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Research article

Estrogen receptor α expression in mice kidney shows sex differences during aging

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Abstract

We have used semi quantitative RT-PCR and western blotting for the analysis of expression of estrogen receptor (ER) α and β mRNA and protein in the kidney of adult and old mice of both sexes. Uterus, a well-known target for estrogen action, was used for comparison. As compared to adult, the expression of both ER α mRNA and protein of old mice decreased in male but increased in female. However, unlike uterus, neither ERβ mRNA nor protein could be detected in the kidney of adult or old mice. Thus the present data reveal that the expression of ERα in mice kidney shows sex differences during aging.

Abbreviation: βME – β mercaptoethanol; ECL – enhanced chemiluminescence; EDTA – ethylenediaminetetraacetic acid; ER – Estrogen receptor; PAGE – polyacrylamide gel electrophoresis; PBS – phosphate buffered saline; PCR – polymerase chain reaction; PMSF – phenyl methyl sulphonyl fluoride; PVDF-polyvinyl difluoride; RT – reverse transcriptase; SDS – sodium dodecyl sulphate

Introduction

Estrogen exerts biological effects in numerous organs throughout the body. The role of estrogen in reproduction as well as in the prevention of age-related disorders is well established. Many studies have suggested that estrogen reduces the risk of development of Alzheimer's disease (Kawas et al. 1997), colon cancer (Nanda et al. 1999), cardiovascular diseases (Grodestein et al. 2000), cataracts (Worzala et al. 2001) and osteoporosis (Nelson et al. 2002). These actions of estrogen correspond with wide spread distribution of estrogen receptor (ER) in various organs (Kuiper et al. 1997).

Estrogen actions are mediated by two genetically different estrogen receptors, ERα and ERβ. These receptors share similar though divergent cellular expression profiles in target cells, and are functionally distinct (Hall et al. 2001; Gustafsson 2003). They are members of the nuclear receptor superfamily and act as a versatile regulator of gene transcription and cell signaling. Upon binding to its ligand, the receptor undergoes conformational rearrangement leading to the formation of a homo- or heterodimer of ERα and ERβ. Activated ER can interact with target genes directly by binding to specific, high affinity estrogen responsive elements within promoters or indirectly through protein-protein interactions with transcription factors such as AP1 or SP1 (Hall et al. 2001). Whereas both receptor subtypes can activate transcription, ERα appears to be more robust activator than ERβ, and when both receptors are expressed, ERβ can modulate ERα activity. Thus, the response to estrogen depends on the relative expression level of ER subtypes (Turgeon et al. 2004).

The uterus, a major target organ of ovarian hormones, undergoes continuous synchronized changes of proliferation and differentiation in response to changes in levels of circulating estrogen.

The level of ERα and ERβ changes in uterus during different phases of the estrous cycle (Weihua et al. 2000). The number of estrogen binding sites and their ability to bind DNA complexes decreases in old uterus (Kaur and Thakur 1991).

Kidney is an important target for estrogen dependent gene expression (Jelinsky et al. 2003) and the incidence of end-stage renal diseases increases after menopause, suggesting that estrogen may prevent development or progression of renal diseases (Shim et al. 2004). Estrogen is also known to be involved in the development of kidney carcinogenesis in Syrian hamsters (Bhat et al. 1993). Apart from diseases arising due to changes in the estrogen level, there are also reports of susceptibility to lupus nephritis in men due to ERα polymorphism (Liu et al. 2002).

One of the main characteristics of aging is decline in circulating estrogen level in females. This may affect the expression of ER and hence estrogen action in different tissues. Though there are several reports on age-dependent changes in the expression pattern of ERα and ERβ in different tissues (Kanungo et al. 1975; Kaur and Thakur 1991; Wilson et al. 2002; Chakraborty et al. 2003; Thakur et al. 2004a), no such data is available for kidney. Therefore, in the present study we have analyzed the expression of ERα and ERβ in the kidney of adult and old AKR mice.

Materials and methods

Animals

Male and female adult (25 ± 5 weeks) and old (65 ± 5 weeks) mice of AKR strain were used for the study. Adult female mice were in diestrus stage of estrous cycle when used for the study. The average life span of these mice under our laboratory conditions is about 75 weeks. They were maintained in a colony at 25 ± 2 °C, with alternating 12-hour dark and light schedule and having free access to standard mice feed and drinking water.

