Differential Expression of Genes Important to Efferent Ductules Ion Homeostasis across Postnatal Development in Estrogen Receptor-α Knockout and Wildtype Mice

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ABSTRACT: Our earlier studies showed that estrogen was involved in the regulation of fluid reabsorption in adult mouse efferent ductules (ED), through estrogen receptor (ER) α and ERβ, by modulating gene expression of epithelial genes involved in ion homeostasis. However, little is known about the importance of ERα in the ED during postnatal development. Based on previous findings, we hypothesized that there should be a difference in the expression of epithelial ion transporters and anion producers in the ED of postnatal wild type (WT) and estrogen receptor α knockout (αERKO) mice. Using absolute, comparative and semi-quantitative RT-PCR along with immunohistochemistry, we looked at expression levels of several genes in the ED across postnatal development. The presence of estrogen in the testicular fluid was indirectly ascertained by immunohistochemical detection of the P450 aromatase in the testis. There was no immunohistochemically detectable difference in the expression of P450 aromatase in the testes and ERβ in the ED of WT and αERKO mice. ERα was only detected in the ED of WT mice. The absence of ERα in the ED of postnatally developing mice resulted in differential expression of mRNAs and/or proteins for carbonic anhydrase II, Na⁺/H⁺ exchanger 3, down-regulated in adenoma, cystic fibrosis transmembrane regulator, and Na⁺/K⁺ ATPase α1. Our data indicate that the absence of ERα resulted in altered expression of an epithelial ion producer and transporters during postnatal development of mice. We conclude that the presence of ERα is important for regulation of the ED function during the prepubertal developmental and postpubertal period. (Key Words: Estrogen Receptor α, Efferent Ductules, P450 Aromatase, Estrogen, Epithelial Ion Transporters)

INTRODUCTION

Efferent ductules (ED), part of the excurrent ducts in the male reproductive tract, are the sites where the majority of testicular fluid is reabsorbed, resulting in a several fold increase in sperm concentration (Clulow et al., 1994). The epithelium of the ED consists of ciliated and nonciliated cells with the latter having the major role in the reabsorption of fluid (Illio and Hess, 1994; Lee et al., 2000; Clulow et al., 1994). As an embryologic homology, the ED share common morphological and functional similarities with proximal tubules in the kidney (Hinton and Turner, 1988).

The presence of high concentrations of estrogen in the rete testis fluid (Free and Jaffe, 1979) and the existence of estrogen receptor alpha (ERα), as well as beta (ERβ), in the ED (Hess et al., 1997b) are well documented. These findings suggest that estrogen has a regulatory role in the ED function during the prepubertal developmental and postpubertal period. (Key Words: Estrogen Receptor α, Efferent Ductules, P450 Aromatase, Estrogen, Epithelial Ion Transporters)
al., 2001). We also demonstrated that estrogen differentially regulates gene expression of ion transporters through both ERα and ERβ, thereby controlling fluid reabsorption in the ED (Lee et al., 2001).

Estrogen is derived from testosterone by the action of cytochrome P450 aromatase (P450arom) (Carreau, 2000). In the male reproductive tract, mRNA and protein expression of P450arom are detected in somatic cells and germ cells in the testis of various species, including the mouse (Nitta et al., 1993), bear (Tsubota et al., 1997), rat (Janulis et al., 1998), and chicken (Kwon et al., 1995). In the mouse, P450arom is first detected in pachytene spermatocytes and then the more mature germ cells (Nitta et al., 1993). In addition, spermatozoa in the efferent ducts possess P450arom (Kwon et al., 1995; Janulis et al., 1996; Janulis et al., 1998). In the developing mouse testis, different germ cell populations appear chronologically at different ages. For example, pachytene spermatocytes appear at postnatal day (PND) 14, round spermatids at PND 18, and first spermatozoa at PND 35 (McCarrey, 1993). Thus, it would be reasonable to expect that the concentration of estrogen in the testicular fluid of the mouse varies during the postnatal development because of the developmentally timed appearance of different germ cells which express P450arom.

