline.

2.4. Tissue preparation and cytokine extraction

Immediately after the physiological measurements were completed, animals were euthanized using intraperitoneal injections containing a commercial euthanasia solution [Sodium pentobarbital 390 mg/mL + sodium phenytoin 50 mg/mL, (Euthasol®, Virbak AH Inc., Fort Worth, TX)] at a dose of 0.1 mg/g of body weight. Thoracotomy was then performed followed by lung and diaphragm tissue dissections. Great care was taken to

maintain a constant positive end-expiratory pressure of 2 cmH₂O during tissue harvesting. Right non-dependent lung samples (highest part of the lungs in relation to gravity) and right diaphragms were dissected, formalin-fixed and prepared for histological and histomorphometrical analysis. Rat's left lungs and diaphragms were dissected, snap-frozen in liquid nitrogen and stored at -80 °C, followed by homogenization at a later time. The levels of rat TNF- α , IL-4, and IL-6 in the supernatant generated from homogenized lung tissue were measured with quantitative enzyme-linked immunosorbent assay (ELISA) using commercially available rat TNF- α , IL-6, and IL-4 ELISA kits (R&D systems, Minneapolis, MN). Tissue samples (50 mg) were washed twice with phosphate-buffered saline and homogenized with a ratio of 10 μ l:1 mg of lung tissue or 15 μ l:1 mg of diaphragm tissue of RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO) at 1:100 dilution. The homogenate was centrifuged at 12,000 $\times g$ for 10 min at 4 °C to obtain the supernatant for measurement of IL-4, IL-6, and TNF- α . Recombinant rat IL-4, IL-6 and TNF- α proteins (R&D systems, Minneapolis, MN) were used as positive controls, and the control range for their respective immunoassays was as follows: rat IL-4 (108–180 pg/mL), rat IL-6 (184–372 pg/mL) and rat TNF- α (73–121 pg/mL). All samples were appropriately diluted to fall within the detection range of each assay, and all samples and standards were assayed in duplicate. The test sensitivity for respective immunoassays was as follows: rat TNF- α (less than 5.0 pg/mL), rat IL-6 (14e16 pg/mL), and IL-4 (less than 5 pg/mL). Inter-assay and intra-assay coefficients of variation were <10%.

2.5. Histopathology protocol

Right lung non-dependent tissue sections ($n = 8$) vs. ($n = 12$) vs. ($n = 6$) and right diaphragms sections ($n = 8$) vs. ($n = 9$) vs. ($n = 6$) of wt, orl and sham-orl rats respectively were stored and removed from 10% formaldehyde after 24–48 h. They were then washed in PBS solution and embedded in paraffin. The embedded tissue was then sectioned (5- μ m) and three samples of each were mounted on Superfrost Plus slides. Tissues were removed from the paraffin, placed in Bouin's fixative, and stained using hematoxylin & eosin (H&E), according to CLIA-approved protocols. The preparations were examined by standard light microscopy (NIS-Elements BR 3.1). A board-certified pathologist (PAK), who was blinded to the group of animals, analyzed each preparation as follows: Four representative fields were selected from each preparation. In each lung field, the degree of peribronchial, perivascular, and alveolar septal inflammation was scored at 200 $\times$ total magnification. An inflammatory injury scoring system was developed; the injury scoring system was based on 3 histological measurements: Perivascular inflammation, peribronchiolar inflammation, and alveolar septal inflammation. Each parameter was scored between 0 and 4 (none-severe), depending on the degree of inflammation 0 = none, 1 – minimal, 2 – mild, 3 – moderate and 4 – severe.

2.6. Histomorphometry protocol

For histomorphometrical analyses, right non-dependent lung samples were step sectioned at 500 μ m intervals and a total of five 5 μ m sections were slide-mounted, and stained with hematoxylin and eosin. Morphometric analysis was performed using a computerized image analysis program (Image Pro Plus®, Silver Spring, MD) with custom-written algorithms

[18]. Using densitometry, digitized images of matched lung samples were measured to determine total gas exchange (V_g) and parenchyma area (V_p) for calculating expansion index ($EI = V_g/V_p$) [19]. The number of exchange units per fixed field, and measurement of alveolar area and alveolar diameter was determined using a modification of the radial alveolar count method and morphometric algorithms, respectively. For the airway dimensions, wall thickness of the conducting zone (primary and terminal bronchiole) was calculated from the differences between luminal and outer wall diameter of the airway measured at 6 points viewed on cross-section under 200 $\times$ magnifications. Wall thickness data results are based on a total of 22 airways in the wild type group vs. 23 airways in the orl group.