Isolation of total RNA

Mice ($n = 2$ per group; two times) were sacrificed by cervical dislocation. In order to avoid any diurnal rhythm in mRNA levels, all animals were killed at the same time of day. The uterus and kidney were removed and immediately processed for total RNA isolation according to acid guanidinium thiocyanate–phenol–chloroform single step extraction method (Chomczynski and Sacchi 1987). The integrity and quality of extracted RNA was monitored by formaldehyde–agarose gel electrophoresis and measurement of A_{260}/A_{280}. Samples showing intact RNA and $A_{260}/A_{280} > 1.7$ was used for further study.

Semi quantitative RT-PCR

RT-PCR was performed as described elsewhere (Kuippers and Beyer 1999; Weihua et al. 2000) with few modifications. For the reverse transcription reaction, 5 μg total RNA together with 2 μg random hexamer was denatured at 65 °C for 5 min, and then added to reverse transcription reaction mix in a final volume of 25 μl containing 0.2 mM dNTPs, 50 U of RNasin RNase inhibitor and 100 U of M-MuLV reverse transcriptase (Amersham Biosciences, USA). The reaction was allowed to proceed for 1 h at 37 °C. After completion of reaction, enzyme was inactivated at 95 °C for 5 min. For PCR amplification, 2.5 μl of total cDNA synthesized in reverse transcription reaction was used with following primers:

ERα forward 5′AATTCTGACAATCGACG CCAG 3′
ERα reverse 5′GTGCTTCAACATTCTCCC TCCTC 3′
ERβ forward 5′ ACAGTCCTGCTGTGAT GAAC3′
ERβ reverse 5′ACTAGTAACAGGGCTGG-CA C3′
βActin forward 5′GGGCACAGTGTGGGT-GA C3′
βActin reverse 5′CTGGCACCACACCTTC-TA C3′

Each reaction mix contains 0.2 mM dNTPs, 0.2 μM specific primers, 2.5 U Taq DNA polymerase and buffer (Amersham Biosciences, USA) in a total volume of 40 μl. Following parameters were used for amplification–denaturation at 95 °C for 60 seconds, annealing at 55 °C for 60 seconds and extension at 72 °C for 60 seconds. At the end

of desired cycles, one final incubation was done at 72 °C for 5 min.

In order to validate linearity of PCR amplification, ERα and β were amplified from uterine cDNA. Ten microliter aliquots of PCR products were removed after 20, 25, 30 and 35 cycles and used for 1.5% agarose gel electrophoresis. Results obtained after analysis of the amplification products showed that ERα and ERβ amplification were below the saturation limit up to 35 cycles. After validation of linearity, all samples were amplified for 30 cycles.

Southern hybridization

Ten microliter of PCR products were electrophoretically separated on 1.5% agarose gel and then blotted onto positively charged nylon membrane (Amersham Biosciences, USA). Random primed DIG-11-dUTP labeled probes were prepared from mice ERα and β cDNA (kind gift from Vincent Giguere, Canada) and β actin cDNA (Sigma, USA). Hybridization and detection were performed according to the instruction provided by the manufacturer in DIG-11-dUTP chemiluminiscent detection kit (Roche Applied Science, Germany).

Preparation of tissue lysate

Tissue lysate was prepared for western detection of ER. The uterus and kidney were lysed in a buffer containing 50 mM Tris-Cl (pH 8.0), 400 mM KCl, 10 mM EDTA and 2 mM PMSF. The lysate was centrifuged at 30,000 × g for 1 h at 4 °C. The supernatant was saved and protein concentration was estimated by Bradford method (Bradford 1976).

Western blotting of ER

Fifty microgram of protein of total tissue lysate was separated by 10% SDS-PAGE (Sambrook et al. 1989) and transferred onto PVDF membrane (Sigma, USA). The membrane was blocked with 5% non-fat milk in PBS for 2 h at room temperature. Then membranes were incubated with either antibody F-10 (Santa Cruz Biotechnology, USA, anti human ERα C-terminus monoclonal antibody raised in mice, 1:2000 dilutions) or L-20 (Santa Cruz Biotechnology, USA, anti human ERβ C-terminus polyclonal antibody raised in goat, 1:1000 dilutions) at 4 °C overnight. Subsequently, the blots were washed two times (5 min each) in PBS, 0.1% Tween-20 and incubated with either anti mouse IgG-HRPO conjugate (1:2000 dilutions, Genei India) or anti goat IgG-HRPO conjugate (1:2000 dilutions, Genei India) for 2 h at room temperature. The blots were washed four times (5 min each) in PBS, 0.1% Tween-20. The signals were detected by ECL method (Amersham Biosciences, USA).