In the present study, based on our previous studies and other observations, we hypothesized that the expression of epithelial ion transporters in the mouse ED would relate with that of P450arom in the testis during postnatal development. We also hypothesized that the presence of functional ERα would be necessary for the expression of epithelial ion transporter in the ED during prepubertal period. To test this hypothesis, we first determined the expression pattern of P450arom in the developing testes of WT and αERKO mice by immunohistochemistry. Immunohistochemistry was used as a means of gauging the likely presence of estrogen as it is not physically practical to measure the concentration of estrogen in the rete testis fluid of such young mice. The presence of ERα and ERβ in the ED of the postnatal developing mice was also detected by immunohistochemistry. Lacking access to Real-time PCR, we used the alternative methods of absolute and comparative RT-PCR or semi-quantitative PCR to measure mRNA abundance of our target genes in the ED of the postnatal developing WT and αERKO mice. In addition, the presence and localization of the epithelial ion producer and transporters in the ED were detected by immunohistochemistry. The target genes tested in the present study were the epithelia ion producer, carbonic anhydrase II (CAII), and the ion transporters, Na+/H+ exchanger 3 (NHE3), a Cl-/HCO3- exchanger, down-regulated in adenaoma (DRA), cystic fibrosis transmembrane regulator (CFTR), and Na+/K+ ATPase (ATPase) α1 subunit.

### MATERIALS AND METHODS

#### Animals and tissue collection

Homoygous αERKO and WT sibling (C57BL65/129SVJ) male mice were obtained from a resident breeding colony maintained at the University of Illinois and University of Missouri (a generous gift from Dr. D. Lubahn). Three experimental groups consisting of both WT and αERKO were used at the following ages: 10 days-old WT (n = 12) and αERKO (n = 11), 18 days-old WT (n = 16) and αERKO (n = 10), and 60 days-old WT (n = 11) and αERKO (n = 12). The criteria for selecting 3 age groups for the present study were based on differences of secretion of the testicular fluid and the appearance of P450arom-expressing germ cells in the testis during the postnatal developmental period. Mice were killed by cervical dislocation. Male reproductive tract from 3-5 mice in each experimental group were fixed for detection of ion transporters, ERα and ERβ in the ED, and P450arom expression in the testes by immunohistochemistry. The kidney was used as a positive control for ion producer and transporters, such as CAII, NHE3, CFTR, and ATPase α1. Male reproductive tract from 7-9 mice in each group were rapidly dissected in ice-cold PBS, and the ED were isolated and frozen in liquid nitrogen for absolute and comparative RT-PCR or semi-quantitative PCR analysis of gene expression of ion transporters. To obtain a sufficient amount of RNA, the ED of WT or αERKO mice in each experimental group were pooled for RT-PCR analysis.

#### Immunohistochemistry

The male reproductive tract was fixed in 10% neutral buffered formalin (NBF) for 24 h or in Bouin’s fixative for 6 h. Then the ED was carefully dissected from other parts of the reproductive tract. The NBF-fixed tissues were dehydrated, cleared, and infiltrated with paraffin using a vacuum infiltration processor (Tissue-Tek VIP, Sakura Finetek USA Inc., Torrance, CA). The Bouin’s fixed tissues were manually processed. The tissues were embedded in paraffin and sectioned at 5 μm thickness. We used polyclonal mouse anti-CAII (a generous gift from Dr. Linser, University of Florida, Gainesville, FL), polyclonal rabbit anti-NHE3 (AB3085; Chemicon International, Temecula, CA), monoclonal mouse anti-CFTR Ab-3 (L12B4) (MS-1248-P0; Lab Vision Corporation, Temecula, CA), monoclonal rabbit anti-NHE3 (AB3085; Chemicon International, Temecula, CA), monoclonal mouse anti-ERα (NCL-ER-6F11; Novocastra, Newcastle, UK), and polyclonal rabbit anti-ERβ (AB1410; Chemicon International, Temecula, CA) as primary
antibodies. First, sections were deparaffinized and rehydrated by exposure to a series of ethanol. Sections were microwaved in 0.01 M citrate buffer, pH 6.0, for 7 min for antigen retrieval and placed in 0.3% hydrogen peroxide in methanol for 15 min to inactivate endogenous peroxidase. After washing in PBS, sections were treated with 10% normal goat serum for 10 min to block nonspecific binding. Diluted primary antibodies were placed on the tissues and incubated overnight at 4°C in a humidified chamber. The dilutions of the primary antibodies were selected after a series of multiple preliminary trials for each antibody. We used dilutions of 1:2 for CAII, 1:400 for NHE3, 1:50 for CFTR, 1:100 for DRA, 1:200 for ATPase α1, 1:400 for ERα, and 1:400 for ERβ. After washing off excess primary antibodies with PBS, tissue sections were then incubated with either biotinylated anti-rabbit IgG secondary antibody (Vectorostain kit, Vector Laboratories, Burlingame, CA) for NHE3, DRA, and ERβ or biotinylated anti-mouse IgG secondary antibody (DAKO Corporation, Carpinteria, CA) for CAII, CFTR, ATPase α1, and ERα for 1 h at room temperature in a humidified chamber. Unbound secondary antibodies were washed off with PBS, and elite avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA) was placed on tissue sections for 30 min at room temperature in a humidified chamber. After washing in PBS, the sections were treated with a mixture of 3,3′-diaminobenzidine (DAB; Sigma, St. Louis, MO), 0.05 M Tris-HCl buffer, and 5% hydrogen peroxide to detect the peroxidase. Then, sections, except those for ERα and ERβ, were counterstained with hematoxylin, dehydrated, and mounted. For negative controls, sections were treated with normal rabbit (DAKO Corporation, Carpinteria, CA) or mouse (Chemicon International, Temecula, CA) serum at the same dilution as the primary antibody in a humidified chamber. The immunostaining was evaluated with digitalized images captured with Olympus-MagnaFire camera (Olympus America, Melville, NY) using Optronics MagnaFire Camera Imaging and Control version 1.1 software (Optronics, Goleta, CA). The photographic images were processed in Photoshop software (Adobe Systems, San Jose, CA).

