Endocrine, *vol. 12, no. 3, 207–213, June 2000* 0969–711X/00/12:207–213/$11.75

Normal Development of Thymus in Male and Female Mice Requires Estrogen/Estrogen Receptor-α Signaling Pathway

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Estrogen receptors (ERs) are expressed in the thymus of both males and females, but their role in thymic development and function is unclear. To determine whether ERα plays a role in thymic function of either males or females, we compared thymuses of male and female wild-type (WT) and ERα knockout (αERKO) mice from birth to adulthood. Although thymic size was similar in both male and female WT and αERKO mice at birth (d 0), by postnatal d 5 and at all subsequent ages, both male and female αERKO mice had significant (30–55%) reductions in thymic weight. Morphometric analysis revealed a reduction in thymic medullary areas in adult αERKO mice compared with age-matched WT controls that paralleled thymic involution. There were changes in relative percentages of $CD4^+$ and $CD4^+CD8^+$ T-cells, and large decreases (70–80%) in overall absolute numbers of $CD4^+$ and $CD4^+CD8^+$ T-cells. Serum corticosterone and testosterone levels were not different in either neonatal or adult male WT or αERKO mice, and serum levels of 17β-estradiol (E_2) were similar in neonatal WT and αERKO males, indicating that increases in these thymolytic hormones are not responsible for the decreased thymic weight in αERKO males. Additionally, delayed-type hypersensitivity was significantly increased in male αERKO mice compared with WT mice. In summary, ERα deficiency does not inhibit initial differentiation or fetal thymic development, but the absence of ERα results in marked decreases in thymic size in both sexes during the postnatal period. These results are the first direct demonstration that the E_2/ERα signaling system is necessary for maintenance of normal postnatal function of the female thymus gland. The similar results obtained in males demonstrate a role for the E_2/ERα signaling system in the male thymus and emphasize that estrogens play a more critical role in the male than previously realized.

Key Words: αERKO mouse; thymolysis; thymic growth; immune; sex steroids.

Introduction

Extensive data indicate that in females estrogen may play an important role in normal thymic development and function *(1,2)*. The thymus is larger postnatally in females compared to males in some strains of mice *(3)*, potentially owing to higher circulating estrogen levels. Additionally, immune responses are sexually dimorphic, with both humoral and cell-mediated responses being more robust in females *(1,2)*. The sexual dimorphism in immune function is also accompanied by sex-related differences in susceptibility to autoimmune diseases. For example, multiple sclerosis and systemic lupus erythematosus are two- and nine-fold more common, respectively, in women than in men *(4)*. Similarly, spontaneous or experimentally induced autoimmune disorders in laboratory animals typically have a greater incidence and an increased severity in females compared to males *(5)*.

The thymus, a primary lymphoid organ, expresses both the classical estrogen receptor-α (ERα) and the recently described estrogen receptor-β (ERβ) in males and females *(6)*. Although the cellular distribution of ERβ has not yet been reported, ERα is present in both stromal cells and some types of lymphocytes in the thymus *(7,8)*. Immature lymphocytes entering the thymus do not express either the CD4 or CD8 surface markers used to classify these cells and are known as double negative. As the lymphocytes develop, they express both CD4 and CD8, becoming double positives. They then subsequently undergo selection and maturation, and before they reenter the circulation, they express only one of these markers and are either $CD4^+$ or $CD8^+$. ERα has been reported to be primarily expressed in $CD8^+$ T-cell lymphocytes *(7)*, indicating that this cell type may respond directly to estro-

Received October 15, 1999; Revised December 16, 1999; Accepted December 22, 1999.

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gen. Estrogen also induces compositional changes in T-cell lymphocyte populations, since 17β-estradiol (E_2) treatment of mice in vivo increases the number of $CD4^+$ and $CD4^+CD8^+$ double-positive T-cells *(9)*.