2.7. Immunohistochemistry (IHC) protocol

Formalin fixed paraffin embedded samples of rat lung and diaphragm were cut at 5 mm on a Leica 2050 microtome and floated onto slides. Slides were heat immobilized for 60 min at 60 °C, cooled to room temperature (RT), deparaffinized and rehydrated to de-ionized water (dH_2O). Samples were heat retrieved in sodium citrate buffer at pH 6.0 for 30 min and allowed to cool to RT. The slides were washed in phosphate buffered saline followed by a brief rinse in dH_2O . The sections were blocked with dual enzyme blocker (DAKO, Carpinteria, CA) for 5 min at RT, rinsed in phosphate buffered saline for 5 min, placed in rabbit polyclonal IL-4 (Abcam Inc., Cambridge, MA) at 1:50 in phosphate buffered saline with 2% bovine serum albumin (BSA) and incubated for 1 h at 4 °C in a humidity chamber. Sections were rinsed and placed in fresh phosphate buffered saline for 5 min. The sections were developed in a polymer//HRP detection for 10 min at RT, rinsed, and washed for 5 min in phosphate buffered saline, then placed in 3', 3'-diaminobenzidine for 5 min. The slides (prior to double staining in the second primary antibody) were rinsed in dH_2O , blocked in double stain blocker for 5 min, and rinsed for 5 min in phosphate buffered saline. Next, sections were placed in the second primary antibody, mast cell tryptase-AA1 (Novus Biologicals, Littleton, CO) at 1:10,000 in phosphate buffered saline with 2% BSA and incubated for 1 h at RT. The slides were washed in phosphate buffered saline for 5 min and placed in the secondary antibody (a rabbit/mouse link) followed by the companion polymer/AP detection for 30 min at RT and rinsed (2 $\times$) for 5 min each in phosphate buffered saline. All slides were then placed in permanent red chromogen for 10 min, washed, and counterstained in Mayer's hematoxylin for 3 min. The slides were blued in phosphate buffered saline for 5 min and placed in a final dH_2O prior to mounting in Advantage (Accurate Chemical, Westbury, NY). A tissue array control slide of normal organs from an 8 week old Sprague–Dawley adult rat (IMAGENEX HISTO-Array, San Diego, CA) lung (Fig. 1a) and abdominal wall muscle (Fig. 1b), was used as the negative control. Pediatric rat lung (Fig. 1c) and diaphragm (Fig. 1d) were also used as negative controls.

2.8. Statistical analysis

Results are presented as the mean and standard error of the mean (SEM), unless indicated otherwise. Unpaired *t*-tests (2-sided) were performed for the physiological, inflammatory markers and histomorphometry outcomes. Confidence intervals (CI) were set at 95% with an alpha significance level of 0.05. The primary end-point is the significant change in the lung function parameter, specifically respiratory resistance, during methacholine challenge. No

differences in AHR, inflammatory biomarkers and structural alterations in the lung and diaphragmatic tissues are our null hypotheses. Each methacholine concentration-response curve was fitted to the Hill equation using the least square method to provide estimates for half maximal effective concentration (EC₅₀) values. Statistical analysis was performed by factorial ANOVA, interaction between methacholine concentration and strain was reported. Paired *t*-test (two-tailed) was performed for the airway dilation test and % of changes were calculated as mean% post-challenge e higher MCh concentration/higher MCh concentration *100. For each histological assessment (peribronchiolar, perivascular, and alveolar inflammation), counts were computed and the overall inflammatory score for each parameter was statistically analyzed using chi-square analysis. All assumptions were tested for normalization. In case of violation of any assumption, appropriate transformations were taken or non-parametric tests were performed. Variances were tested using Levene's test. Transformed variables are presented by means of geometric means (GM). The groups were compared using *t*-test or the Wilcoxon Rank Sum test. Continuous non-normal dependent variables were reported using medians. Statistical analysis was completed using a combination of statistical software's (Graph Pad Prism version 5.02, San Diego, CA, USA and SPSS version 19; IBM, Chicago, IL, USA). Unchallenged orl rats served as controls.