**Absolute and comparative RT-PCR analysis**

**Total RNA isolation**: The total RNAs were prepared by RNAeasy Mini kit (Qiagen Inc., Valencia, CA) using a Polytron homogenizer (Fisher Scientific, Pittsburgh, PA). The isolated RNAs were stored at -80°C until used for relative and comparative RT-PCR or semi-quantitative RT-PCR analysis. The purity and concentration of the total RNAs were determined spectrophotometrically, and the qualities of the total RNAs were checked by gel electrophoresis prior to proceeding RT reaction.

**Absolute and comparative RT-PCR analysis**: The RT and PCR procedures were performed according to the instructions in IntraSpec comparative RT-PCR kit (Ambion Inc., Austin, TX) (Figure 1). Briefly, 2 μg of isolated total RNA were reverse-transcribed, radio-labeled with [α-P32]dATP (Amersham Pharmacia Biotech, Piscataway, NJ), and tagged with one of two reverse transcription primers, possessing common PCR primer binding sites (PBSs), and distinct spacers of different sizes, 10 or 50 nucleotides for WT or αERKO mice, respectively (Figure 1). Unincorporated free radioisotopes were removed from labeled cDNA using NucAway Spin Columns (Ambion Inc., Austin, TX). One μl of labeled cDNA from WT and αERKO mice at same ages was used to measure radioactivity in a scintillation counter and equalized with nuclease-free H2O to achieve the same cpm/μl. To quantify PCR products, we labeled the PBS-specific primer with [γ-P32] ATP (Amersham Pharmacia Biotech, Piscataway, NJ) using KinaseMax kit (Ambion Inc., Austin, TX). The PCR mixture consisted of 2 μl of each cDNA population from WT and αERKO, a [γ-P32] ATP-labeled PBS-specific primer, and a gene specific primer (GSP). Sequences of these GSPs for an ion producer and ion transporters tested in this study were chosen based on criteria of IntraSpecᵀᴹ comparative RT-PCR kit (Ambion, Austin, TX) (Table 1).
Utilizing a hot start, PCR cycles were set up as following: denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and final extension at 72°C for 10 min. After PCR, amplified DNAs were fractionated by 2% agarose gel electrophoresis and visualized with ethidium bromide staining. Two distinct bands were detected in the gel due to the different sizes of the linkers in the RT primers, i.e. the upper band for αERKO having extra 50 nucleotides in the linker and the bottom band for WT possessing extra 10 nucleotides in the linker (Figure 1). Under UV light, bands were excised with a razor blade and immersed in separate scintillation cocktail (Fisher Scientific, Pittsburgh, PA). Radioactivity of each band was counted in a scintillation counter. Cyclophilin was used as an internal PCR control for this assay.

Semi-quantitative relative PCR analysis for NHE3:

For this study because insufficient 3'-end sequence information was available for NHE3 to use the comparative RT-PCR method. We used the same cDNA made from the IntraSpec comparative RT-PCR kit (Ambion Inc., Austin, TX) for this analysis. Sequences of NHE3 primers used in this study are shown in Table 1. For the semi-quantitative analysis, RT-PCR reactions were analyzed prior to the plateau phase of amplification. The RT-PCR products were subjected to electrophoresis on a 2% agarose gel. The gels were stained with ethidium bromide and the image of each gel was photographed under UV. The optical density at each position was measured with a densitometer.
densities of the RT-PCR products were measured and quantified using a PDI scanner and RFLPrint software (both from Bio-Rad Laboratories, Hercules CA). In this assay, we included S15, a ribosomal RNA, which served as an internal PCR control.