The effects of E_2 on the thymus are complex and dependent on the age of exposure. In addition to its putative role in normal female immune development and function, exogenous estrogen perturbs immune development in males and females when administered at high levels during early life. In developing rodents, E_2 has thymolytic effects in both males and females *(10)*. However, exogenous E_2 given to neonatal female mice was recently reported to cause thymic enlargement *(11)*. The administration of the synthetic estrogen diethylstilbestrol during development has also been shown to result in immune abnormalities during adulthood *(12)*.

The increased levels of estrogen in females may act through thymic ER to produce the sexually dimorphic pattern of thymic growth, normal adult immune function, and susceptibility to T-cell-mediated autoimmune disorders. However, the relative contributions of ERα, ERβ, and the nongenomic membrane effects that have been reported for E_2 in thymic cells *(13)* in mediating the normal, basal effects of E_2 as well as susceptibility to autoimmune diseases in females are unknown. Additionally, it has been unclear whether or not ERα and/or ERβ play any physiological role in the normal development and function of the male thymus. Likewise, the mechanism by which prenatal or neonatal exposure to E_2 alters thymic involution and adult immune function in males and females is not well understood.

The recent generation of ERα-knockout (αERKO) mice *(14)* has provided a powerful tool to examine the role of the E_2/ERα signaling pathway in thymic development and function. In this study, we have used both male and female αERKO mice to explore the role of ERα in the thymus. Our results indicate that the lack of ERα results in changes in thymic size in both the postnatal male and female thymus, and results in structural, compositional, and functional changes in the adult male thymus. These data emphasize the importance of ERα in the development of the female thymus and also directly implicate the E_2/ERα signaling in maturation of thymic function in males.

Results

The role of the E_2/ERα signaling pathway in thymic size and function was examined using WT and αERKO mice (Figs. 1 and 2). The thymic index (thymic weight/body weight) in wild-type (WT) mice at birth (d 0) was not significantly different between males and females. In males, the thymic indices at birth was not significantly different in WT compared with αERKO mice, but by neonatal d 5, and at all subsequent time points examined, thymic indices were significantly reduced by about 30–45% (Fig. 1; $p < 0.05$ at all time points). Similarly, thymic indices at birth were not different in female WT and αERKO mice, but as in the males, there was a significant decrease by d 5 and all later ages (Fig. 2). The magnitude of the decreases in the αERKO females (30–55%; $p < 0.05$ at all time points) was similar to that seen in the males. The gross morphology of the thymuses from adult αERKO males was normal except that the thymuses were smaller. Kidney weights in αERKO and

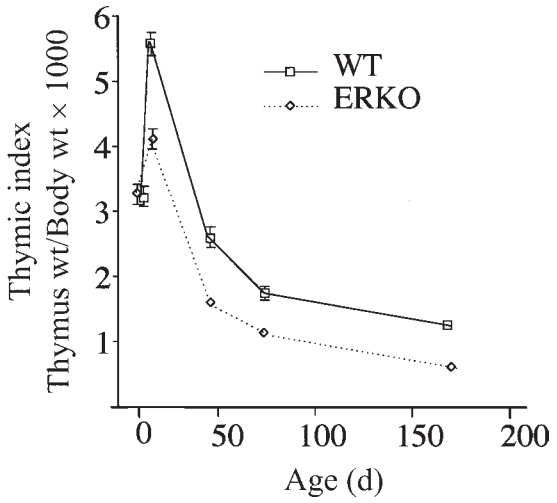

Fig. 1. Thymic indices in male WT and αERKO mice during development. Data are expressed as mean ± SEM. Thymic indices in WT and αERKO mice were significantly different at all ages examined except for d 0 (day of birth). For WT males, $n = 8$ for all points except for d 70, when $n = 5$. For αERKO males, $n = 8$ to 9 for d 0, 5, and 35, and $n = 6$ for d 70 and 160.