3. Results

3.1. Baseline outcomes

The orl and wild type groups had similar body weights ($p > 0.05$), while the body weight of the sham-ork group differed from the orl ($p < 0.001$) and wild type ($p < 0.05$) weights, however all groups had similar oxygen saturations, heart rates, R and Cdyn values (Table 1).

3.2. Pharmacologic outcomes

The respiratory resistance concentration-response curves (Fig. 2) demonstrate differences between the strains, expressed by the left shift and steeper slope of the orl respiratory resistance curve. An immeasurable plateau was seen in orl vs. wild type rats, indicating an exaggerated maximal response. The estimated provocative concentration of methacholine causing a 40% increase in baseline respiratory resistance (PC₄₀) was lower for orl rats (3.1 mg/mL) compared with wild type rats (6.2 mg/mL). No differences in R measurements were found between orl genders, $p = 0.97$.

The Cdyn concentration-response curves (Fig. 3) were not different between the strains nor were their EC₅₀ and CI estimates: orl 3.2 (1.3, 7.6) mg/mL vs. wild type 5.09 (1.6, 15.0) mg/mL. The PC₃₀ was slightly lower for orl rats (1.5 mg/mL) compared with wild type rats (3.1 mg/mL) but the difference was not statistically significant.

Following treatment with albuterol sulfate, orl rats showed a reduction in respiratory resistance, whereas the fall in wild type rats was not significant (Fig. 4). The proportion of adverse events of tachycardia and bradycardia in the wild type group were the same, 2 out of 9 (22%). The orl rats demonstrated 30% of bradycardia events, one tachycardia event (8%), and one animal exhibited tachycardia followed by bradycardia.

3.3. Lung and diaphragmatic quantitation of inflammatory biomarkers

In comparison with wild type rats, concentrations of IL-4 and IL-6 in lung tissue were lower in orl rats (Fig. 5a–b), with no differences in levels of TNF- α (Fig. 5c). IL-4 was significantly higher in diaphragmatic tissue homogenates in the orl group (Fig. 5d). No differences in diaphragmatic levels of IL-6 (Fig. 5e) or TNF- α (Fig. 5f) were found in orl rats compared with wild type rats.

3.4. Lung and diaphragm histological outcomes

Lung histology results at high magnification (200 $\times$) demonstrated regional differences in lymphocytes, histiocytes, and eosinophils. Wild type rat lungs were characterized by patchy mild to moderate perivascular, peribronchiolar, and alveolar septal cellular inflammation composed of lymphocytes/histiocytes (Fig. 6a). In orl rats, there was minimal perivascular and peribronchiolar presence of lymphocytes/histiocytes/eosinophils, and none-mild alveolar septal cellular inflammation composed of lymphocytes/histiocytes (Fig. 6b).

These score differences (Fig. 7) were supported by analysis of the relationship between the rat strain and the overall degree of inflammation. These results indicated that there was a relationship between the overall degree of tissue inflammation and the rat strain, $p = 0.0019$. No severe inflammation was seen in any tissue section, but thickened vascular smooth muscle was seen in orl rats.

The diaphragm tissues of the orl and wild type rats demonstrated occasional perivascular inflammation composed of mast cells, normal cytosolic muscle fiber, and normal endomysium hematoxylin and eosin (H&E) staining.

Histomorphometry parameters, mainly the expansion index or the ratio between gas-filled spaces to tissue, and the number of opened gas exchange units/fixed fields (#GE units), which collectively reflect gas exchange regions, demonstrated that the expansion index and #GE units in orl and wild type rats were alike (Table 2). No differences in mean alveolar area, alveolar diameter, or airway wall thickness were observed between orl and wild type (Table 2).

3.5. Immunohistochemical outcomes

Anti-rat antibodies detected IL-4 antigens localized mainly in the bronchiolar epithelium (mostly apical), alveoli (mild to moderate staining of pneumocytes and capillaries), and vascular endothelium (minimal staining). Vascular smooth muscle showed no IL-4 staining in the lungs of orl rats (Fig. 8a) or wild type rats (Fig. 8b). Occasional perivascular and peribronchiolar mast cell tryptase was seen in both strains. In diaphragmatic tissue, IL-4 staining was detected inside the muscle fiber, with the staining pattern of a secreted protein, and in the vascular endothelium of orl rats (Fig. 8c) and wild type rats exposed to methacholine (Fig. 8d). In both strains, rare-occasional perivascular mast cells were detected whereas vascular smooth muscle (mostly larger vessels) was negative for IL-4 staining.