**Data presentation**: We repeated the RT reaction and PCR for each experimental group five times to obtain an intra-variation mean and standard deviation. For an absolute and comparative RT-PCR assay, radioactivities of PCR products were normalized by comparison to abundance of cyclophilin between WT and αERKO mice at each age. Data are presented as percentage of changes of mean values of αERKO mice as compared to those of WT mice at the same age. For a semi-quantitative assay for NHE3, densitometry values were normalized to those of S15, and like the absolute/comparative RT-PCR assay, percentage changes of mean values of NHE3 in αERKO mice were compared with those in WT mice at same age.

**RESULTS**

**Immunohistochemical localization of P450 aromatase in the testes**

There were no recognizable differences in the immunohistochemical localization of P450arom in the testes of WT and αERKO mice at all experimental time points. No visible immuno-staining of P450arom was detected at 10 days of age in testes of WT and αERKO mice (Figure 2a; b). At 18 days of age, Leydig cells in the testes of WT and αERKO were weakly immuno-positive for P450arom (Figure 2c and d). However, Sertoli cells in the testes were immuno-negative. Moderate immuno-reactivity of P450arom was found in the cytoplasm of pachytene spermatocytes (Figure 2c and d). Secondary spermatocytes were occasionally seen and also showed strong immuno-activity in the cytoplasm (data not shown in Figure 2). No visible difference in the immunolocalization pattern of P450arom was found between the testes of WT and αERKO mice. In adult testes at 60 days of age, intensive immuno-staining for P450arom was found in the cytoplasm of pachytene spermatocytes (Figure 2e and f). Secondary spermatocytes were occasionally seen and also showed strong immuno-activity in the cytoplasm (data not shown in Figure 2).

**Immunohistochemical localization of ERα and ERβ in the efferent ductules**

ERα was localized in nuclei of ciliated and nonciliated cells of the ED at all ages of WT mice (Figure 3A, a and c).
Smooth muscle and connective tissue cells of the ED were always immuno-negative (Figure 3A, a and c). No positive staining for ERα was detected in αERKO mice (Figure 3A, b and d), as expected. However, the ERβ was detected in nuclei of ciliated and nonciliated cells in epithelia of the ED in both WT (Figure 3B, a and c) and αERKO (Figure 3B, b and d) mice at all experimental ages. The smooth muscle layer and connective tissues surrounding the ED were also immuno-positive for ERβ at all ages (Figure 3B).

Expression of mRNA and immunohistochemical localization of CAlI in the efferent ductules

Expression of CAII mRNA was not detected in the ED of WT and αERKO at 10 and 18 days of age (Figure 4A), while CAII mRNA was expressed in WT and αERKO mice at 60 days of age (Figure 4A). Level of CAII mRNA in WT mice was 54% higher than that in αERKO mice at 60 days of age (Figure 4A).

Immunohistochemical localization of CAII in the ED is shown in Figure 4B. No visible difference in the staining at light microscopic level was seen in the ED of WT and
We detected a very weak positive reaction in the cytoplasm of the ED epithelia of WT (Figure 4B, a) and αERKO (Figure 4B, b) mice at 10 days of age. At 18 days of age, the ED of WT (Figure 4B, c) and αERKO (Figure 4B, d) mice showed more intensive reaction for CAII (Figure 4B, d). Strong positive immuno-staining for CAII was detected in the ED of WT and αERKO mice at 60 (Figure 4B, e and f, respectively) days of age.

Expression of mRNA and immunohistochemical localization of NHE3 in the efferent ductules

Using semi-quantitative analysis, NHE3 mRNA was detected in the ED of WT and αERKO mice at 60 days of age (Figure 5A), but not at 10 and 18 days of age (Figure 5A). At 60 days of age, αERKO mice showed a decrease by 54% in the level of the NHE3 mRNA compared to that in WT mice (Figure 5A, graph).

Immunohistochemical study showed that NHE3 was localized on the brush border of nonciliated cells in the ED epithelia (Figure 5B). In WT mice, no positive immuno-staining for NHE3 was detected in the ED at 10 days of old.
Figure 5B. a and b). Weak immuno-reactivity of NHE3 was observed at 18 days of age (Figure 5B, c). Strong immuno-reactivity of NHE3 was found in the ED of WT mice at 60 days of age (Figure 5B, e). The ED of αERKO mice at 10 (Figure 5B, b) and 18 (Figure 5B, d) days of age showed no positive immuno-reactivity of NHE3. The ED of αERKO mice showed weakly positive staining for NHE3 at 60 days of age (Figure 5B, e).