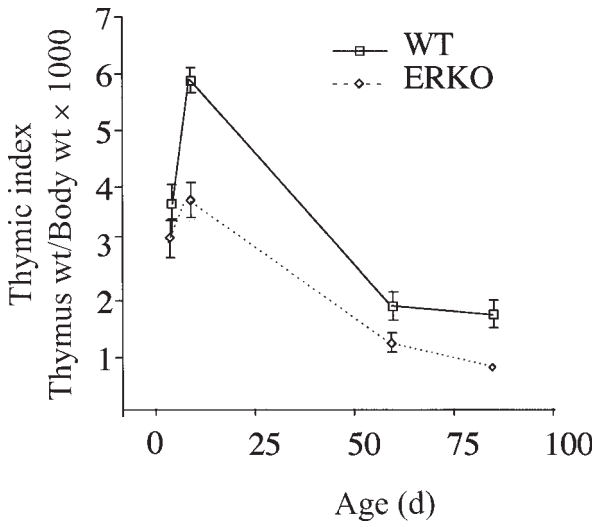

Fig. 2. Thymic indices in female WT and αERKO mice during development. Data are expressed as mean ± SEM. Thymic indices in WT and αERKO mice were significantly different at all ages examined except for d 0. For WT females, $n = 6$ for d 0 and 5, and $n = 3$ for d 55 and 85. For αERKO females, $n = 6$ for d 0, $n = 5$ for d 5 and 55, and $n = 3$ for d 85.

WT mice were not significantly different at any age examined (data not shown). Body weights in αERKO mice were equal to or greater than those of controls at all ages examined (data not shown).

The overall area of histological sections of the αERKO thymuses was reduced by approximately 35% compared with controls, and this decrease closely paralleled the observed decreases in wet weight in αERKO thymuses at this age. Morphometric analysis revealed that the medullary area of male αERKO thymuses was reduced 55% compared with WT thymuses (αERKO = 3.08 ± 0.60 mm^2 and WT = 5.60 ± 0.55 mm^2; $p < 0.05$). The cortical area in αERKO and WT male mice thymuses was not significantly different ($p > 0.05$).

Concentrations of serum testosterone in αERKO mice were not significantly different from WT mice in either 2- to 6-day-old neonates (WT = 1.71 ± 0.23 ng/mL and αERKO = 2.22 ± 0.44 ng/mL; $n = 9$ and 7, respectively) or 55- to 60-d-old young adults (WT = 7.82 ± 4.79 ng/mL and αERKO = 9.39 ± 3.15 ng/mL; $n = 7$ for both groups). Similarly, concentrations of serum corticosterone were also not significantly different (neonatal: WT = 8.31 ± 1.62 ng/mL and αERKO = 6.64 ± 3.34 ng/mL; $n = 7$ for both groups; young adults: WT = 80.62 ± 16.12 ng/mL and αERKO = 79.56 ± 8.33 ng/mL; $n = 6$ and 7, respectively). Serum concentrations of E_2 were uniformly low in neonatal WT and αERKO males ($n = 8$ and 5, respectively), and did not differ between the two groups (data not shown).

Flow cytometric analysis was performed using 60-d-old male αERKO and WT mice to determine whether the decreases in thymic weight were accompanied by decreases in overall numbers of T-cells and specific changes in the relative proportions of T-cells in the thymus and spleen. In the thymus of αERKO males, there was a significant reduction in the proportion of $CD4^+$ cells and an increase in the proportion of $CD4^+CD8^+$ cells compared with that of WT males (Table 1). Absolute numbers of $CD4^+$ and $CD4^+CD8^+$ cells in αERKO males were reduced by 80 and 70%, respectively (Table 1); the magnitude of these reductions exceeded the decreases observed in wet weight. The absolute number of $CD8^+$ and $CD4^-CD8^-$ cells also showed decreases, but these did not reach the threshold of statistical significance. Neither the proportion nor the absolute number of T-cells in the spleen differed between αERKO and WT males (data not shown).

