Identification of the receptor isoforms that mediate estrogen and progestagen action in the female lower urinary tract

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Abbreviated title: ER and PR expression in the female lower urinary tract

Keywords: Bladder, estrogen receptor, Progesterone receptor, Urethra, Urothelium

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Word count (text): 2,500

Word count (abstract): 246

Abstract

Purpose

Bladder symptoms can be ameliorated by sex steroids but the mechanism of action is unknown. Previous work examining steroid receptor expression in the bladder did not examine receptor subtype expression. We now report the distribution of estrogen ($ER\alpha$, $ER\beta$) and progesterone receptor isoforms (PR-A, PR-B) in the female lower urinary tract.

Materials & methods

Prospectively recruited women undergoing routine urogynaecological or gynaecological surgery provided cold cup biopsy samples from bladder dome, trigone, proximal and distal urethra. These were immediately frozen or fixed in formalin. Following RNA extraction, transcripts for $ER\alpha$, $ER\beta$, PR-A and PR-B by RT PCR were demonstrated using isoform-specific primers. The precise cellular localisation of receptor protein and their relative levels were assessed by immunochemistry on formalin fixed tissue sections with isoform-specific antibodies.

Results

Nine pre-menopausal and 10 post-menopausal women were recruited to the study. Two post-menopausal women were taking hormone replacement therapy. $ER\alpha$, $ER\beta$, PR-A and PR-B transcripts were detected in whole bladder extracts. Nuclear $ER\alpha$ immunoreactivity was present in squamous epithelia, but absent from transitional epithelium. $ER\beta$ immunoreactivity was expressed in squamous epithelia, and also in transitional cell epithelium. Nuclear PR expression was present within urethral squamous epithelia only. PR expression was greater in pre-menopausal women and the post-menopausal women taking oestrogen.

Conclusions

ER α and ER β genes are transcribed within bladder tissue but only ER β is translated into protein suggesting that the urothelium responds to endogenous estrogen via ER β . PR expression is confined to urethral squamous epithelium, and the major isoform is PR-A.

Introduction

The female lower urinary tract is partially derived from the urogenital sinus and is dependent upon the gonadal hormones estrogen and progesterone reproductive life. After the menopause, bladder capacity, detrusor contractility and flow rates are reduced and estrogen supplementation relieves the symptoms of urinary urgency in postmenopausal women¹. Additionally, estrogen significantly reduces the re-infection rate in post-menopausal women with recurrent urinary tract infections².

Estrogen and progesterone action is mediated by high affinity intracellular receptors³. Ligand-bound receptors translocate to the nucleus to act as transcription factors regulating gene expression. Recent evidence points to multiple isoforms of estrogen receptors (ER) and progesterone receptors (PR) being generated from each gene. The main ER isoforms studied in other tissues have been ER α and ER β ⁴, and the main PR isoforms studied have been PR-A and PR-B⁵.

ER and PR have previously been demonstrated in the human female bladder^{3,6,7} and found to localise to the epithelium of the urethra, but both receptors have been reported as absent from transitional epithelium⁸, despite this tissue binding radio-labelled estradiol and progesterone⁹. These studies were completed before the existence of different isoforms for ER and PR was known, so it may be possible that ER β or other PR isoforms may be the mediators of sex steroid action. We report here the distribution of the PR and ER isoforms in the female adult human bladder and lower urinary tract.

Materials & Methods

Patients

Women having gynaecological surgery where urethral catheterisation was required were eligible to participate. A single cold cup biopsy was taken from the following sites: bladder

dome, bladder trigone, proximal urethra, and distal urethra. Biopsies were immediately fixed in 10% formal saline for 16-24 hours, and then embedded into paraffin wax. Local research ethics committee approval was obtained and all patients provided signed, informed consent. For RNA samples, bladder dome biopsies were taken from four patients whereas RNA purified from archived endometrial and ovarian tissues, obtained for other studies, was used as positive controls.