Expression of mRNA and immunohistochemical localization of DRA in the efferent ductules

Expression of DRA mRNA in the ED was detected in all experimental groups (Figure 6A). At 10 days of age, a decrease of 37% in DRA mRNA was detected in αERKO mice, compared with the mRNA abundance in WT mice (Figure 6A, graph). However, in the ED of αERKO mice at 18 days of age, the mRNA abundance of DRA showed an
increase of 54% over WT mice (Figure 6A, graph). After puberty, the degree of increase of DRA mRNA in αERKO mice was somewhat decreased, but DRA mRNA abundance was still 16% higher than that of WT mice at 60 days of age (Figure 6A, graph).

Localization of DRA was limited in the brush border of nonciliated cells (Figure 6B). Positive immuno-staining of DRA was detected in the ED epithelia of both WT and αERKO mice as early as 10 days of age (Figure 6B). Weakly positive staining was seen on a few nonciliated cells in the ED of 10 days old (Figure 6B, a and b inserts).

At 18 days of age, immuno-reactivity of DRA became strong and more prevalent along the ED epithelia (Figure 6B, c and d inserts). Similar staining pattern was observed in the ED of WT and αERKO mice at 60 (Figure 6B, e and f, respectively) days of age. However, unlike NHE3, not all but some of nonciliated cells in the ED epithelia were only immuno-positive (Figure 6B).

Expression of mRNA and immunohistochemical localization of CFTR in the efferent ductules

Expression of CFTR mRNA was detected in the ED of all experimental mice (Figure 7A). At 10 days of age, a level of CFTR mRNA in αERKO mice was 51% lower than that in WT mice (Figure 7A, graph). However, compared to WT mice, the abundance for CFTR mRNA in αERKO mice increased 78% at 18 days of age (Figure 7A, graph). Similarly, the abundance of CFTR mRNA in αERKO mice was 61% higher than that in WT mice at 60 days of age (Figure 7A, graph).

Immunohistochemical study showed the localization of CFTR at the brush border and apical cytoplasm of nonciliated cells (Figure 7B). At 10 days of age, the ED of WT mice showed a weak immuno-staining in a limited number of nonciliated cells (Figure 7B, a), while there was no visible positive reaction for CFTR in αERKO mice (Figure 7B, b). Detectable immuno-reactivity of CFTR in the ED of αERKO mice was seen at 18 days of age, even though a few nonciliated cells exhibited positive staining for CFTR (Figure 7B, d). More intensive immuno-reaction for CFTR was evident in the ED epithelia of WT mice at 18 days of age (Figure 7B, c). Strong immuno-reactivity for CFTR was detected in the ED of WT mice at 60 days of age (Figure 7B, e). Positive immuno-staining for CFTR in αERKO mice at 60 days of age was found in some of the nonciliated cells (Figure 7B, f).

Expression of mRNA and immunohistochemical localization of Na⁺/K⁺ ATPase α1 subunit in the efferent ductules

Expression of ATPase α1 subunit mRNA was detected in the ED of all experimental mice (Figure 8A). Compared to WT mice, abundance of ATPase α1 subunit mRNA in αERKO mice was lower by 65% at 10 days of age, whereas there was negligible change between WT and αERKO mice at 18 days of age (Figure 8A, graph). An increase of 21% in ATPase α1 subunit mRNA abundance was detected in the ED of αERKO mice at 60 days of age (Figure 8A, graph).

Immunohistochemical study showed the localization of ATPase α1 subunit on lateral (red arrow in insert) and basal (black arrow in insert) sides of epithelial cells in the ED of WT and αERKO mice during postnatal development. Bar = 50 μm. Bar in insert = 25 μm.
DISCUSSION

A functional ERα is essential to maintain normal morphology and function of the ED of adult mice (Iguchi et al., 1991; Eddy et al., 1996; Lee et al., 2000; Lee et al., 2001). However, little is known about the role of estrogen and ERα in the ED of prepubertal mice. The aim of the current study was to investigate the expression of epithelial ion transporters and their proteins in the ED of postnatally developing WT and αERKO mice at 10 and 18 days of age (prepubertal), and 60 days of age (postpubertal).