To assess the role of the E_2/ERα signaling pathway in T-cell function in αERKO mice, we used an in vivo hapten-induced DTH reaction. The αERKO males exhibited a significantly greater delayed-type hypersensitivity (DTH) reaction compared with WT mice (Fig. 3). However, histologically the enhanced swelling was associated with increased edema and neutrophil infiltration (Fig. 4), suggesting that the increased DTH response may also involve an enhanced inflammatory response.

Discussion

Although the thymolytic effects of exogenous E_2 in female and male mice have been extensively described *(9,10)*, it has not been possible to delineate what role, if any, the endogenous, basal E_2/ERα signaling pathway plays in thymic development and function. The present results are the first demonstration that maintenance of normal thymic size and function in females requires ERα, and that thymic involution is increased in the absence of ERα. The magnitude of the decrements in thymic weight in the absence of ERα (30–55%) suggest that the E_2/ERα signaling pathway is of major importance during the neonatal period, in agreement with recent observations that thymic ER levels are increased in rats at 5 d postnatal relative to later ages *(15)*. Likewise, our results indicate that ERα is also essential for the normal postnatal development of the male thymus and are in agreement with the recent observations of Staples et al. *(16)* that thymic size is reduced in 10-wk-old αERKO males. As in females, the magnitude of the changes in male thymic weight and T-cell number in the absence of ERα attests to the importance of ERα in male thymic development. In addition, the similar thymic weight decreases in both sexes in the absence of ERα suggest that this receptor may be equally important for maintenance of thymic size in both sexes.

The present results show that the E_2/ERα system does not appear to play a critical role in the initial differentiation and fetal development of the thymus, because thymic weights in both male and female WT and αERKO mice are similar at birth. However, the clear difference in thymic indices between WT and αERKO mice by 5 d postnatal suggests that the E_2/ERα signaling pathway is critical for the maintenance of postnatal thymic size in both sexes. The changes in thymic indices that arise in αERKO mice during the neonatal period appear to be permanent, because they persist during all subsequent time points in αERKO animals of both sexes. The decreased thymic indices in αERKO males and females represented a specific decrease in this tissue, rather than a generalized decrease in body weight and organ size, since neither body nor kidney weights were decreased even in αERKO animals that showed large decreases in thymic weights.

Estrogen has historically been considered the female hormone, and androgen the male hormone. For many years, it was unclear whether estrogen played any physiological role in the development and function of male reproductive and other organ systems. However, ERs are widely distributed in males, and estrogen treatment, especially during development, causes significant pathological changes in males. This, in conjunction with evidence that estrogens were present in low but measurable quantities in male mice, suggests that estrogen could play some critical role in males.

Recent insights from both knockout mice lacking ERα and clinical observations of humans lacking either ERα or

Table 1
Flow Cytometric Analysis of Thymocytes of Adult WT and αERKO Male Mice[a]

Cell type	Relative percentage		Absolute numbers ($\times 10^6$)	
	WT	αERKO	WT	αERKO
$CD4^+$	9.46 ± 0.88	6.06 ± 0.34[b]	4.16 ± 0.65	0.77 ± 0.20[b]
$CD8^+$	1.60 ± 0.56	0.72 ± 0.26	0.65 ± 0.24	0.11 ± 0.06
$CD4^+CD8^+$	85.65 ± 1.66	91.80 ± 0.79[b]	35.22 ± 5.28	11.36 ± 2.52[b]
$CD4^-$ $CD8^-$	3.28 ± 0.31	2.08 ± 0.31	1.41 ± 0.31	0.29 ± 0.10

[a]Thymocytes (1×10^6) from six mice each in WT and αERKO groups were stained with MAbs against CD4 and CD8 and analyzed by flow cytometry to determine relative percentages. For absolute numbers, thymocytes were counted in the entire thymus, and this value was multiplied by the relative percentages of the various subtypes to obtain total number of the different cell types in the thymus. The relative percentages and absolute numbers (mean ± SEM) of the various cell types in the WT and αERKO thymuses were compared by unpaired Student's *t*-test.
[b]$p < 0.05$.