After detection of ER, blots were stripped in the presence of 82 mM Tris-Cl (pH 7.5), 2% SDS and 100 mM β ME at 65 °C for 30 min. After blocking in 5% non-fat milk in PBS for 2 h, membranes were reprobed with actin antibody (A2668, Sigma, USA, 1:4000 dilutions) and anti rabbit-HRPO conjugate (Sigma, USA, 1:2000) by the method described above.

Densitometry and statistical analysis

Densitometry was done with the help of Fluorchem version 2 software (Alpha Innotech Corporation, USA). Results obtained from two independent experiments after normalization with β actin (for semi quantitative RT-PCR) and actin (for western blots) were analyzed statistically. The level of significance was determined by '*t*' test according to the method described by Williams (1993). The values are expressed as mean ± SEM. A $P < 0.05$ was considered as statistically significant.

Results

In order to ensure the accuracy of measuring the quantity of the transcript, the PCR amplification of cDNA obtained from reverse transcribed uterine RNA was carried out for 20, 25, 30 and 35 cycles (Figure 1). The linearity was observed up to 35 cycles. However, amplification was done for 30 cycles for further experiments.

ERα mRNA level decreased significantly in old uterus and old male kidney, but increased in old female kidney as compared to its adult counterpart (Figure 2a). Except in adult uterus, we were unable to find any amplification signal for ERβ either in adult or old kidney of both sexes (Figure 2b).

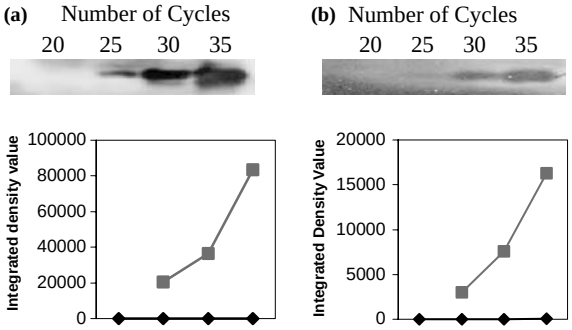

Figure 1. RT-PCR amplification of Estrogen receptor α (a) and β (b) during different number of cycles (number given in top panel) and their density plotted against cycle number.

To evaluate whether these changes in mRNA level are reflected at the translation stage, we measured ERα and ERβ protein level through western blotting. Apart from normal ~67 kDa band of ERα, we noted one extra band of ~36 kDa which appears to be the product of ERα after proteasomal degradation. Like ERβ mRNA, signal for ERβ protein could not be detected in the kidney though it was downregulated in the uterus of old mice (Figure 3b). When compared to uterus, the kidney showed similar level of ERα mRNA but a significantly decreased expression of ERα protein. Interestingly, sex differences were noted in the expression of ERα mRNA and protein in kidney. The female kidney expressed relatively higher ERα protein than male, and the level was upregulated in female but downregulated in male with age (Figure 3a).

Discussion

The existence of two ER isoforms raises the possibility that variation in the presence and relative extent of ER expression mediates tissue and age specific actions of estrogen. In this study, we have examined the expression pattern of ERα and ERβ mRNA and protein in the kidney of male and female mice during aging. We have used semi quantitative RT-PCR method to study changes in the expression of these receptors because of low abundance of their transcript. However, the results obtained by semi quantitative RT-PCR have greater chances of error if analysis of the end product is performed after plateau effect. To rule out this probability, a pilot experiment was performed. For this purpose, we used uterine RNA because it was already reported that uterus expresses more ERα than the kidney (Kuiper et al. 1997). Thus RT-PCR of uterine RNA having more copy number of transcript will reach plateau earlier than kidney.

Our results show that ERα mRNA and protein level decreased in old uterus and old male kidney while it increased in old female kidney. Male kidney in general showed lower expression of ERα