Direct measurement and analysis of testicular fluid in mouse is unfeasible. The formation of the Sertoli cell junction and canalization of the seminiferous tubules, is indicative of active testicular fluid secretion, and develops between 10 and 16 days of age (Nagano and Suzuki, 1976; Gondos and Berndston, 1993). Thus, it is likely that the mouse testis does not actively secrete testicular fluid at 10 days of age. Consequently, it is assumed that differential expression of epithelial transporters between the ED of WT and αERKO mice at this age would be due not to testicular factors secreted from the testis but from systemic factors and/or a developmental defect of αERKO mice which lack a functional ERα from conception. At 18 days of age, the time at which testicular fluid is secreted and the Sertoli cell junction and lumen is present (Nagano and Suzuki, 1976; Gondos and Berndston, 1993), we speculated that the function of the ED would be influenced by factors in testicular fluid. At this age, the testis of the mice has a high number of pachytene spermatocytes (Bellve et al., 1977), which possess P450arom mRNA and protein (Nitta et al., 1993; Carreau, 2000). Thus, it is reasonable to consider that fluid secreted from the testis at this age would contain estrogen produced by pachytene spermatocytes. The testis at 60 days of age is fully functional and actively secretes testicular fluid. In addition, all types of germ cells and spermatozoa, known as sources of estrogen in male reproductive tract, are present in the testis (Bellve et al., 1977). Accordingly, we considered that relatively high level of estrogen would be present in testicular fluid produced from the testis of mouse at 60 days of age.

The presence and expression of P450arom in the male reproductive tract of various species have been demonstrated from a number of laboratories (Nitta et al., 1993; Kwon et al., 1995; Janulis et al., 1996; Tsubota et al., 1997; Janulis et al., 1998; Carreau, 2000). In the mature rat testis, the presence of P450arom is localized in Leydig and Sertoli cells (Papadopoulos et al., 1986), whereas the Sertoli cells synthesize estrogen in the immature rat testis (Carreau et al., 1999). In our present study, no specific immunostaining for P450arom was found in any of the cell types in the testes of WT and αERKO mice at 10 days of age. In addition, the Sertoli cells of the testis showed no immunopositive reaction for P450arom at any postnatal age. Such differences between the mouse and the rat would be due to species-difference on localization and expression of P450arom during the postnatal development (Carreau et al., 1999) and/or difference in methods used to detect expression of P450arom. The finding of no visible difference on P450arom immuno-staining pattern in the testes of WT and αERKO mice throughout postnatal development would indicate that the presence of functional ERα is not critically important for the expression of P450arom in specific cell types in the mouse testis. The expression of P450arom in the Leydig and germ cells is up-regulated by testosterone (Bourguiba et al., 2003). Because αERKO mouse has higher blood testosterone level than WT mouse (Eddy et al., 1996), it is possible to postulate that the level of P450arom might be higher in the testes of αERKO mice than those of WT mice.

Immunohistochemical study showed the presence of both ERα and ERβ in the ED in the mouse throughout the postnatal development period. The expression of ERα was limited in only the nuclei of the ED epithelia of WT mice and not in other cell types of the ED, agreement with previous findings (Iguchi et al., 1991). Immuno-activity for ERβ was detected in nuclei of several cell types in the ED of both WT and αERKO mice, including connective tissues and smooth muscle layer. A similar distribution of ERβ in the ED has also been reported in the adult rat (Hess et al., 1997b). However, Rosenfeld et al. (1998) showed cytoplasmic, as well as nuclear, localization of ERβ in the ED of adult WT and αERKO mice. The different immunostaining pattern for ERβ in Rosenfeld et al. (1998) and our present findings may be the result of using different antibodies and procedures.

CAII is a cytoplasmic enzyme which helps regulate intracellular pH and HCO3− concentration (Alper, 2002). Estrogen regulates the expression of CAII mRNA in the ED of adult mouse through ERα (Lee et al., 2001). The mRNA for CAII was not detectable in the ED of WT and αERKO mice at 10 and 18 days of age, in spite of positive cytosolic immuno-staining for CAII in the ED epithelia. Such disagreement in RNA and protein expression could be due to post-transcriptional regulation of CAII expression, such as a rapid degradation of CAII mRNA and/or enhanced translational efficiency of CAII mRNA, during early postnatal period. At this point, it is not clear if the expression of the mRNA and protein for CAII is directly regulated by testis-derived estrogen during early postnatal development, even though our recent study showed estrogen-regulation of mRNA expression for CAII in adult mice (Lee et al., 2001). Further detailed studies are required to determine transcriptional and translational mechanisms.
of CAII in the mouse ED at these ages. A dramatic decrease of CAII mRNA level was found in the ED of αERKO mice at 60 days of age, which is in agreement with our previous findings (Lee et al., 2001). However, no visible difference of immuno-staining between adult WT and αERKO mice was detected. Such a paradox could be explained with reduction of cytoplasmic area along with decreased epithelial height in the ED of αERKO mice. Further quantitative studies are needed to directly determine the level of CAII protein in the ED of WT and αERKO mice.