Antibodies

Monoclonal mouse anti-bovine ER α antibody directed against SDS-solubilised calf uterus ER α (Chemicon International Ltd, Harrow, UK), and monoclonal mouse anti-human ER β antibody developed against the N-terminal region of the human ER β sequence (Clone 14C8; Abcam, Cambridge, UK.) were used. Anti-human PR-B antibody (San27) directed at B-isoform specific epitopes within the first 164 amino acids of the PR gene and the anti-human PR-A antibody (clone 16) directed at N-terminal region of the PR-A isoform were purchased from Novacastra Laboratories, Newcastle-upon-Tyne, UK.

RNA isolation and RT-PCR

Bladder, endometrium and ovary biopsies were flash-frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated by adding one ml of TRIzol reagent (Invitrogen, Paisley Scotland) to each 100 mg of frozen tissue and allowing it to briefly de-frost. Samples was homogenised using a fine Ultra-Turrax probe (ten five second bursts on ice with ten seconds between each). Complete homogenisation was performed using glass Dounce homogenisers after allowing the TRIzol reagent to work for 5-10min at room temperature. The resulting TRIzol/cell mixture was transferred to a 1.5ml microfuge tube, chloroform (0.5ml) was added, the tubes shaken vigorously for 15 sec and then incubated at room temperature for 3min. After centrifugation (15min, 7000rpm, 4°C), the supernatant was transferred to fresh

1.5ml microfuge tubes, 0.5ml isopropanol added and the RNA precipitated over 10min at room temperature. RNA was pelleted (10min, 13000rpm, 4°C), and washed with 1ml of 75% ethanol/DEPC-treated dH₂O. The RNA was air-dried inverted over a paper towel for 7min. The pellet was re-dissolved in 50µL DEPC-treated dH₂O at 56°C for 5min and then RNA was stored at -80°C for RT-PCR analyses.

First strand synthesis was performed on one µg of total cellular RNA using AMV-RT (Promega Corp., Southampton, UK) in the presence of 25 Units of RNase inhibitor (RNasin; Promega Corp.) 0.4mM dNTP mixture and 2.8 µM anchored dT primer (Sigma-Aldrich, Poole, UK) for one hour at 42°C, followed by 2 min at 95°C to denature the reverse transcriptase enzyme. Amplification of GAPDH and ERα was performed in a Genius thermal cycler (Techne Corp., Duxford, UK) using 1µl of cDNA, (2µl cDNA for ERβ and PR). Reactions included 5 µl of 10 x AJ Buffer (450mM Tris-HCl (pH8.8); 110 mM NH₄SO₄; 45mM MgCl₂; 2 mM of each dNTP; 1.1 mg/ml acetylated BSA (Roche Diagnostics Ltd., Lewes, UK; 110 mM β-mercaptoethanol; 4.4 µM EDTA), 10 pmol of GAPDH-, ERα- or ERβ- or PR-specific primers combined with 1.0 Unit of Taq polymerase (Promega Corp.) diluted in 1 x AJ Buffer. Thermal cycler conditions for GAPDH were: initial denaturation step at 95°C for 2 min; ; 35 cycles of 95°C for 45 sec, 60°C for 1 min, 72°C for 1 min, with a final extension time of 10 min at 72°C. The absence of genomic DNA was confirmed using samples where the AMV-RT enzyme had been omitted. Optimal cycle conditions for ERα were 10 cycles of 94°C, for 30 sec, 60°C for 30 sec, 68°C for 1min, then 25 cycles with increased extension times of 5 sec/cycle, followed by a final extension for 5 min at 68°C. The amplification conditions for ERβ were the same as those for ERα but 40 cycles were required. PR was amplified with optimised cycle conditions of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min for 40 cycles followed by a final extension for 10 min at 72°C. The

amplification profiles for each gene were all within the linear range of detection (data not shown). Completed reaction mixtures were resolved through 3% TAE-agarose gels impregnated with ethidium bromide (50ng/mL) at ~5V/cm for 1 hr and fluorescent images captured on GeneGenius gel documentation and analysis system (Syngene, Cambridge, UK). Deoxyribonucleotide primers were obtained from Sigma-Genosys, (Pampisford, Cambridgeshire, UK) and purified by gel electrophoresis prior to use. The mRNA specific primer sets (Table 1) gave expected sizes for the amplicons of 381 bp for ER α , 279 bp for ER β , 396 bp for PR-A/B and 276 bp for PR-B. Levels of transcript for the house-keeping gene GAPDH to evaluate any variation in the mRNA content and cDNA synthesis in the different preparations showed no difference between any of the samples (data not shown). No PCR products were detected when the reverse transcriptase was omitted (data not shown).