3.6. Sham-ori outcomes

Lung tissue homogenate experiments on non-constricted lungs were performed on samples obtained from six unchallenged ori animals, harvested immediately after mechanical ventilation and acclimation, termed sham-ori rats. These animals were studied following the initial two cohorts of animals. The results demonstrated (Fig. 9a–c) that IL-4, IL-6, and TNF- α levels in lung tissue were higher in sham-ori rats than ori rats. In the diaphragm homogenates, the absence of IL-4 was distinctive in sham-ori rats (Fig. 8d). No difference in IL-6 (Fig. 9e) or TNF- α levels (Fig. 9f) were found in sham-ori rats compared with ori rats.

Sham-ori rats had mainly minimal alveolar septal inflammation, none peribronchiolar, and rarely demonstrated perivascular mast cell tryptase expression. Sham-ori demonstrated normal cytosolic muscle fiber and normal endomysium H&E staining.

4. Discussion

The use of experimental animal models of asthma, mainly murine models, provides information on the mechanisms of AHR at the organ, cellular, and molecular levels [20–22]. The models are being used as surrogate models for human diseases. However, the majority of these are targeted to understand allergic asthma. The ori strain provides a model of AHR with characteristics of both native and augmented hyperreactivity that are novel in comparison with previous mouse models [23,24]. The endpoint used to quantify the airway response in clinical settings is usually a lung function measurement, as used in this pre-clinical study.

To our knowledge, this is the first study to compare the effects of a muscarinic receptor agonist and short-acting β_2 -adrenoceptor agonist in related rat strains as a pre-clinical model of AHR. The concentration-response curve in the ori group indicated increased sensitivity, hyperreactivity, and an exaggerated maximal response in comparison with the wild type group. Potency refers to the concentration of a ligand required to yield an effect, and efficacy refers to the quantitative ability of a drug to produce a biological effect [25]. In this regard, the C_{dyn} concentration-response curves demonstrate no differences in potency and efficacy in ori vs. wild type, suggesting negligible small airway reactivity involvement. Methacholine is a cholinergic synthetic analogue of acetylcholine (ACh) and produces smooth muscle contractions of the airways and increased tracheobronchial secretions through the M3 muscarinic postganglionic parasympathetic receptors located in the tracheobronchial tree. Methacholine has more predominant cardiac effects (reflex tachycardia at low dose/bradycardia at higher dose) in comparison with ACh, due to the β -methyl substitution and resistance to enzymatic hydrolysis [24], which may explain the proportion of cardiac events seen in this study. Cyclic guanosine monophosphate (cGMP)-induced bronchoconstriction is increased by direct stimulation of muscarinic receptors. Although reciprocal changes in cyclic-3',5'-adenosine monophosphate (cAMP) and cGMP appear to determine bronchial smooth muscle tone, dysfunction of the non-adrenergic non-cholinergic system, a system that also innervates bronchial smooth muscle, has been associated with AHR in pre-clinical studies [27].

Our unpublished data suggest that multiple loci may contribute to the risk of cryptorchidism in this strain but no linkage analysis data are available to elucidate the susceptibility loci for AHR in orl rats. The position of male gonads in mammals is dependent on differential development of the gubernaculum in response to androgen and insulin-like 3 (INSL3) [28]. INSL3 is a specific ligand for the relaxin family receptor RXFP2, which is expressed in the ovary, testis, and gubernaculum [29] but not in the lung. The gubernaculum contains striated muscle and our data suggest altered muscle-specific gene expression in the orl fetal gubernaculum [2]. Although the pathogenesis of AHR may also involve abnormal airway smooth muscle function and/or muscle-specific gene expression, pointing towards some mechanical intrinsic differences in airway smooth muscle irritability/relaxation or intrinsic differences in small airway stability, further studies are needed to detect these airway smooth muscle differences and to define candidate genomic loci for asthma susceptibility in this strain.