NHE3 is a member of NHE (Na+/H+ exchanger) family, which mediates Na⁺ absorption by an electroneutral countertransport of H⁺ for Na⁺ across the plasma membrane (Hayashi et al., 2002). It is believed that NHE3 is involved in contributing to the microenvironment in the ED by the reabsorption of Na⁺ from the fluid (Lee et al., 2001). From the present study, no detectable mRNA for NHE3 was found in the ED of WT and αERKO mice at 10 and 18 days of age. However, a weakly positive immuno-staining for NHE3 was detected in the ED of WT mice, but not in the ED of αERKO mice at 18 days of age. As we previously found no direct regulatory effect of estrogen on the mRNA expression of NHE3 through ERα in the ED of adult mice (Lee et al., 2001), we must speculate that failure to detect NHE3 protein in the ED of αERKO mice at 18 days of age is due not to the lack of functional ERα control of NHE3 mRNA expression but to effects on post-transcription and/or translation of NHE3. A considerable decrease of the mRNA level for NHE3 in the ED of αERKO mice at 60 days of age strongly agrees with our previous study (Lee et al., 2001). Correlatively, a positive immuno-staining of NHE3 in the ED of αERKO mice was weaker than that of WT mice. Adult αERKO mice have short and disorganized microvilli on nonciliated cells of the ED (Lee et al., 2000). Thus in the present study, the low levels of detectable NHE3 in the ED of αERKO mice at 60 days of age is likely the result of defective developmental morphology resulting from a lack of functional ERα. Direct transcriptional regulation of NHE3 mRNA expression by estrogen through ERα is still controversial (Lee et al., 2001; Zhou et al., 2001). Even so, results from our present study clearly imply that a presence of functional ERα is required to maintain adequate level of NHE3 in the ED during the postnatal development.

DRA is an apical Cl⁻/HCO₃⁻ exchanger which closely works with NHE3 and CFTR to facilitate NaCl absorption and HCO₃⁻ secretion in intestine (Jacob et al., 2002; Rossmann et al., 2005). Even though the presence and physiological function of DRA in gastrointestinal system are relatively well-studied, the existence of DRA in male reproductive tract has not been determined. For the first time, the present study showed the expression of DRA mRNA and apical localization at nonciliated cells in the ED of mouse. Interestingly, the level of DRA mRNA in the ED of αERKO mouse was greatly lower at 10 days of age but higher at 18 days and 60 days of ages, compared to WT mouse. An increase of DRA mRNA level in the ED of adult αERKO mouse was also observed from our earlier study (Lee et al., 2001). A molecular mechanism leading into such transient change of DRA mRNA expression in the ED of αERKO mouse is not identified at this point. However, it is clear that a lack of functional ERα leads into abnormal expression of DRA mRNA in the ED. Our previous study demonstrated that estrogen down-regulates the expression of DRA mRNA in the ED of adult mouse mainly through ERβ (Lee et al., 2001). Results from present study indicate that ERα would also play a role in the expression of DRA mRNA in the ED. In addition, a transient increase of DRA mRNA level after 18 days of age, at which active secretion of testicular fluid begins (Nagano and Suzuki, 1979; Gondos and Berndston, 1993), implies that a testicular factor(s) would be involved in the regulation of DRA expression in the ED.