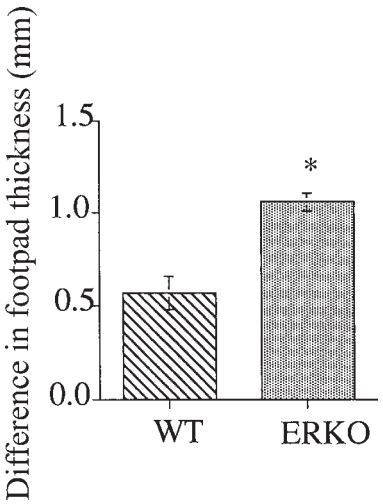

Fig. 3. Difference in footpad thickness in male αERKO and WT mice (hapten-injected foot minus vehicle injected foot) following the DTH test. n = 6 and 4 for αERKO and WT, respectively. *, Significantly ($p < 0.05$) different from WT value.

aromatase have conclusively demonstrated previously unrecognized important actions for estrogen in the normal male. Estrogen is critical for fluid reabsorption in the efferent ductules of the male mouse, and the absence of this reabsorption in the αERKO male is a critical contributor to the infertility in these mice *(17)*. In addition, recent results with the aromatase knockout mouse, which lacks endogenous estrogen, have also indicated that estrogen plays a role in male germ cell development *(18)*. Observations of humans lacking ERα or aromatase have revealed a necessary role for estrogens in key physiological processes in males such as closure of epiphysial plates *(19–21)*. The loss of ERα function through either lack of the receptor or ligand also results in insulin resistance and altered lipid profiles, further indicating a normal role for estrogen in males *(21)*. In addition, deficiency in ERα in a human male has been reported to be accompanied by early onset of cardiovascular disease *(22)* and dysfunctions in vasodilation *(23)*, findings supported by recent observations that male αERKO mice are more adversely affected by cardiac ischemia/reperfusion injury than their control counterparts *(24)*. This suggests that cardioprotective effects of estrogen, long known in females, may also be important in males. These recent findings, in conjunction with the present results indicating an important role of ERα in development of the male thymus, indicate that estrogen plays a far more important role in males than recognized only a few years ago, and it is likely that additional physiological roles for estrogen in the developing and adult male will be discovered.

The decreased size of the neonatal thymus in αERKO males and females raises obvious questions concerning the mechanism of this effect. It is well known that steroid hormones such as estrogens, androgens, glucocorticoids, and progesterone can cause thymic involution *(25–27)*. One potential mechanism for the decrease in thymic size in αERKO mice would be an increase in one or more thymolytic hormones such as E_2, testosterone, or corticosterone. Testosterone levels are increased by approx 50% in older αERKO males *(28)*. Our findings that testosterone concentrations appeared to be somewhat higher in neonatal and young adult αERKO males are in basic agreement with the observations in older αERKO males. However, androgens are less effective than either estrogens or glucocorticoids in inducing thymic involution *(29)*. In addition, other work has shown that chronic administration of 200 μg/d of dihydrotestosterone to intact male mice did not induce thymic involution despite the presumed increase in circulating androgens caused by this relatively high dose *(30)*. Thus, it seems unlikely that the relatively modest increases in testosterone seen in αERKO males result in the observed decreases in thymic size in the αERKO males. Likewise, the similar levels of corticosterone in αERKO and WT mice indicate that it also is not responsible for the thymic decreases in the αERKO mice.