Short-acting β_2 -adrenoceptor agonist such as albuterol has been the standard therapy for the symptomatic treatment of asthma [30]. The pharmacological effect of albuterol, relaxation of bronchial smooth muscle and an anti-inflammatory effect, is at least in part attributable to the stimulation of intracellular adenylyl cyclase with increasing cAMP levels, and the activation of β_2 -adrenoceptors [31]. The significant reduction in respiratory resistance in the orl group following albuterol (Fig. 3) demonstrated the reversible component of the airway mechanical factor of AHR. Thus, methacholine/albuterol-associated airway reactivity/reversibility differences in this study may be mediated by different receptors, and may reflect a quantitative or qualitative difference in receptor function or in signal transduction [26]. Other possible mechanisms may include a combination of anatomical, developmental, and biochemical differences in airway smooth muscle between these rats, which were not addressed in this study. We are aware of the bronchodilator effect of ketamine and believe that this drug has not influenced the bronchoconstrictor and bronchodilator responses evaluated herein, in this regard; during methacholine challenge and albuterol delivery, if any bronchodilator effect is elicited by the doses of ketamine used in this report, this effect would be unrelated because ketamine was administered to all rats. We evaluated also the bronchodilation response after the bronchoconstriction response in this pre-clinical study because, clinically, methacholine airway challenge is followed by bronchodilator administration [32].

With regard to inflammatory biomarkers, lower expression of IL-6 and IL-4 in lung homogenates in orl compared with wild type rats (Fig. 4) may be due to the β_2 -adrenoceptor agonist inhibitory effect of plasma exudation in the airways by its action on β_2 -receptors on endothelial cells in postcapillary venule endothelial cells [33]. β_2 -Adrenoceptor agonist also inhibited the secretion of AHR mediators from airway mast cells [31] and the release of inflammatory mediators from inflammatory cells through receptor or post-receptor mechanisms [34,38]. As demonstrated in this study, there is an inflammatory cell component to the AHR; there was a predominant minimal degree of inflammation and the presence of eosinophils in orl rats in comparison with wild type rats, which demonstrated higher degrees of inflammation (Fig. 5b). Also, the sham-orl results suggest that differences in cytokines between sham-orl and orl animals may be related to the effect of β_2 -adrenoceptor agonist after methacholine challenge (Fig. 7). Sham-orl rats had mainly minimal alveolar septal

inflammation, whereas mainly none was seen in orl rats. With regard to immunohistochemical findings, since IL-4 in the lung was localized mainly in the airway epithelium (Fig. 6b), we speculate that the airway epithelium may be a target of the inflammatory pathway or an important determinant of airway inflammation.

Peripheral lung alveolar dilatation was seen mainly in orl rats (Fig. 5d) compared to wild type rats, which demonstrated peripheral and central dilatation. Alveolar spaces on non-constricted lungs were homogeneous in sham-orl rats (Supplementary Fig. 1). The variability in responsiveness to these pharmacological aerosol exposures is likely strain-dependent, as demonstrated by the concentration-response and dilatation plots, and in part attributable to the pattern of tissue constriction induced by methacholine regardless of group, possibly via selective stimulation of muscarinic receptors. On the other hand, normal quantitative pulmonary morphology based on histomorphometric analysis was observed in both groups. Therefore, we speculate that the pulmonary function changes seen in orl rats were not linked to histological pulmonary structural changes.

The diaphragm muscle is composed of fibers of different types. In the adult human, slow fibers and fast fibers are present in equal proportion whereas in rats the proportion of fast muscle fibers is higher [39]. There are β_2 -adrenoceptors in the diaphragm, the concentration of these receptors is greater in slow fibers than in fast fibers, and therefore the properties of the diaphragm can also be modified by β_2 -adrenoceptor agonists. Acute administration of this agonist has shown a variable effect in fast and slow diaphragmatic fibers in other rat strains and the mechanism appears to be in part mediated by cAMP [40]. Also, in a different rat strain, the up-regulation of cytokines including IL-6 and IL-4 in the diaphragm has been reported; those rats were exposed to strenuous respiratory resistive loads [14]. In our study using wild type and orl strains, we did not detect significant differences in IL-6 at the protein level in diaphragmatic tissue homogenates between the groups or in comparison with the sham-orl rats in response to these types of pharmacological challenges, but IL-4 was highly elevated in the orl rats (Fig. 7). In this study, albuterol sulfate did not appear to inhibit the levels of IL-4 in the diaphragm, as demonstrated by the higher levels of IL-4 in diaphragmatic tissue homogenates in orl rats compared with wild type rats, and may support the inflammatory theory of a post- β_2 -adrenoreceptor inhibitory mechanism in lung tissue. Furthermore, the observed absence of IL-4 in the diaphragms of sham-orl rats (Fig. 7), animals that were subjected to the same surgical and mechanical ventilation procedures but without pharmacological interventions, indicates that diaphragmatic cytokine up-regulation may be due to the airway challenge tests in the orl animals.