Immunohistochemistry

Immunohistochemistry was performed on 5 μ m thick sections attached to silane-coated slides dried for 48-72 h at 37°C before use. After dewaxing in xylene and re-hydration through graded alcohol to water, antigens were retrieved using microwaving at 850W in 10mM citrate buffer (pH 6.0) for either 30 min (PR-A and ER β), 26 minutes (PR-B), or 12 minutes (ER α) and allowed to cool in this buffer for exactly 20 min. After a brief wash in distilled water, endogenous peroxidase activity was quenched by incubation with 6% (v/v) hydrogen peroxide for 10 minutes. Non-specific binding sites were blocked with 10% (v/v) normal rabbit serum (NRS; DAKO, Glostrup, Denmark) diluted in phosphate buffered saline (PBS) for PR-B and ER α , or Tris-buffered saline (TBS) for PR-A and ER β . Sections were then incubated with the primary mouse monoclonal antibodies (anti PR-B, 0.17 μ g/ml, anti PR-A 1.8 μ g/ml, anti ER α 1.2 μ g/ml and anti ER β 2 μ g/ml diluted in 10% NRS in the appropriate buffer), in a humidified container for 18 hours at 4°C. After washing with the appropriate

PBS or TBS buffer for 20 min, sections were then incubated with biotinylated rabbit anti-mouse immunoglobulins (1:400, DAKO) for 30 minutes at room temperature. After another wash with buffer for 20 min, specimens were incubated with Vectastain ABC-linked horseradish peroxidase reagent (ABC Elite; Vector Laboratories, Peterborough, UK) for 30 min at room temperature in a humidified chamber. After a further wash with buffer for 30 min, immunoreactivity was visualised with exposure to 3,3'-diaminobenzidine (DAB substrate; Vector Laboratories, Peterborough, UK) for 5 min, sections briefly counterstained with Mayer's haematoxylin (Sigma), dehydrated and cleared to xylene before being permanently mounted in XAM mountant (BDH, Poole, UK). Mouse IgG (Vector) was used in the place of the primary antibody for the negative controls and endometrium and breast cancer specimens were used as positive control tissues.

Analysis

The distribution of positively stained cells was determined qualitatively by light microscopy without formal cell counting. For epithelia, a semi-quantitative assessment of the proportion of epithelium stained (all, extensive, some, scanty, none) was recorded, and a note made of whether the staining was cytoplasmic or nuclear. For sub-epithelial tissues (lamina propria) the localisation and number of positively stained cells was recorded in a similar way. Squamous or transitional epithelia were identified by their typical histological appearance.

Results

Nineteen women were recruited, of whom 9 were pre-menopausal and 10 were post-menopausal. Two post-menopausal women were taking combined estrogen and progesterone hormone replacement therapy.

RT-PCR Transcript Levels

The presence of transcripts for human ER α , ER β and PR were confirmed in whole bladder extracts by RT-PCR (Figs. 1 and 2). All gonadal steroid receptor transcript levels were significantly lower than that found in representative reproductive tissues. Further analysis with primers which detect transcripts for both PR-A and PR-B isoforms (Fig. 2c) or the PR-B transcript alone (Fig. 2d), indicated that the major PR transcript present in the human female bladder was for the A-isoform (Fig. 2c).

Immunohistochemistry

11 out of the 76 biopsies did not contain enough tissue to be analysed. The remaining 65 biopsies were analysed (Table 2). Results demonstrated that immunochemical staining was dependent on the histological epithelial type present rather than anatomical site. Therefore, results are presented on the basis of histological epithelium rather than on anatomical localisation (Table 3). Detrusor muscle cells (where present) were devoid of any immunoreactivity for any of the antibodies.