Circulating concentrations of E_2 are approx 10-fold greater in adult αERKO females than in WT females *(31)*, raising the possibility that the decreases in thymic weights

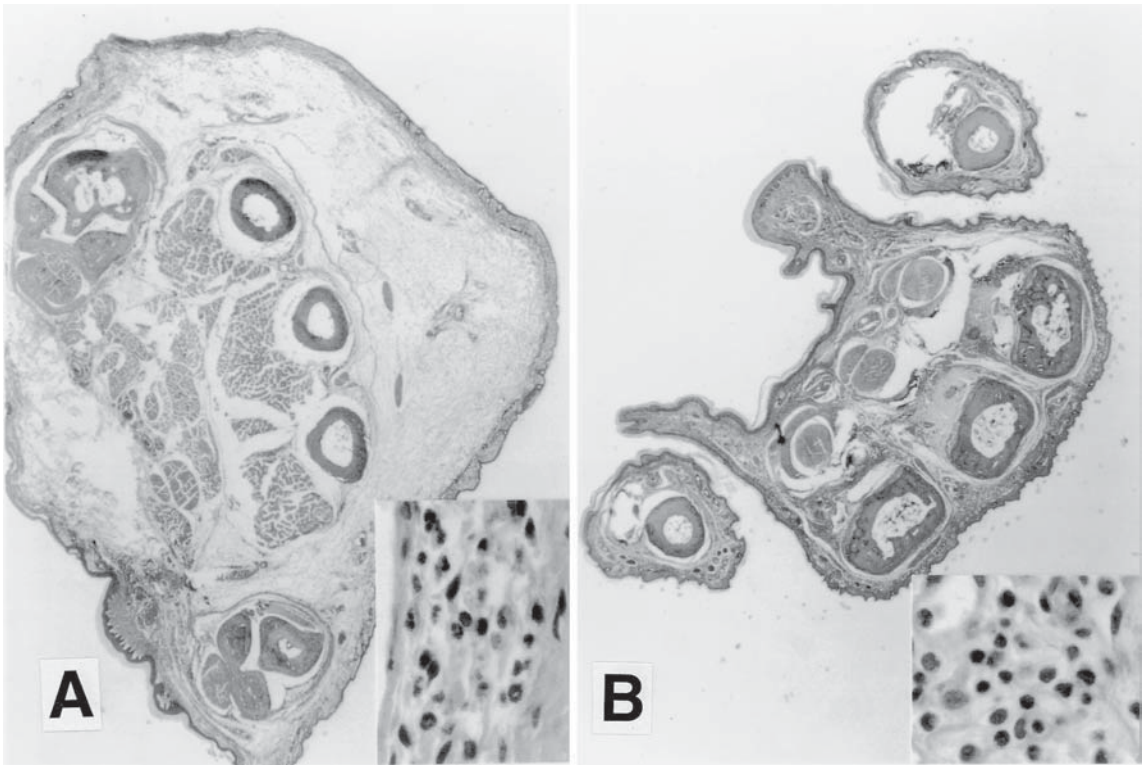

Fig. 4. Histological sections of footpads from **(A)** αERKO and **(B)** WT adult male mice showing excessive edema in αERKO compared with WT following the DTH test (×40). The insets in (A) and (B) illustrate neutrophil infiltration in the connective tissue (×100). In both cases, hapten-injected feet are shown.

in females could be owing to increased signaling of E_2 through ERβ, which is expressed normally in the thymus of the αERKO mouse despite the lack of ERα *(6)*. Although concentrations of E_2 have been reported to be normal in adult αERKO males *(32)*, serum E_2 concentrations have not previously been examined in neonatal αERKO males, the time when thymic changes first become obvious in these animals. Our results indicating that serum E_2 is similarly low in neonatal WT and αERKO males suggest that a neonatal increase in serum E_2 is not the causative factor in the observed thymic decreases in αERKO males, and all of our endocrine measurements *in toto* suggest that the observed decreases in thymic weight in male and female αERKO mice during the neonatal period are induced by some mechanism other than increases in the levels of thymolytic hormones.