The immunohistochemical findings of mainly intradiaphragmatic IL-4 expression and occasional mast cells with no infiltrating inflammatory cells in both, wild type and orl strains are consistent with in vivo animal results demonstrating that diaphragmatic myocytes are capable of producing IL-4 secondary to increased load on the diaphragm [14]. Although we did not analyze locally the rest of the cytokines with IHC or draw blood samples to detect plasma cytokine levels (which are limitations of this study), we speculate that the IL-4 up-regulation seen in tissue homogenates was due to diaphragm-derived cytokine. This speculation emphasises the important role that the diaphragm may play in the augmentation of the stimuli in AHR and the need for further studies in this field. In general, the direct

challenges mainly have an effect on airway smooth muscle and, as such, are predominantly reflected by changes in airway mechanics, whereas AHR assessed from indirect tests correlates better with the degree of airway inflammation [41,42].

5. Conclusions

This study suggests that after aerosol administration of methacholine, in comparison with wild type, orl rats have greater airway hyperresponsiveness and higher induction of IL-4 cytokine in diaphragmatic tissue homogenates. We have also identified that exposure of orl rats to short-acting β_2 -adrenoreceptor agonist is associated with down-regulation of IL-4 and IL-6 in lung tissue. Predominant immunohistological lung intraepithelial/intradiaphragmatic IL-4 staining as opposed to vascular endothelial IL-4 staining - with normal lung histomorphometry - was seen in both, wild type and orl rats. The demonstration of a relationship between the aerosol interventions and inflammatory outcomes showed that, indeed, the AHR seen in the orl group may be due to a combination of both factors: inflammation and abnormal respiratory functional mechanics.

In summary, this model offers advantages over other pre-clinical bronchoconstriction and bronchodilation murine models for studying potential mechanistic links between cryptorchidism and asthma. The shift of the concentration-response curve to the left, in combination with increased inflammatory cell number/cell activity points towards pre-junctional mechanisms involve in airway hyperresponsiveness. Based on these study findings and future studies to expand our understanding of local and systemic cytokine responses and receptor distribution/function, this novel animal model of native airway hyperresponsiveness, may be useful for further testing of compounds/therapeutics options for treating AHR.

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Abbreviations

AHR	airway hyperresponsiveness
R	respiratory resistance
Cdyn	dynamic compliance

EI	expansion index
GM	geometric means
IL	interleukin
IHC	immunohistochemistry
TNF α	tumor necrosis factor- α
V_g	total gas exchange area
V_p	parenchyma area
UDT	undescended testis

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Fig. 1. Rat tissue negative controls for IL-4 and mast cell tryptase IHC double-staining. a) Adult rat lung; b) Abdominal wall muscle; c) Pediatric rat lung; d) Diaphragmatic tissue. IL = interleukin; IHC = immuno-histochemistry. Original magnification: 200 $\times$.

Fig. 2.

Stimulation effects of nebulized methacholine. Schematic representation of the log concentration-response curves observed in wt and orl rats in R. Provocative concentrations (dotted lines) causing a 40% increase in R (PC_{40}) from baseline. Y-axis ordinate shows responses expressed as average % of change from baseline. R = respiratory resistance; wt = wild type. Data are mean $\pm$ SEM; *** $p < 0.001$ for significant interaction in orl ($n = 9$) vs. wt ($n = 13$) rats; SEM = standard error of the mean.

Fig. 3.

Potency and efficacy of methacholine related to schematic representation of the log concentration-response curves observed in wt ($n = 13$) and orl rats ($n = 9$) in Cdyn. Provocative concentrations (dotted lines) causing a 30% decrease in Cdyn (PC_{30}) from baseline. Data are mean $\pm$ SEM; Y-axis ordinate shows responses expressed as average % of change from baseline. Cdyn = dynamic compliance; SEM = standard error of the mean; NS = not significant; wt = wild type.

Fig. 4.

Percentage of change in *R* before and after albuterol aerosol treatment. a) *wt* group (*n* = 9) and b) *orl* group (*n* = 13). †*p* = 0.057 and **p = 0.009 for comparisons between MCh and albuterol; *R* = resistance; *wt* = wild type; MCh = methacholine.

Fig. 5.