ER α immunoreactivity

ER α expression was observed as discrete nuclear immunoreactivity in all squamous epithelia, regardless of site (Fig. 3a). Transitional epithelium within the bladder was uniformly negative (Fig. 3b), but uniformly positive when transitional epithelium was present within the urethra (9 out of 16 samples) (Fig. 3c). Immunoreactive cells were sparsely present within the lamina propria of all epithelia in both organs (Fig. 3c & 3d). Expression was not affected by estrogen status.

ER β immunoreactivity

ER β staining was similar to ER α staining within the urethra and in squamous epithelium within the bladder (Fig. 4a and b). However, ER β was uniformly expressed in all nuclei

within transitional epithelium in the bladder (Fig. 4b & d). Hormonal status had no effect on the staining pattern of ER β (data not shown).

PR-B and PR-A immunoreactivity

PR-A and PR-B immunoreactivity were confined to nuclei and were almost identical to each other, with expression only in squamous epithelium within the urethra (Fig 5). Here, staining appeared to be affected by hormonal status, with extensive nuclear staining in the premenopausal samples, and those of women taking HRT, but not in post-menopausal samples without HRT (not shown). Immunoreactive cells were also seen in the lamina propria of all epithelia. Cell numbers appeared to be greater beneath transitional epithelium of the bladder (compare Fig 5a & c with 5b & d) than elsewhere.

Discussion

We have demonstrated expression of ER β protein within transitional epithelium of the female human bladder. Previous work before the identification of the ER β isoform suggested that transitional epithelium was unable to respond to oestrogen⁸, mainly because expression of ER could not be demonstrated. The lack of ER α immunoreactivity in transitional epithelium of the present report supports some of the previous findings⁶. However, the finding of ER α by RT-PCR (Fig.1) indicates that transcripts for ER α are present in the bladder suggesting that the levels of protein are below the detection limits for the available assays.

The lack of appreciable amounts of ER α protein suggests that oestrogens act on transitional epithelium via the beta isoform of the receptor. The absence of any demonstrable ER immunoreactivity in the bladder muscle cell probably indicates that the detrusor is not a target for oestrogens in the woman, although previous studies have indicated the presence of ER α in the human male bladder⁴.

Immunohistochemistry indicated that the expression of the two PR isoforms (A and B) was more uniform than the ER isoforms, with PR almost exclusively confined to squamous epithelia (Fig 5). Previous data using an antibody that detects both PR isoforms also demonstrated a similar pattern of expression³. However, by examining the relative levels of bladder transcript for PR isoforms it is apparent that the major PR isoform present in the human female bladder is the PR-A isoform. The PCR conditions for amplification of both isoforms together and the B-isoform alone were the same and used a common 3' primer. Since the levels of A+B grossly exceeded that of B alone, we can conclude that isoform A must be present at a higher level.

The PR-A isoform is considered the weaker transcriptionally active form of the receptor although recent evidence suggests that PR-A may have PR-B-independent transcriptional roles in the in the uterus and thus PR-A may play a similar independent role in normal bladder physiology. Indeed, experiments with the human bladder cell line HUC E6 indicate that physiologically relevant concentrations of progesterone stimulate urothelial cell proliferation through an EGF-dependent pathway¹⁰, although these authors have not yet described which PR isoform is responsible for that effect. It is also interesting to note that the HUC E6 cells also proliferate in response to oestrogen, but this time via an NGF-dependent pathway¹¹.

Our observations suggest that the observed menstrual cycle fluctuation of urinary symptoms¹², could be mediated through ER β and PR-A. Rising levels of both estrogen and progesterone in mid-cycle are associated with increased urethral sphincter tone¹³. A meta-analysis of 11 randomised trials of estrogen treatment of urinary incontinence demonstrated that symptoms were improved by oestrogen, although there were no differences in urodynamic measurements of bladder function¹⁴. It seems likely that oestrogens act on the bladder by modulating the sensory functions of the urothelium, with secondary indirect

effects upon the motor functions of the bladder, or by an increase in the expression of the PR receptor, such as is seen in Syrian hamsters¹⁵.