The observed thymic changes in αERKO mice could be due to decreases in the structural elements and/or in T-cells; the latter effect could result from reduced numbers of thymocyte precursors in the bone marrow, altered thymocyte proliferation, increased apoptosis, and/or accelerated release of mature T-cells into the periphery. Our data suggest that the decreased thymic weight in αERKO males is, in large part, due to a concomitant decrease in the absolute number of thymocytes, and is in agreement with similar findings recently reported for the αERKO mouse *(16)*. Furthermore, the decrease in thymocyte numbers in αERKO males is accompanied by specific shifts in T-cell populations. The decrease in $CD4^+$ cells in male αERKO thymuses is consistent with the previous report that E_2 treatment increases the proportion of these cells in the thymus *(9)* and suggests that the E_2/ERα signaling pathway may be critical for the development of $CD4^+$ T cells in males. In addition, the observed decreases in $CD4^+$ T cells are consistent with the decreases in medullary area measured in the αERKO thymuses, because these $CD4^+$ T cells are located in the medulla.

Despite the fact that decreases in T-cell numbers in the thymus appear to be a major contributor to the overall thymic weight decreases in αERKO mice, the decreased thymic size in these mice may be predominately due to the lack of ERα in thymic stroma. Recent reports using both the progesterone receptor knockout and αERKO mouse indicate that the stromal receptor in the thymus may be critical for steroid effects on these organs *(16,28)*. This suggests that the decreases in T-cell populations in αERKO mice may be owing to an alteration in paracrine signaling from the stromal cells to the lymphocytes, although both the normal nature of this communication and how it is affected by steroids are presently not well understood.

Because E_2 has been shown to alter $CD4^+$ T-cell function *(32)*, we assessed the role of the E_2/ERα signaling pathway in these cells by using an in vivo hapten-induced DTH reaction. Although αERKO males exhibited a significantly greater DTH reaction than WT mice, this response appeared

to involve enhanced swelling, edema, and neutrophil infiltration, indicating that the increased DTH reaction seen may not be solely owing to the effects of the E_2/ERα signal transduction system in $CD4^+$ T-cells; in may also involve additional components of the inflammatory response.

In summary, our results indicate the maintenance of normal thymic size is dependent on the E_2/ERα signaling pathway in female mice. This pathway appears to play an equally important and previously unappreciated role in the maintenance of male thymic size and function. Although additional work is required to clarify the exact role of the E_2/ERα signaling pathway in the thymus, these results and similar recent results in several other organs emphasize that estrogen is a key regulator of both reproductive and nonreproductive organs in males.

Materials And Methods

Animals

All animal experiments were approved by the Laboratory Animal Care Advisory Committee of the University of Illinois. Homozygous αERKO and WT mice were obtained by mating mice that were heterozygous for the ERα gene disruption *(14)*. Parental heterozygotes used to generate the WT and αERKO mice used in this study were derived by backcrossing the C57BL6/129SV *(14)* to C57BL/6J for seven generations. Pup genotypes were determined by multiplex polymerase chain reaction *(14)*.

αERKO and WT male mice 0, 5, 30, 70, and 160 d of age ($n \geq 5$ for both groups at all ages) were used to determine the effects of ERα deficiency on the weight of the thymus and other organs. Similarly, female αERKO and WT mice 0, 5, 55, and 85 d of age (n = 3–6 for both groups at all ages) were used. All mice were initially weighed. For 0- and 5-d-old mice, pups were killed by CO_2 inhalation, and the thymus and kidneys were removed and immersion fixed. A tail sample was taken for subsequent retrospective genotyping. For animals at later ages, pups were genotyped first, then subsequently anesthetized and perfused with 4% glutaraldehyde in 0.2 *M* cacodylate buffer; kidneys and thymuses were then removed and weighed. Thymus from αERKO and WT mice were processed for histology, paraffin embedded, and sectioned at 4 μm, and then stained with hematoxylin and eosin (H & E).