CFTR, an apical Cl⁻ channel in epithelial cells, is responsible for secretion of Cl⁻, providing a driving force for NaCl and water transport. The expression of DRA in tracheal epithelial and pancreatic duct cells is induced by, CFTR (Wheat et al., 2000; Greeley et al., 2001). Such a tight correlation of expression between CFTR and DRA was also observed in our present study. A decrease of CFTR expression was found in the ED of αERKO mice at 10 days of age, followed by a transient over-expression of CFTR mRNA in αERKO mice at 18 days and 60 days of ages. Over-expression CFTR mRNA in the ED of adult αERKO mice was also detected from our previous study (Lee et al., 2001). Moreover, the immunolocalization and presence of CFTR at the apical region of nonciliated cells in the ED were observed for the first time and were similar to those of DRA and NHE3. These results in our present study imply that NHE3, DRA, and CFTR could work together to regulate NaCl and HCO₃⁻ movement in the ED, as found in the intestine (Jacob et al., 2002; Rossmann et al., 2005). Our earlier study demonstrated that the expression of CFTR mRNA is mainly under ERβ regulation, and the CFTR would play a role in regulating intracellular concentration of Cl⁻ in the ED of adult mouse (Lee et al., 2001). However, a function of the CFTR in the ED of prepubertal mouse has not been determined yet. Results from our current study indicate that a lack of functional ERα results in aberrant expression of CFTR mRNA in the ED of not only adult mouse, but also prepubertal mouse. A transient change of CFTR expression in the ED at 18 days of age seems to relate to the time at which the testicular fluid is secreted from the testis. Thus, it is reasonably speculated that a
factor(s) in the testicular fluid, such as estrogen, would affect the expression of CFTR in the ED of mouse.

A passive movement of water from the lumen in a reabsorptive epithelium is secondary effect of the active transport of Na⁺, resulted from a removal of intracellular Na⁺ by basolaterally localized Na⁺ transporters (Skou, 1988). Our earlier study demonstrated the expression of ATPase α1 subunit and regulation of its expression in the ED of adult mouse by estrogen through ERα (Lee et al., 2001). In the present study, a lack of functional ERα led into a dramatic decrease of ATPase α1 subunit expression in the ED of the mice at 10 days of age. However, the level of ATPase α1 subunit mRNA in the ED of αERKO mice returned close to the level of its WT mice at 18 days of age, followed by a transient increase of mRNA level at 60 days of age. Immuno-activity of ATPase α1 subunit was exclusively localized at basolateral region of nonciliated cells in the ED of mouse, in agreement with the finding in the ED of rat (Byers and Graham, 1990). It is presently not certain which factor(s) regulate the expression of ATPase α1 subunit in the ED of mouse during postnatal development. However, results from the present study clearly imply that normal expression of ATPase α1 subunit in the ED of mouse demands the presence of functional ER, as found in our previous study (Lee et al., 2001). Combined with results from our current and previous studies (Lee et al., 2001), it is thought that estrogen derived from the testicular fluid largely influences the mRNA expression of ATPase α1 subunit in the ED of mouse. Nonetheless, it seems that a non-testicular factor(s) would involve in a regulation of mRNA expression of ATPase α1 subunit in the mouse ED at 10 days of age, because an active secretion of the testicular fluid begins after 16 days of age (Nagano and Suzuki, 1976; Gondos and Berndston, 1993). Thus, it is speculated that mRNA expression of ATPase α1 subunit mRNA in the ED of early postnatally developing mice would be regulated by a circulatory factor(s) and/or the ED-driven endogenous factor(s) through ERα.

The results from the present study are summarized as following; 1) there is no observable difference on expression pattern of P450arom between WT and ERKO testes throughout the postnatal developmental period; 2) ERα and ERβ are present in the ED of WT mice during the entire postnatal development whereas only ERβ are in the ED of αERKO mice during the same period; 3) differential expression of epithelial ion transporters in the ED of αERKO mice, compared to those of WT mice, during the postnatal development is likely due to the combined effect of a developmental defect and lack of estrogen regulation through ERs. To our knowledge, this is a first report of the expression of the mRNAs and their proteins for CAII, NHE3, DRA, CFTR, and ATPase α1 subunit in the mouse ED during postnatal development. Also, the present study provides the first evidence of the presence of ERβ in the ED of the postnatal developing mice. Future studies need to concentrate on the developmental effects of ERα at the cellular and tissue level as well as the regulatory interplay of ERα and ERβ as controllers of ED ion homeostasis.

In conclusion, our present study shows that a lack of functional ERα influences the expression of epithelial ion transporters in the ED of the mice during postnatal development. In addition, this current study clearly provides evidence, demonstrating that a presence of functional ERα is necessary to maintain normal morphology and function of the ED of postnatally developing mice. Such roles of ERs in the ED are likely due to regulation of the expression of epithelial ion transporters by testis-derived factor(s), for example, estrogen, and/or a circulatory factor(s).

ACKNOWLEDGMENT

This study was supported in part by Biogreen 21 program (20050401-034-712) provided by Rural Development Administration, Korea and a research grant provided by University of Illinois at Urbana-Champaign.

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