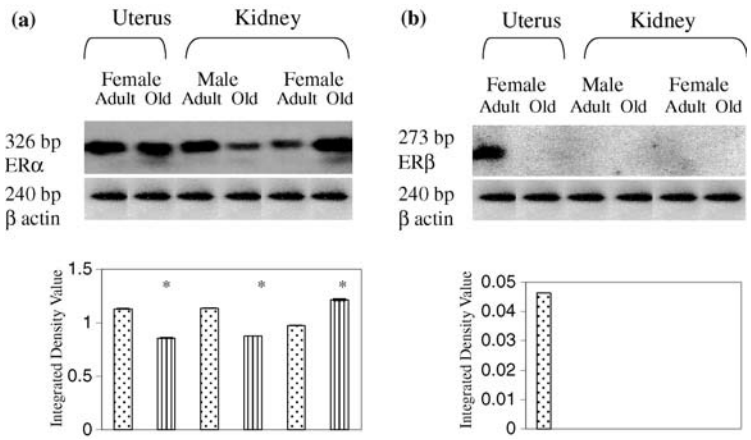

Figure 2. Expression of estrogen receptor α (a) and β (b) mRNA in uterus and kidney of adult and old male and female mice. The upper panel shows amplified product after southern blotting, and lower panel shows densitometric data after normalization (ER/ β Actin ratio). Values are represented as mean of two experiments ± SEM. * are the significant values $P < 0.05$.

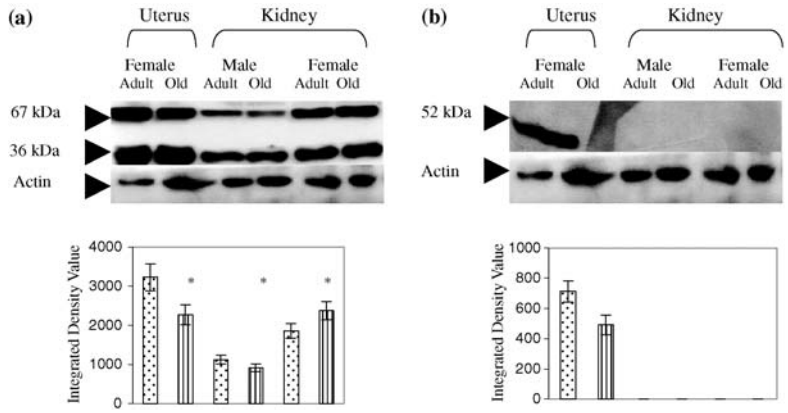

Figure 3. Expression of estrogen receptor α (a) and β (b) protein in uterus and kidney of adult and old male and female mice. The upper panel shows ECL detected blots, and lower panel shows densitometric data after normalization (ER/Actin ratio). Values are represented as mean of two experiments $\pm$SEM. * are the significant values $P < 0.05$.

than the female kidney. In agreement to the results of Kuiper et al. (1997), we were unable to find any transcript of ERβ in kidney. This may be either due to absence or less copy number of transcript.

The complex expression pattern of ERα may be due to multi hormonal regulation. These regulations are known to be tissue and sex specific (Shupnik et al.1988). Grandien et al. (1997) reported that growth hormone and thyroid hormone are important in the maintenance of ERα expression in liver but not in uterus. Apart from growth hormone and thyroid hormone, androgen, progesterone and estrogen are also responsible for the regulation of ERα. Both testosterone and estrogen are known to be responsible for the downregulation of ER mRNA and protein in the brain of mice and rat (Clancy and Michael 1994; Asaithambi et al. 1997; Zhou et al. 2000; Thakur et al. 2004b). Although the level of sex steroids changes at different stages of the life span, the change in the level of estrogen in females is drastic than androgen in males in old age. The androgen can get converted to estrogen locally by aromatization, thus regulating the ERα level in males differently than females. Similar sex, age and region dependent expression of ERα and ERβ was reported in rat brain and developing rat heart (MacLusky et al. 1997; Jankowski et al. 2001; Wilson et al. 2002).

Western blot results also show one extra band of $\sim$36 kDa. Similar small size bands were also reported when the whole cell extract of rat pituitary was used (Alarid et al. 1999). The $\sim$36 kDa band represents protease dependent degradation of ERα (Maeda et al. 1984; Horigome et al. 1988), rather than a general degradation because no degradation product was seen in case of uterine ERβ. One of the well-documented mechanisms of ER degradation is proteasome-mediated degradation (Nawaz et al. 1999), which helps in the maintenance of optimal level of ERα in a cell.

Renal diseases tend to be less severe among premenopausal women compared with male patients of same age (Neugarten and Silbiger 1995; Gross et al. 2004), but susceptibility of disease in female increases with decline in circulating estrogen level during aging. These results when combined with our finding of higher amount of ERα protein in old female kidney and lower amount of ERα in old male kidney raise a possibility that all ERα protein may not be fully functional, either due to the absence of appropriate concentration of estrogen or loss of ability to bind target DNA during aging.

In conclusion, ERα mRNA and protein level of mouse kidney alters with age and shows sex differences. The precise mechanism for such alteration is not known so far. Nevertheless, these findings suggest sex specific estrogen action in the kidney of aging mice.

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