Radioimmunoassays

To determine whether changes in thymic weight observed in αERKO mice could reflect changes in thymolytic hormones, the concentrations of serum testosterone, corticosterone, and E_2 were determined at various ages by radioimmunoassay. Neonatal (2- to 6-d-old) and young adult (55- to 60-d-old) WT and αERKO males (n = 4–9 per genotype and age) were decapitated, and the blood from the cervical stump was collected in microfuge tubes. Blood was allowed to clot at 4°C, and then serum was separated for RIA. Concentrations of testosterone, corticosterone, and E_2 were determined as described previously *(33–35)*. All concentrations of serum testosterone were determined in a single RIA; the intraassay coefficient of variation (CV) was 5%, with a sensitivity of 3.9 pg/tube. Concentrations of serum corticosterone were determined in a single RIA; the intraassay CV was 7%, with a sensitivity of 3 ng/tube. Similarly, serum E_2 concentrations were determined in a single radioimmunoassay, with an intra-assay coefficient of variation of 5% and a sensitivity of 2 pg/tube.

Morphometric Analysis

Thymuses from adult age-matched αERKO and WT mice (n = 3 for both groups) were longitudinally embedded in paraffin. Histological cross-sections were cut from the middle of each gland, and stained by H & E. Histological sections were photographed using a SPOT digital camera and a Power Macintosh G3 computer. Cortical and medullary areas in the captured images were then measured using the public domain NIH Image program, which was developed at the National Institutes of Health (NIH) and is available over the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, VA, part no. PB95-5001295GEI.

Flow Cytometric Analysis

Direct immunofluorescence was used to analyze lymphocyte subsets in both the thymus and spleen of 60-d-old male WT and αERKO animals ($n \geq 5$) to determine whether there were alterations in lymphocyte populations in immune organs as a result of ERα deficiency. Cells (1×10^6) were incubated with cy-chrome conjugated antimouse CD4 (L3T4, Pharmingen, San Diego, CA) monoclonal antibody (MAb) and phycoerythrin conjugated antimouse CD8 (LY-2 Pharmingen) MAb for 45 min at 4°C. After two sequential washes, cells were fixed with 4% paraformaldehyde and 10,000 cells were examined by flow cytometry (Coulter EPICS XL, Hialeah, FL). Appropriate isotype controls were run with each sample.

Delayed-Type Hypersensitivity

To determine whether the morphological changes observed in thymus of αERKO mice were accompanied by functional changes in immune responses, the in vivo DTH responses of αERKO and WT mice were compared. Young adult (60-d-old) αERKO and WT male mice (n = 6 and 4, respectively) were primed by subeutancously injecting 0.05 mL of *o*-nitrophenyl succinate (NP-O-SU) (7 g in 100 mL of dimethyl sulfoxide [DMSO]) in the flanks. Immediately after injecting the antigen, 0.1 mL of borate-buffered saline (pH 7.8) was injected subeutancously (this solution facilitates tissue coupling of succinimide esters). Six days after sensitization, mice were challenged with the NP-O-SU at 2 g% (w/v) in DMSO *(36)*. For challenge, the haptenic solution

was prepared by adding 20 μL of NP-O-SU to 400 μL of phosphate-buffered saline (PBS). The hapten (0.25 mL) was injected into the right foot, and a control solution containing the DMSO and PBS vehicles was injected into the left foot. One day later, the footpad thickness was measured in triplicate using a peacock dial gage, after which the animals were euthanized and both footpads processed for histopathology.

Statistical Analysis

Differences between means in all experiments were tested by using the two-tailed student's *t*-test. In all cases, $p < 0.05$ was considered significant.

Acknowledgments

The authors thank Eman Jassim for help with animal perfusion. We also thank Drs. Rex Hess and David Bunick for their helpful discussion, and Colette Abbey for assistance with hormone assays. This work was supported by NIH grants AG 15500, ES 08272, AI 407123, NS 36526, and AI 41236. Portions of this work were presented in abstract form at the 31st Annual Meeting of the Society for the Study of Reproduction, College Station, TX, 1998, and the 27th Annual Autumn Immunology Conference, Chicago, IL, 1998.

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