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Essential Intracrine Androgenic Action in Lung Development for Both Sexes

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ABSTRACT

Albeit their recognized negative effects on lung maturation, androgens have been proposed to play an essential positive role in lung development. This work aimed to evaluate the impact of blocking endogenous androgen and estrogen actions and to study the effect of an excess of androgen and estrogen during the end of saccular stage and the beginning of the alveolar stage on lung development. This was performed with normal oxygen atmosphere and with hyperoxia, a model of alveolar simplification, which is observed in new bronchopulmonary dysplasia. Mouse lung samples were collected on postnatal day 9 after exposure to 21% or 80% oxygen (postnatal days 1 to 4), and after administration (postnatal days 3 to 8) of vehicle, pure antiandrogen (flutamide), dihydrotestosterone, pure antiestrogen (fulvestrant), or 17β-estradiol. With 21% oxygen, the major effects on morphometric parameters were induced by flutamide. In contrast, with hyperoxia, both flutamide and dihydrotestosterone had similar effects on several morphometric parameters. For instance, a decrease in the relative frequency of closed areas (mainly composed of saccules/alveoli) < 1000 μm^2 and an increase for those > 2500 μm^2 were observed after flutamide administration. In conclusion, during the junction between the saccular and the alveolar stages, endogenous androgens play an essential intracrine role in lung development for both sexes while an excess of androgens are deleterious when combined with a hyperoxia treatment, but not with normal oxygen levels. Endogenous estrogens have no effects on the lungs during the developmental window studied, while exogenous estrogens had only isolated effects on some morphometric parameters.

1. Introduction

Our previous results suggested that androgens play a positive physiologic role in the mouse developing lung for both sexes [1]. This postulate was surprising based on the recognized negative effects of androgens, delaying lung maturation in males [2,3] and being responsible for the higher incidence and severity of respiratory distress syndrome in males of the population of premature babies [4,5]. Nevertheless, based on the expression of androgen synthesizing enzymes in the lungs of both sexes (17β-hydroxysteroid dehydrogenase type 5 (17β-HSD5) [1] and 5α-reductase 1 [6], for a review see [7]), a positive role for androgens in lung development was also proposed. In support of this observation, immunohistochemistry with an anti-androgen receptor antibody on mouse lungs isolated on gestation day (GD) 15.5, 16.5, and 17.5 revealed the sub-localization of the androgen receptor protein within the nucleus of several epithelial cells of the conducting and the respiratory zones for both sexes [8], which confirmed androgen receptor activation. Based on the affinity of the androgen receptor for its ligands [9], the amounts of androgens present in lungs isolated on GD 16.5 and 17.5 [10] are insufficient to explain the observed activation of the androgen receptor in female developing lungs. Androgen receptor activation in spite of very low local androgen levels is compatible with an intracrine action of androgens where the active hormones are synthesized within the cells where they exert their action and are then inactivated before reaching the circulation and this, without accumulation of active sex steroids within the tissue [11–13].

Nuclear-translocated androgen receptors were also observed within postnatal mouse lungs of males and females, more precisely in epithelial cells of the conducting epithelium and the respiratory epithelium on postnatal day (PND) 1 and 3 in the saccular stage, and on PND 15 in the

Abbreviations: 5α-DH-DOC, 5α-dihydro-deoxycorticosterone; AKR, aldo-keto reductase; DHT, dihydrotestosterone; DOC, deoxycorticosterone; GD, gestation day; HSD, hydroxysteroid dehydrogenase; NMR, nuclear magnetic resonance; PND, postnatal day

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alveolar stage [14]. In parallel with these observations, we have also determined that there is not enough androgens to activate the androgen receptor in postnatal female lungs of the saccular (PND 0 and 2) and the alveolar (PND 5 and 10) stages and postnatal male lungs of the alveolar stage (PND 5 and 10) [10]. Thus, postnatal mice constitute an excellent model to study the putative intracrine action of sex steroids.

In the present study, we have evaluated the effect of endogenous androgens and estrogens on male and female postnatal lung development using specific steroid receptor antagonists. Theoretically, one major beneficial effect of the intracrine way is that cell specificity of the action allows avoiding putative negative effects that would appear if the entire lungs were exposed to sex steroids. Therefore, the effect of an excess of androgens and estrogens on saccularization/alveolarization has also been studied. Because a new form of bronchopulmonary dysplasia (BPD) was reported in which alveolarization is impaired, our study was conducted both in the presence of a normal atmosphere (21% oxygen), and of 80% oxygen, which is an accepted model of impaired alveolarization [15].

2. Materials and methods

2.1. Animals

Protocol and procedures were in compliance with guidelines from the Canadian Council on Animal Care and approved by the animal care and use committee and the institutional review board of the Centre de recherche du CHU de Québec (protocol no. 2014-053). Animals were kept under a 12 h light/dark cycle and received water and feed ad libitum. Balb/c (*Mus musculus*) females at the estrus stage were mated overnight. The beginning of post-natal day 0 (PND 0) corresponds to parturition.