Levels of cytokines in rat tissue homogenates after MCh aerosol challenge and albuterol aerosol exposure. a) GM differences in lung IL-4 levels. b) Mean $\pm$ SEM differences in IL-6 levels. c) Mean $\pm$ SEM differences in TNF- α levels, $p = 0.207$. d) Differences in medians of diaphragmatic IL-4 levels. e) GM differences in IL-6 levels, $p = 0.746$. f) Differences in medians of TNF- α levels, $p = 0.956$. IL = interleukin; MCh = methacholine; wt = wild type; TNF- α = tumor necrosis factor- α ; GM = geometric mean; * $p < 0.05$; ** $p < 0.01$ vs. wild type.

Fig. 6.

Representative section of nondependent right lung tissues from the Long Evans wild type rats and orl rats. a) Tissue inflammation in wild type rats. b) Tissue inflammation in orl rats. *pb* = peribronchiolar; *p**v* = perivascular; *as* = alveolar septal. Hematoxylin and eosin staining; original magnification: 100 .

Fig. 7.

Histological scores of pulmonary inflammation in wild type and orl rats. Data are counts of the overall inflammation scores (perivascular, peribronchiolar, and alveolar septal combined). wt = wild type.

Fig. 8.

Tissue distribution of IL-4 antigens and mast cell tryptase antigens, as assessed by IHC double-staining analysis in lung and diaphragmatic tissues of rats. a) orl lung; note the thickness of *v*. b) wt lung. c) orl diaphragm. d) wt diaphragm. IL-4 antigens (brown color/arrows); mast cell antigens (red color/arrow head). Original magnification: 200 $\times$. IL = interleukin; IHC = immunohistochemistry; wt = wild type; *v* = vascular smooth muscle; *pb* = peribronchiolar; *as* = alveolar septal; *ve* = vascular endothelium; *as* = alveolar septal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 9.

Levels of cytokines in tissue homogenates of sham-ori rats vs. ori rats. a) GM differences in IL-4 levels. b) Mean $\pm$ SEM differences in IL-6 levels. c) Mean $\pm$ SEM differences in TNF- α levels. d) GM differences in diaphragmatic IL-4 levels. e) Mean $\pm$ SEM differences in IL-6 levels, $p = 0.377$. f) Differences in medians of TNF- α levels, $p = 0.608$.

Table 1

Baseline outcomes and respiratory profile in wild type, orl, and sham-orl rats.

Parameter, Units	Wild type (<i>n</i> = 9)	orl (<i>n</i> = 13)	Sham-orl (<i>n</i> = 6)	<i>p</i> Value
Body weight, g	107 (103–112)	86 (76–113)	157 (146–173)	***
Oxygen saturation, %	94 (84–98)	97 (92–98)	94 (92–97)	0.63
Heart rate, beats/min	335 (221–755)	469 (292–608)	290 (280–310)	0.30
<i>R</i> , cm H ₂ O/mL/s	0.78 (0.61 – 1.6)	0.92 (0.81–1.2)	0.94 (0.86–1.6)	0.56
Cdyn, mL/cm H ₂ O	0.01 (0.01–0.02) ^a	0.01 (0.01–0.01)	0.01 (0.01–0.01)	0.12

Data are presented as median (25%–75% percentiles) unless otherwise indicated. *R* = respiratory resistance; Cdyn = dynamic compliance.***
p < 0.001 for significant variation among the groups.^aCdyn was normalized per 10 g of body weight.

Table 2

Histomorphometry outcomes in wild type ($n = 5$) and orl ($n = 12$) rats.

Parameter, Units	Wild type	orl	Difference in means (95% CI)
Expansion index	178.3 $\pm$ 38.9	241.7 $\pm$ 24.1	63.4 (-160, 33.8)
# GE units	218.4 $\pm$ 45.6	168.3 $\pm$ 19.8	50.1 (-39.4, 139.7)
Alveolar area, μm^2	319.0 $\pm$ 84.7	321.4 $\pm$ 40.3	-2.4 (-177.8, 173)
Alveolar diameter, μm	14.11 $\pm$ 1.8	14.45 $\pm$ 0.9	-0.3 (-4.19, 3.51)
Airway wall thickness, μm	59.8 $\pm$ 11.9	64.5 $\pm$ 9.2	-4.7 (-37.86, 28.39)

Data are presented as mean $\pm$ SEM unless otherwise indicated. # GE units = number of opened gas exchange units/fixed fields; SEM = standard error of the mean.