Among post-menopausal women, the use of estrogen improves symptoms of urgency, frequency and nocturia¹⁶⁻¹⁸, although one study suggested this effect was only present in women without detrusor overactivity¹⁹. Sensory symptoms of urgency and frequency are improved by oestrogen¹, in association with improvements in the sensory activity of the bladder (an increase in volume of first desire and of bladder capacity). Thus, it does appear that exogenous estrogen influences the sensations of bladder filling and voiding to some degree, and our data suggest that the ER β receptor is the mediator of this effect. It is interesting to note that oestrogens stimulate nerve regeneration in the uterine smooth muscle through an ER β -dependent mechanism, suggesting that the bladder could be subjected to the same control mechanisms²⁰.

Conclusion

The data presented here suggest for the first time that the human urinary bladder may be responsive to oestrogens, via ER β , and explain the observed clinical effect of estrogen on some urinary symptoms (mainly sensory). Thus, the ER β receptor appears to modulate the sensory pathways within the bladder wall, but further work is required to elucidate the precise pathways involved.

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Figure legends

Figure 1 RT-PCR for ER α and ER β in bladder extracts. A representative ethidium bromide gel for RT-PCR products generated from bladder and ovary cDNA samples which were reverse transcribed in the presence (+) or absence (-) of AMV-RT (**A**). After amplification in PCRs with ER isoform-specific primers (see Table 1) that detect ER α (**left panel**) or ER β (**right panel**) aliquots were separated in 3% agarose gels and visualised under UV-light.

Figure 2 RT-PCR for PR and PR-A/B and PR-B in bladder extracts. A representative ethidium bromide gel for RT-PCR products generated from bladder and ovary cDNA samples which were reverse transcribed in the presence (+) or absence (-) of AMV-RT (**A**). After amplification in PCRs with PR-specific primers (see Table 1) that detect all isoforms of PR aliquots were separated in 3% agarose gels and visualised under UV-light. (**B**). To determine which PR isoform is the major isoform in bladder tissue, PCR performed with primers that detect the PR-A and PR-B isoform and the PR-B isoform alone (**C**) were performed and compared to the levels generated for endometrial samples. The data indicates that the major isoform present in the bladder and the endometrium is the PR-A isoform.

Figure 3 Staining pattern of ER α in different tissues. Immunoreactivity for ER α is shown for normal female urethra sample (**A**) and bladder dome (**B**). Immunoreactive ER α can be found in the nuclei of all epithelial cells in the transitional epithelium (**c**) and in the squamous epithelium (**s**). Immunoreactivity is also present in many cells within the lamina propria (**lp**), either densely stained (**arrows**) or more faint staining (**arrow heads**). In the squamous epithelium the most intense nuclear staining was confined to parabasal cells (*)

close to the basement membrane and was absent from cells near the luminal surface. Panels A and B were obtained at x100 magnification and are representative of 7/8 positive samples.

Figure 4 Staining pattern of ER β in different tissues. Immunoreactivity for ER β is shown for normal female urethra (**A**) and bladder dome (**B**). Immunoreactive ER β can be found in the nuclei of all epithelial cells in the transitional epithelium and in the squamous epithelium. Immunoreactivity is also present in some cells within the lamina propria. ER β is mostly confined to the nuclei, but cytoplasmic staining can be seen in all transitional epithelial cells (**arrows**) and ER β immunoreactivity extends from the parabasal cells to the surface umbrella cells (**arrowheads**). Panels A and B were obtained at x100 magnification and are representative of 7/8 positive samples.

Figure 5 Staining pattern of PR-A and PR-B in different tissues. Immunoreactivity for PR-A is shown for normal female bladder (**A**) and urethra (**B**) and immunoreactivity for PR-B in bladder (**C**) and urethra (**D**). Immunoreactive PR-A and PR-B were absent from bladder urothelium, but were easily detected in the lamina propria. In the urethra, both isoforms were present in the cuboidal and squamous epithelia, with the most intense nuclear staining confined to cells close to the basement membrane, with PR-B positive nuclei found close to the luminal surface (arrowed). All images were obtained at x100 magnification and are representative of 7/8 positive samples.

Fig 1

Fig 2A

Fig 2B

Fig 2C

Fig 3

Fig 4

Fig 5 A & B

Fig 5 C & D