Mice were exposed for 72 hours from PND 1 to PND 4 to room air (21% O_2) or to hyperoxia (80% O_2) in a sealed plexiglas chamber using an oxygen controller device (ProOx P110 Oxygen Controller with E702 Oxygen Sensors and ProCO_2 carbon dioxide sensors) from BioSpherix (Lacona, NY). Animals were daily injected with 50 µl (s.c.) of the following substances /5 g body weight/day: flutamide (kindly provided by Dr. F. Labrie, CEO of EndoCeutics) (250 µg/50 µl, similar to [16]); dihydrotestosterone (DHT) (Steraloids, 25 µg/50 µl as in [17]), fulvestrant (Sigma-Aldrich, 150 µg/50 µl (similar to [18]) and 500 µg/50 µl), 17β-estradiol (Sigma-Aldrich, 12.5 µg/50 µl as in [19]), or vehicle only (0.9% NaCl containing 1% gelatin and 10% DMSO) from PND 3 to PND 8. To certify the results, the purity and integrity of flutamide was assessed by nuclear magnetic resonance (NMR) at the end of the experiments. NMR values were similar to those reported in the literature [20]. Because no effect of fulvestrant was observed using 150 µg/newborn daily, the amount was increased up to 500 µg/newborn in order to confirm the data. All the results presented were obtained with 500 µg of fulvestrant /newborn. Mice were anesthetized on PND 9 with ketamine (100 mg/kg) and xylazine (10 mg/kg) (i.p.). Then, newborns were transcardially perfused with saline and lungs were fixed by instillation under constant pressure via a tracheal cannula, as described [21]. Lungs were then harvested and postfixed for two days at 4 °C.

2.2. Morphometry

Tissue preparation was performed as described [21]. According to our novel technical approach [21], each lung tissue sample was cut in slices of 2 mm from the apical to the caudal regions. Then, after embedding in glycol methacrylate, tissue sections of 3 µm were prepared and stained with 0.1% toluidine blue (Caledon Laboratories Ltd, Georgetown, ON, Canada) before being scanned with a NanoZoomer 2.0-HT C9600-13 digital slide scanner (Hamamatsu). Following this procedure, one section of 3 µm was obtained every 2 mm from the apical to the caudal regions for each lung and all these sections were included into the morphometric analysis. The resulting files were processed using our high-throughput image analysis workflow developed in Matlab® Matwork environment (version 2014a), as previously reported [21]. Of importance, for the present work, our code was deployed as a standalone application (CentOS 6.6) for distributed computing on Colossus, a cluster of servers managed by Calcul Québec (Université Laval). Virtual slides were transferred on Colossus server cluster using Globus Connect network (The University of Chicago and Argonne National Laboratory), and results were retrieved as comma separated value files for subsequent statistical analysis. One hundred percent of the surface of all the resulting slices was integrated in the morphometric study.

According to our new technical approach, “Closed area” and “tip” are basal morphometric parameters. Closed areas correspond mainly to saccules/alveoli (98%), the remaining 2% being any respiratory bronchioles, blood vessels and alveolar ducts [21]. Tips are mainly composed of secondary septa, but a few primary septa cut transversally may also be recorded as tips [21].

At least 4 males and 4 females were studied for each group.

2.3. Statistical analysis

Statistical analyses were performed as described [21]. The significance of the differences between experimental groups was determined using the mixed ANOVA followed by Bonferroni post-test. Data were expressed as means ± SEM. The significance of the differences between groups with 21% oxygen/injection of vehicle and 80% oxygen/injection of vehicle was determined using the unpaired t-test procedure. Values of $P < 0.05$ were considered as statistically significant. Statistical analyses were performed using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA) and GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA).

3. Results

Mouse neonates of both sexes were exposed to 21% oxygen or 80% oxygen from PND 1 to 4. Each of these groups was subjected to subcutaneous injections of flutamide (a pure antiandrogen), DHT (the most potent androgen), fulvestrant (a pure antiestrogen), 17β-estradiol, or vehicle only from PND 3 to 8. Neonates were sacrificed on PND 9. DHT was selected instead of testosterone because it cannot be transformed into estrogen in contrast to testosterone.

It should be noted that this experiment was first performed with an injection window extending from PND 1 to PND 8. Unexpectedly, for the pups injected with flutamide (PND 1 to 8) and exposed to 80% oxygen from PND 1 to 4, a rate of mortality of 75% was observed before PND 9. Moreover, the survivors were lean and their lungs showed very pronounced alveolar simplification (data not shown). When flutamide injections were initiated on PND 3 instead of PND 1, the mortality rate observed with hyperoxia decreased down to the basal value observed with injection of vehicle and the pups' body weight were not statistically different from controls. Therefore, all the injections were performed with the injection window extending from PND 3 to PND 8. As demonstrated in Table 1, the use of this injection window was not associated with a statistically significant difference in body weight and this for each of the injected compound. Moreover, when the values of males and females were analyzed separately, there was still no statistically significant difference in body weight. The only significant difference in body weight was induced by the hyperoxia treatment (21% oxygen, vehicle: 6.0 +/- 0.2 g; 80% oxygen, vehicle 5.2 +/- 0.2 g; $P = 0.001$) as reported [22,23].

3.1. General observations

Representative photographs of lungs are shown for each of the experimental groups in Fig. 1. All of them are from females, but no sex difference was observed within groups (data not shown). Alveolar